BIOAEROSOL DISPERSAL MODELS AND THE *IN SILICO* DESIGN OF A SYNTHETIC STRAIN OF *BACILLUS SUBTILIS* WITH STRINGENT GROWTH REGULATION

by

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of
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Biodefense

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DEDICATION

This dissertation is dedicated to my parents, who always stressed the importance of education and have always pushed me to achieve my full potential, and my amazing wife Cristin, whose love and support all these years has meant more than I could ever express.

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LIST OF ABBREVIATIONS AND SYMBOLS

Acquired Immune Deficiency Syndrome ..... AIDS
Adenosine triphosphate ..... ATP
Advisory Committee on Human Radiation Experiments ..... ACHRE
Aerodynamic diameter ..... D_a
Air Force Regulation ..... AFR
Alaska ..... AK
American Society of Heating, Refrigerating, and Air-Conditioning Engineers ..... ASHRAE
American Type Culture Collection ..... ATCC
Amino acid ..... aa
Anhydrotetracycline ..... aTc
Anthrax (Army code designation) ..... N
Antibiotic resistance gene ..... Ab^R
Army Regulation ..... AR
Atmospheric Dispersion Model ..... ADM
Avenue ..... Ave.
Bacillus ..... *B.*
Bacillus atrophaeus/*Bacillus globigii* ..... BG
Base pairs ..... bp
Biological computer-aided design ..... BioCAD
Biological warfare/Biological weapons ..... BW
Biological Weapons Convention ..... BWC
Biosafety Cabinet ..... BSC
Boulevard ..... Blvd.
Centers for Disease Control and Prevention ..... CDC
Central Intelligence Agency ..... CIA
Chapter ..... Ch.
Coding sequence ..... CDS
Codon adaptation index ..... CAI
Colony forming units ..... CFU
Computational fluid dynamics ..... CFD
Computed tomography ..... CT
Computer-aided design ..... CAD
Coxiella burnetti ..... OU
Cyclic adenosine monophosphate ..... cAMP
Defence Research Establishment Suffield ..... DRES

Degrees (angular) ..... °
Degrees Celsius ..... °C
Delta (gene deletion) ..... Δ
Department of Defense ..... DOD
Department of Homeland Security ..... DHS
Department of Veterans' Affairs ..... VA
Deoxyribonucleic acid ..... DNA
Deseret Test Center ..... DTC
Destroyer (Naval vessel designation) ..... DD
District of Columbia ..... DC
Doctor ..... Dr.
Dollars (US) ..... $
Double-stranded DNA ..... dsDNA
Downstream homologous region ..... DSHR
Eastern equine encephalitis ..... EEE
Escherichia ..... *E.*
Edema factor ..... EF
Effective dose for 50% of exposed population ..... ED_{50}
et alii ("and others") ..... *et al.*
exempli gratia ("for example") ..... *e.g.*
Feedback-inhibited glutamine synthetase ..... FBI-GS
Fluorescent particles ..... FP
Formula Translator ..... FORTRAN
Francisella tularensis ..... UL
Francisella tularensis (wet) ..... TT
Francisella tularensis (dry) ..... ZZ
Freedom of Information Act (5 USC § 552) ..... FOIA
Food and Drug Administration ..... FDA
Generally Regarded as Safe ..... GRAS
Geographic Information System ..... GIS
Glutamine synthetase ..... GS
Gram (SI Unit) ..... g
Hawaii ..... HI
Heating, ventilation, and cooling ..... HVAC
High-efficiency particulate air ..... HEPA
Highly-expressed genes ..... HEG
Human Immunodeficiency Virus ..... HIV
id est ("that is") ..... *i.e.*
Immunoglobulin G ..... IgG
Institutional Review Board ..... IRB
International Genetically Engineered Machine ..... iGEM
Isopropyl β-D-1-thiogalactopyranoside ..... IPTG
Java Codon Adaptation Tool ..... JCat
Kanamycin ..... kan

kilo (10^3) (SI prefix) ..... k
Kilobase pairs ..... kb
Kilodaltons ..... kDa
Lambda bacteriophage ..... λ
Large Area Coverage ..... LAC
Large aircraft carrier (Naval vessel designation) ..... CVB
Laser-induced fluorescence ..... LIF
Le Système international d'unités ..... SI
Less than or equal to ..... ≤
Lethal dose for 50% of exposed population ..... LD_{50}
Lethal factor ..... LF
Liters L
Massachusetts Bay Transportation Authority ..... MBTA
MBTA system ..... T
Messenger RNA ..... mRNA
meter (SI base unit) ..... m
Methicillin-resistant *Staphylococcus arueus* ..... MRSA
micro (10^{-6}) (SI prefix) ..... μ
milli (10^{-3}) (SI prefix) ..... m
Minimum efficiently processed segment ..... MEPS
Minutes ..... min
Mister ..... Mr.
Mole ..... mol
nano (10^{-9}) (SI prefix) ..... n
National Capital Region ..... NCR
National Military Establishment ..... NME
Operational Multiscale Environment Model with Grid Adaptivity ..... OMEGA
Open reading frame ..... ORF
Pentagon Force Protection Agency ..... PFPA
Percent ..... %
Personal protective equipment ..... PPE
Polymerase Chain Reaction ..... PCR
Protective antigen ..... PA
Protein data bank ..... PDB
Puccinia graminis var. *tritici* ..... TX
Quantile coefficient of variation ..... qCV
Quick Urban and Industrial Complex ..... QUIC
Registered trademark ..... ®
Registry of Standard Biological Parts ..... RSBP
Research & development ..... R&D
Reynolds number (turbulence) ..... Re
Ribonucleic acid ..... RNA
Ribosome binding site ..... RBS
RNA polymerase ..... RNAP

Saint ..... St.
Science and Technology Directorate (DHS) ..... S&T
Serratia ..... *S.*
Serratia marcescens ..... SM
Seventh Day Adventist ..... SDA
Severe acute respiratory syndrome ..... SARS
Shipboard Hazard and Defense ..... SHAD
Sigma-A ribosomal subunit ..... σ^A
Sigma-B ribosomal subunit ..... σ^B
Sigma-C ribosomal subunit ..... σ^C
Sigma-H ribosomal subunit ..... σ^H
Simulant Induction Media ..... SIM
Single-stranded DNA ..... ssDNA
Special Operations Division ..... SOD
Square kilometers ..... km^2
Staphylococcus enterotoxin B ..... SEB, PG2
Submarine (Naval vessel designation) ..... SS
Subspecies ..... subsp.
Synthetic Biology Open Language ..... SBOL
T-3 coliphage ..... P
Termination efficiency ..... TE
Times (multiplication symbol) ..... ×
Time point ..... *t*
Toxin-antitoxin ..... TA
Trademark ..... ™
Transcription start site ..... TSS
Transfer ribonucleic acid ..... tRNA
Tryptic soy agar ..... TSA
Tryptic soy broth ..... TSB
Ultraviolet ..... UV
Union of Soviet Socialist Republics ..... USSR
United Kingdom ..... UK
United Nations ..... UN
United Nations Monitoring, Verification, and Inspection Commission .... UNMOVIC
United States ..... US
United States Army Medical Research Institute for Infectious Diseases ..... USAMRIID
United States Code ..... USC
United States Code of Federal Regulations ..... CFR
United States Postal Service ..... USPS
United States Ship ..... USS
University of California, San Francisco ..... UCSF
Upstream homologous region ..... USHR
Utah ..... UT

Variant ..... var.
Venezuelan Equine Encephalitis ..... VEE
Versus ..... *v.*
Western equine encephalitis ..... WEE
Wild-type ..... wt
World Medical Association ..... WMA
Zinc cadmium sulfide ..... ZnCdS, FP

ABSTRACT

BIOAEROSOL DISPERSAL MODELS AND THE *IN SILICO* DESIGN OF A SYNTHETIC STRAIN OF *BACILLUS SUBTILIS* WITH STRINGENT GROWTH REGULATION

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A critical component of biodefense research is the modeling and simulation of the spread of *Bacillus anthracis* spores. Of the three primary approaches used to study the dispersal of such bioaerosols – mathematical modeling, physical simulation, and biological simulation – the direct dispersal of biological simulants offers the most accurate results. However, the dispersal of viable simulants risks colonization of undesired environments, such as immunocompromised persons within exposed populations. During its Cold War biological weapons research program, the US government dispersed biologically viable simulants, exposing human subjects (often unbeknownst to them) to these ostensibly safe but potentially pathogenic organisms. An analysis of these open-air tests illustrates three improvements to ensure that future biodefense research is conducted ethically: reduced pathogenicity of the biological simulant, obtaining informed

consent (either standard or *de facto*) from potentially exposed populations, and ensuring that the unique concerns regarding military bioethics are addressed for any military service members who may be exposed.

One way to mitigate the potential pathogenicity of viable simulants is to use synthetic biology to design a novel strain of *Bacillus subtilis* with both essential and lethal genes regulated by multiple inducible and repressible promoters. Because of the stringent growth regulation of this strain, only bacteria germinating in the presence of the proper combination of six inducing chemicals would be able to survive, providing the system with multiply-redundant safeguards against colonization of undesired environments.

Due to its extremely stringent growth restrictions, the designed *B. subtilis* system behaves as a biologically viable organism only within a specifically-defined, artificially-supplied chemical environment where growth is desired and as an unviable physical simulant in environments where growth is undesired. By combining the robust research possibilities afforded by the former with the safety of the latter, the *B. subtilis* strain described here can be used as a safe simulant for direct analysis of bioaerosol dispersal. This novel synthetically-designed *B. subtilis* strain could be an ideal bioaerosol dispersal model, particularly for simulating dispersal of *B. anthracis* spores, and a valuable tool for the biodefense research community.

1. INTRODUCTION

Bacillus anthracis causes the deadly disease anthrax and is a major concern for biodefense and national security. This dissertation first reviews the three primary methods for simulating the dispersal of *B. anthracis* spores and shows that for simulating bioaerosol dispersal, biological simulants are more suitable than mathematical modeling or physical simulation. However, the advantages of biologically viable simulants are tempered by their potential pathogenicity, making their dispersal an unacceptable risk. After a historical chronology of open-air simulant dispersals conducted by the US government, this work demonstrates the bioethical deficiencies of these experiments, many of which secretly jeopardized the health of exposed Americans. If critical biodefense dispersal research is to continue, a safer biological simulant is required. This dissertation culminates in the *in silico* synthetic biology design of a strain of *Bacillus subtilis* with extremely stringent growth restrictions, which allow it to survive only in a specifically-defined, artificially-supplied chemical environment where growth is desired. This virtually eliminates the possibility of pathogenicity or viability where undesired, making this strain an ideal simulant for *Bacillus anthracis* spores and a valuable tool for the biodefense research community.

1.1. Biological Weapons, Bioterrorism, and Biodefense

Biological warfare is the use of weaponized biological agents to cause deadly or incapacitating diseases for offensive or defensive military purposes. These biological agents can be delivered through various methods, including inhalation, ingestion, injection, and absorption. Modern biological warfare is generally considered to be the product of state actors and governments. Bioterrorism, on the other hand, is the use of biological agents by non-state actors to affect the politics or policies of others, or simply to strike terror into a target group. Because a terrorist group could likely acquire dangerous biological agents, develop an appropriate means of delivery, and use such weapons to infect a target population with a lower probability of detection and reprisal than could a nation, bioterrorism is generally considered the greater threat to US national security.

1.2. *Bacillus anthracis* and the Anthrax Threat

Perhaps one of the most feared biological agents with respect to biological warfare and bioterrorism is the bacterium *Bacillus anthracis*, the etiological agent of the disease anthrax. One of the microbiological characteristics of *B. anthracis* that differentiates it from most other organisms of concern to biodefense is also what makes it such a deadly threat: when the bacterium encounters conditions that are unfavorable to its survival, it forms a hardy spore that is impervious to

most environmental stresses, including most temperature extremes, humidity, exposure to ultraviolet radiation, and at least 200 years of nutritional deprivation.[1]

Bacillus anthracis, depending on the site of inoculation, can cause cutaneous, gastrointestinal, or inhalational disease.[2] The most serious form of the disease – and the form that is of greatest concern to biodefense – is inhalational anthrax, which is caused by the deposition of aerosolized spores into the alveoli of the lungs. Spores of *B. anthracis* can range in size between approximately 0.5 μm – 2.5 μm.[3] These spores are easily aerosolized and settle in the alveoli, where they become phagocytized by macrophages and begin the inhalational anthrax infection. The LD_{50} for inhalational anthrax is 2,500 – 55,000 spores.[4] However, extrapolations from primate data suggest that an individual's infectious/lethal dose may be as low as 1 – 3 spores.[5] The incubation period is usually less than 1 week. Although there is some data suggesting that the

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[1] MJ Hudson *et al.*, "*Bacillus anthracis*: balancing innocent research with dual-use potential," *Int J Med Microbiol*, 298(5-6), 2008.

[2] A more recent presentation of infection, first reported in 2000, has been referred to as injectional anthrax. While clinically and microbiologically interesting, this method of infection is of little relevance from a biodefense perspective. Interested readers may refer to numerous publications: MG Booth *et al.*, "Anthrax infection in drug users," *Lancet*, 375(9723), 2010; AG Powell *et al.*, "A case of septicaemic anthrax in an intravenous drug user," *BMC Infect Dis*, 11, 2011; SH Ringertz *et al.*, "Injectional anthrax in a heroin skin-popper," *Lancet*, 356(9241), 2000; DA Sweeney *et al.*, "Anthrax infection," *Am J Respir Crit Care Med*, 184(12), 2011. Although injectional anthrax could conceivably be used for bioterrorism in a manner similar to the Soviet assassination of Bulgarian dissident Georgi Markov with ricin, it seems highly unlikely that this particular biological agent would be selected for delivery in this manner when a chemical agent or biological toxin would be much more effective and fast-acting for this purpose.

[3] M Carrera *et al.*, "Difference between the spore sizes of *Bacillus anthracis* and other *Bacillus* species," *J Appl Microbiol*, 102(2), 2007.

[4] TV Inglesby *et al.*, "Anthrax as a Biological Weapon: Updated Recommendations for Management," in Bioterrorism: Guidelines for Medical and Public Health Management, Ed. DA Henderson, TV Inglesby, and T O'Toole (Chicago: AMA Press, 2002).

[5] CJ Peters and DM Hartley, "Anthrax inhalation and lethal human infection," *Lancet*, 359(9307), 2002.

incubation period may be as long as two months[6], statistical modeling of anthrax incubation suggests that more realistic maxima for incubations periods are in the range of 10 days.[7] The mortality of inhalational anthrax is 80 – 100% in patients who remain untreated.[8] Of the 11 cases of inhalational anthrax reported from the 2001 anthrax attacks, 5 patients (45%) died from the illness. An important observation from these cases is that all 4 patients who exhibited signs of fulminant disease prior to the initiation of antibiotic therapy died.[9] Although the number of data points is small, this statistic further supports previous findings that radiographic abnormalities are associated with a poor prognosis[10] and that "treatment at this stage of the disease would be unlikely to alter the outcome of the illness.[11]"

A report by the US Office of Technology Assessment reported that an attack on Washington, DC, with one metric ton of sarin would produce up to 8,000 deaths, while an attack with a one-megaton hydrogen bomb – very large on the scale of nuclear weapons deployed today – would produce between

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[6] Centers for Disease Control and Prevention (CDC), "Fact Sheet: Anthrax Information for Health Care Providers," http://www.bt.cdc.gov/agent/anthrax/anthrax-hcp-factsheet.asp.

[7] R Brookmeyer, E Johnson, and S Barry, "Modelling the incubation period of anthrax," *Stat Med*, 24(4), 2005; DA Wilkening, "Sverdlovsk revisited: modeling human inhalation anthrax," *Proc Natl Acad Sci U S A*, 103(20), 2006; DA Wilkening, "Modeling the incubation period of inhalational anthrax," *Med Decis Making*, 28(4), 2008.

[8] A Mehta, "Anthrax (*Bacillus anthracis*)," in Agents of Bioterrorism: Pathogens & Their Weaponization, Ed. G Zubay (New York: Columbia University Press, 2005); RS Weinstein and K Alibek, Biological and Chemical Terrorism: A Guide for Healthcare Providers and First Responders (New York: Thieme, 2003).

[9] JA Jernigan *et al.*, "Bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States," *Emerg Infect Dis*, 7(6), 2001.

[10] TA Mayer *et al.*, "Clinical Presentation of Inhalational Anthrax Following Bioterrorism Exposure: Report of 2 Surviving Patients," in Bioterrorism: Guidelines for Medical and Public Health Management, Ed. DA Henderson, TV Inglesby, and T O'Toole (Chicago: AMA Press, 2002).

[11] TV Inglesby *et al.*, "Anthrax as a biological weapon: medical and public health management. Working Group on Civilian Biodefense," *JAMA*, 281(18), 1999.

570,000 and 1.9 million deaths. The estimated number of deaths from an attack using 100 kilograms of *B. anthracis* spores would be between 1 and 3 million.[12] According to these estimates, the number of deaths caused by anthrax could be 50% greater than with a massive thermonuclear weapon and 375 times greater than with the highly toxic nerve agent sarin. Worst of all, not only could the total number of casualties be greater, but this could be accomplished in a more cost-effective manner using biological weapons. A United Nations study published in 1969 examined the cost per casualty per square kilometer. In this assessment, an attack using conventional weapons requires $2,000 per casualty/km^2; attacks with nuclear and chemical weapons require $800 and $600, respectively. However, to cause the same effect with biological weapons would only require $1.[13] This places the capability for such devastation within the reach of both nations and bioterrorists.

Indeed, during the 2001 anthrax attacks, approximately five grams of powdered *B. anthracis* were placed in envelopes and mailed through the US Postal Service (USPS); twenty-two people were confirmed or suspected as having contracted either inhalational or cutaneous anthrax, five of whom died, and approximately $320 million was spent on facility decontamination alone.[14] The total cost of the medical response and the multi-year decontamination efforts

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[12] US Congress. "Proliferation of Weapons of Mass Destruction: Assessing the Risks," Office of Technology Assessment, US Government Printing Office, (Washington, DC: 1993).
[13] United Nations Secretary General, "Chemical and Bacteriological (Biological) Weapons and the Effects of Their Possible Use," (Geneva: United Nations, 1969).
[14] K Schmitt and NA Zacchia, "Total decontamination cost of the anthrax letter attacks," *Biosecur Bioterror*, 10(1), 2012.

have been estimated at close to $1 billion.[15] As this estimate does not include the lost productivity due to closures of affected buildings or the infrastructure upgrades made to the USPS system and government buildings to detect and protect against future attacks, the full cost of these five grams of powdered anthrax is likely well over $1 billion. Thus, it becomes clear that even a minute amount of a biological agent can exact a tremendous toll in terms of lives, economic impact, and psychological terror.

Since inhalational anthrax is highly lethal, dispersal of *B. anthracis* spores is the primary way this bacterium can be used to cause mass casualties. This makes it an attractive choice for those seeking to engage in biological warfare or bioterrorism. This is supported by evidence that most state-sponsored BW programs and terrorist groups that have developed or sought to develop such a capability have turned to *B. anthracis* as one of their first choices. Historically, the major powers with pre-BWC biological weapons programs are all known to have developed *B. anthracis* as a weapon: Canada, France, Germany, Japan, the UK, the US, and the USSR/Russia.[16] Iraq and South Africa were at one time known to possess *B. anthracis* weapons.[17] Although some estimates have

[15] C Campbell *et al.*, "Decontamination after a release of *B. anthracis* spores," *Biosecur Bioterror*, 10(1), 2012.
[16] AH Cordesman, Terrorism, Asymmetric Warfare, and Weapons of Mass Destruction: Defending the US Homeland (Westport, CT: Praeger, 2002).
[17] EM Eitzen, Jr. and ET Takafuji, "Historical Overview of Biological Warfare," in Medical Aspects of Chemical and Biological Warfare, Ed. FR Sidell, ET Takafuji, and DR Franz, *Textbook of Military Medicine* (Washington, DC: Office of The Surgeon General, Department of the Army, United States of America, 1997); United Nations Monitoring, Verification, and Inspection Commission (UNMOVIC), "Unresolved Disarmament Issues: Iraq's Proscribed Weapons Programmes," (New York: United Nations, 2003).

suggested that between six and thirteen nations had offensive BW programs[18], the most recent unclassified assessment by the US Department of State suggests that five nations may still have or be researching a BW capability: China, Iran, North Korea, Russia, and Syria.[19] Notable terrorists or terrorist groups who have sought to use *B. anthracis* as a weapon include Aum Shinrikyo[20], Larry Wayne Harris[21], al-Qaeda[22], the group known currently as the Islamic State[23], and of course the perpetrator of the 2001 anthrax attacks (Amerithrax).[24]

Because inflicting inhalational anthrax on a target population requires the inhalation of aerosolized of *B. anthracis* spores, research into the aerosol

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[18] GD Koblentz, Living Weapons: Biological Warfare and International Security, *Cornell Studies in Security Affairs* (Ithaca: Cornell University Press, 2009); M Leitenberg. "Assessing the Biological Weapons and Bioterrorism Threat," Strategic Studies Institute: US Army War College, (Carlisle, PA: 2005).

[19] US Department of State. "Adherence to and Compliance with Arms Control, Nonproliferation, and Disarmament Agreements and Commitments," (Washington, DC: 2013).

[20] DE Kaplan, "Aum Shinrikyo (1995)," in Toxic Terror: Assessing Terrorist Use of Chemical and Biological Weapons, Ed. JB Tucker (Cambridge, MA: MIT Press, 2000); M Leitenberg, Assessing the Biological Weapons and Bioterrorism Threat.

[21] JE Stern, "Larry Wayne Harris (1998)," in Toxic Terror: Assessing Terrorist Use of Chemical and Biological Weapons, Ed. JB Tucker (Cambridge, MA: MIT Press, 2000).

[22] S Salama and L Hansell, "Does Intent Equal Capability? Al-Qaeda and Weapons of Mass Destruction," *The Nonproliferation Review*, 12(3), 2005. Additionally, the final report of the Silberman-Robb Commission states that al-Qaeda was interested in obtaining a dangerous strain of "Agent X". The Commission on the Intelligence Capabilities of the United States Regarding Weapons of Mass Destruction. "Report to the President of the United States," (Washington, DC: 2005). Although the agent could not be publicly identified in the unclassified report, it is believed that "Agent X" most likely refers to *B. anthracis* (or possibly botulinum toxin). "Al-Qaeda," in Jane's World Insurgency and Terrorism (IHS, 2014); M Leitenberg, Assessing the Biological Weapons and Bioterrorism Threat.

[23] A laboratory in Fallujah, Iraq, operated by the terrorist group known as Jamaat al-Tawhid wal-Jihad, was found to contain notebooks with details about anthrax. The group later affiliated with al-Qaeda and changed its name to al-Qaeda in Iraq, which later spawned the Islamic State in Iraq and the Levant (ISIL). ISIL recently adopted its current name: al-Dawla al-Islamiyya, or the Islamic State. "Islamic State," in Jane's World Insurgency and Terrorism (IHS, 2014).

[24] J Guillemin, American Anthrax: Fear, Crime, and the Investigation of the Nation's Deadliest Bioterror Attack, 1st ed. (New York: Times Books, 2011); D Willman, The Mirage Man: Bruce Ivins, the Anthrax Attacks, and America's Rush to War, 1st ed. (New York, NY: Bantam Books, 2011).

dispersal of this first-line BW agent is a critical component of biodefense. Thus, it is necessary to conduct simulations into how such spores are spread under various conditions and their potential effects. As will be detailed in the next chapter, these simulations can take the form of mathematical or computational models, physical simulations dispersing non-viable chemicals or particles, and dispersal of viable biological simulant organisms. In a 1977 report to Congress entitled "US Army Activity in the US Biological Warfare Programs," the Army defined biological simulants as "living microorganisms, not normally capable of causing infection, representing the physical and biological characteristics of potential microbiological agents and considered medically safe to operating personnel and surrounding communities.[25]" However, as will be seen in the coming chapters, any naturally-occurring viable organism can be considered potentially pathogenic. Thus, for simulating the dispersal of *B. anthracis* spores, the three types of simulations all have significant flaws: mathematical modeling suffers from questionable accuracy and a lack of flexibility, physical simulation suffers from questionable fidelity, and biological simulation is the superior method in terms of accuracy and fidelity but risks pathogenicity to exposed populations. The result is that existing methods for modeling the dispersal of *B. anthracis* spores are insufficient or potentially hazardous.

The purpose of this dissertation research is threefold: 1) to determine the technical insufficiencies in current dispersal models, 2) to determine the

[25] US Army. "US Army Activity in the US Biological Warfare Programs," (Washington, DC: 1977).

bioethical flaws in the historical open-air experimentation by the US government, and 3) to use synthetic biology to develop a *Bacillus subtilis*-based simulant system which can be utilized with virtually no chance of pathogenicity. This dissertation will utilize multiple different methodologies to accomplish these goals. First, a review of the literature on mathematical, physical, and biological dispersal models will illustrate that all three simulation types as currently employed are insufficient (Ch. 2). Second, a historical chronology of open-air dispersal tests by the US government will show that many of these experiments were conducted on US military service members or the US civilian population (Ch. 3). Third, a critical analysis of the ways in which these experiments were performed in most (but not all) cases shows clear bioethical flaws with regard to pathogenicity, informed consent, and military coercion; technical and policy improvements that could be incorporated into simulant systems and their use in order to avoid these problems are suggested (Ch. 4). Focusing on a method to reduce pathogenicity, this dissertation's research culminates in using design principles from the cutting-edge field of synthetic biology to develop specific rationally-designed genetic enhancements that result in the *in silico* design of a novel, artificially-created biological simulant system designed to be viable only in a specifically predetermined narrow set of conditions (Ch. 5). Chapter 6 provides an integrative discussion of the results from the previous chapters and describes the potential practical applications of the novel *B. subtilis* simulant system.

The rationale behind this research is the *in silico* development of a strain of *Bacillus subtilis* that could be used as a safe biological simulant for direct analysis of bioaerosol dispersal. Due to its extremely stringent growth restrictions, this *B. subtilis* system behaves as a biologically viable organism only within a specifically-defined, artificially-supplied chemical environment where growth is desired and as an unviable physical simulant in all other environments. Because this essentially eliminates the possibility of viability or colonization in undesired environments, this novel synthetically designed *B. subtilis* strain is an ideal bioaerosol dispersal model, particularly for simulating dispersal of *B. anthracis* spores, and could be a valuable tool for the biodefense research community.

1.3. Nomenclature and Terminology

In order to address the material covered by this dissertation in the clearest and most accurate manner possible, it is necessary to address various issues relating to nomenclature and terminology.

First, two of the bacterial species that are the primary focus of this dissertation – *Bacillus subtilis* and *Bacillus atrophaeus* – have been the subject of numerous name changes that are likely to cause confusion if not addressed in advance. A brief historical chronology of the nomenclature associated with these organisms is useful.

Bacillus subtilis was one of the earliest identified bacteria, first described in 1835.[26] In 1900, a new species was discovered and called *Bacillus globigii*.[27] This species became widely used as a dispersal simulant, particularly by the military, where it was typically referred to by the acronym BG. In 1952, further study of *B. globigii* showed that while there are differences between *B. globigii* and *B. subtilis*, those differences were slight enough to "downgrade" *B. globigii* from a separate species to a variant of *B. subtilis*. It was given the name *Bacillus subtilis* variant *niger*.[28] The discovery of DNA and genetic sequencing gave scientists the ability to examine bacteria at the genetic level, allowing the determination of the real differences between bacteria. Based on differences in DNA-DNA hybridization, it was realized that strains of "*Bacillus subtilis* var. *niger*" actually were different enough from *Bacillus subtilis* that they should constitute a new species. Thus, *Bacillus atrophaeus* was recognized as a separate species in 1989.[29] Based on analyses of archived military BG strains, the BW simulant strains were determined to be most similar to the species now known as *Bacillus atrophaeus*. Because the military strains appear to have been subcultured and distributed to many different military laboratories, most of these likely originated

[26] CG Ehrenberg, "Dritter Beitrag zur Erkenntniss grosser Organisation in der Richtung des kleinsten Raumes," *Physikalische Abhandlungen der Koeniglichen Akademie der Wissenschaften zu Berlin aus den Jahren 1833–1835*, 1835.
[27] W Migula, "System der Bakterien," in Handbuch der Morphologie, Entwicklungsgeschichte und Systematik der bacterien (G. Fischer Verlag Jena, 1900).
[28] NR Smith, RE Gordon, and FE Clark, Aerobic Sporeforming Bacteria. Agriculture Mongraph No. 16 (Washington, DC: United States Department of Agriculture. US Government Printing Office, 1952).
[29] LK Nakamura, "Taxonomic Relationship of Black-Pigmented *Bacillus subtilis* Strains and a Proposal for *Bacillus atrophaeus* sp. nov.," *Int J Syst Bacteriol*, 39(3), 1989.

from the same original strain, probably the Detrick-1 strain.[30] Thus, any of the organisms named in the US BW documents are most likely what is now known as *B. atrophaeus*, regardless of what they were called at the time.

In an effort to maintain cohesiveness throughout this work and reduce confusion, the currently accepted nomenclature will be used in all cases when discussing historical tests involving biological agents, except when an agent is referred to in a direct quotation. (The currently accepted nomenclature will, however, be provided parenthetically.) Thus, as an example, the US Army's 1950 simulated BW attack on the San Francisco Bay Area dispersed an organism referred to in the study as *Bacillus globigii*.[31] In this work, the organism will be referred to as *Bacillus atrophaeus*, except in the case of a direct quote from a historical document. Because various strains of *B. atrophaeus* used in many of these biological warfare tests and research were previously known as *B. globigii*, it has been suggested that these strains be classified under the new name "*Bacillus atrophaeus* subsp. *globigii*". The assignment of such a name to these strains would "realign the name of this group with the historical and present use of its members throughout the biodefense research and industrial communities.[32]" However, it is as of yet not a validly published name according

[30] HS Gibbons *et al.*, "Genomic signatures of strain selection and enhancement in *Bacillus atrophaeus* var. *globigii*, a historical biowarfare simulant," PLoS One, 6(3), 2011.
[31] US Army. "Special Report 142: Biological Warfare Trials at San Francisco, California, 20-27 September 1950," PD Division, Biological Department, US Army Chemical Corps, (Camp Detrick, Frederick, Maryland: 1951).
[32] SA Burke *et al.*, "Detection of molecular diversity in *Bacillus atrophaeus* by amplified fragment length polymorphism analysis," Appl Environ Microbiol, 70(5), 2004.

to the rules set forth by the International Code of Nomenclature of Bacteria.[33] Therefore, the name *Bacillus atrophaeus* remains the currently accepted nomenclature and will be used throughout this work when referring to any of these nomenclatural designations.

Because *Bacillus subtilis* (proper) has been a model laboratory organism for over a century, it is generally considered the best understood organism after *E. coli*. Because of this, a vast repository of knowledge about this organism exists, e.g., which genes are essential and the structural and regulatory characteristics of operons. The complete genome sequence of *B. subtilis* was released in 1997.[34] However, *B. atrophaeus* was not sequenced until 2011[35], and it is not as well understood. Thus, it was felt that *Bacillus subtilis* was the better choice for the base organism upon which to design the genetic improvements. Not only does more information exist, but the decades of *B. subtilis* characterization likely results in an increased likelihood of success for the synthetically designed genetic modifications.

Second, a further complication to the nomenclature for *Bacillus* species is potential inaccuracy when using historical sources of information. In many cases, nonpathogenic *Bacillus* species were automatically classified as *Bacillus*

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[33] S Lapage *et al.*, Eds., International Code of Nomenclature of Bacteria: Bacteriological Code, 1990 Revision (Washington, DC: ASM Press,1992).
[34] F Kunst *et al.*, "The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*," *Nature*, 390(6657), 1997.
[35] HS Gibbons *et al.*, "Genomic signatures of strain selection and enhancement in *Bacillus atrophaeus* var. *globigii*, a historical biowarfare simulant."

subtilis.[36] Until relatively recently, species differentiation relied on morphological characteristics and biochemical assays that may have yielded incorrect designations through identical results. These tests are still used today, but genetic tests such as PCR and DNA sequencing have helped clarify the relationships between species and strains. As such, some of the "*Bacillus subtilis*" seen in older research may actually be from another species, such as *Bacillus cereus*. Without modern genetic testing of historical bacterial strains (which in most cases are unlikely to have been archived), determining with absolute certainty what species of bacteria was used for any given test may not be possible at this time. In these cases of potential ambiguities in the nomenclature from historical sources, the best educated guess as to the identity of the organism will be made from the available information.

Third, the latter chapters of this dissertation discuss the insertion of lethal genes into *B. subtilis*. Although the technical discussions in those chapters should make this point quite apparent, the potentially concerning nature of this term warrants an upfront clarification: within the context of this dissertation, "lethal genes" refers to genes that encode proteins that, when expressed, are lethal to the *Bacillus subtilis* simulant itself, not to any humans that may exposed to the bacterial strain.[37]

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[36] NA Logan, "*Bacillus* species of medical and veterinary importance," *J Med Microbiol*, 25(3), 1988.

[37] Aside from being illegal according to both federal and international law, intentionally increasing the pathogenicity of *B. subtilis* by addition of genes lethal to humans would be wholly antithetical to the principles of bio*defense*.

2. BIOAEROSOL DISPERSAL SIMULATION AND MODELING

2.1. The Necessity of Simulation

In order to defend against the threat of infectious or toxic bioaerosols, the physical and biological dynamics inherent to the dispersal of biological agents via aerosols must be understood. The only way to analyze the spread of pathogenic *Bacillus anthracis* spores with absolute accuracy is to release these deadly spores into the environment in which the analysis is desired. Obviously, such a release would be unreasonable, unethical, and (of course) highly illegal. Thus, as with most scientific quandaries that cannot be tested directly, the next best alternative becomes simulation.

Historically, research into the subject of pathogenic bioaerosol dispersal has been conducted using simulations from three different classes: mathematical/computational models, physical simulations using nonbiological simulants, and physical simulations using biological simulants. However, simulations of all types can prove inadequate in a variety of ways. In order to fully understand the requirements that an ideal simulant should meet, a critical analysis of previously employed simulant systems provides invaluable information by illustrating the deficiencies with these systems. This chapter discusses the various types of simulant systems and provides a critical analysis of specific models and simulants. The dispersal of bioaerosols such as bacterial

spores can be broken down into a wide variety of components, including deposition, interparticulate interaction, etc. Some of the models discussed in this chapter examine a subset of the available considerations. Some of the models (particularly in the mathematical models section) are not geared toward the simulation of bioaerosols specifically, but rather just generic particles, which depending on the parameters used could simulate chemical contaminants or bioaerosols.

A review of the primary literature on particulate aerosol dispersal was conducted. The literature was obtained primarily through PubMed, available online at http://www.ncbi.nlm.nih.gov/pubmed/. Utilized search terms included "aerosol", "aerosolization", "anthrax", "*Bacillus*", "*Bacillus anthracis*", "*Bacillus atrophaeus*", "*Bacillus subtilis*", "bioaerosol", "biodefense", "dispersal", "dispersion", "modeling", "simulant", "simulation", "spores", and combinations thereof. Retrieved papers were evaluated for relevance. Additionally, relevant second- and third-order references were obtained by examining the citations and references of all reviewed literature. The initial set of most relevant literature was selected based on the general applicability of the research and/or methodology to the aerosol dispersal of *Bacillus* spores, simulation or modeling of dispersal of *Bacillus* spores, development of a simulant for *Bacillus* spores, or any combinations of the above. The initial set of most relevant literature is listed in Table 2-1.

Table 2-1: Literature evaluated for review

Type	First Author	Particulate Studied	Size (µm)	Simulated Environment	Primary Focus	Secondary Focus
Mathematical	Nicogossian	*Bacillus anthracis* spores	2 – 6	Open-air	Model outdoor dispersal over an urban region	Effect on NCR infrastructure
	Hathway (2008)[38]	Unspecified particulate	5, 14, 20	Hospital room	Model dispersal from an activity-created zonal source	n/a
	Reshetin	*Bacillus anthracis* spores	0.1 – 5	Building, high-rise, 50 floors	Develop model to predict time required for dispersal	Determine the numbers of people exposed and infected
	Sohn[39]	Unspecified pollutant	n/a	Building, office, 5 floors	Develop a prototypical building taxonomy	Rank contributions to uncertainty of model
	Wein[40]	*Bacillus anthracis* spores	< 5*	Open-air	Compare emergency responses to anthrax attack	Age-dependent, dose-response model
	Sextro	*Bacillus anthracis* spores	2 – 10	Building, office, single floor	Model the transport of spores throughout a building	Effect of human activities
	Lai	Unspecified particulate	10	Building	Model deposition onto vertical surfaces	n/a
Physical	Farrell	BG Bugbeads	0.7 – 0.9	n/a	Develop a nonviable simulant with antigenic surface properties similar to *Bacillus atrophaeus*	n/a
	Thatcher (2004)/ Finlayson	Uranine dye	≈ 0.02**	Large atrium	Model dispersal	Investigate scale modeling; CFD comparison
	Thatcher (2002)	Olive oil/isopropanol	0.5 – 10	Large atrium	Particle deposition, effect of room furnishings and air velocity	n/a
	Fischer[41]	Methane	0.000399	Large atrium	Develop a LIDAR/CT system for mapping tracer dispersal	n/a
Biological	Garza	*Bacillus amyloliquefaciens*	0 – 10; ≤150	Open-air	Model outdoor dispersal from release outside the Pentagon	Evaluate efficacy of sampling equipment and techniques
	Wong	*Staphylococcus aureus* subsp. *aureus*, *Micrococcus luteus*	1	BSC chamber	Model dispersal in indoor environments	n/a
	Kournikakis (2009)	*Bacillus atrophaeus*	2.5 – 10†	Office building	Dispersal from an anthrax letter	Effect of responses by letter opener
	Duncan[42]	*Bacillus atrophaeus*	0.75 – 9	Office building?	Model the number of viable spores in a bioaerosol particle	n/a
	Hathway (2007)	*Serratia marcescens*	0.5 – 2‡	Hospital room	Model dispersal from an activity-created zonal source	Comparison to CFD results
	Agranovski	*Bacillus thuringiensis*	0.5 – 10	Office building?	Dispersal from an anthrax letter	Effect of responses by letter opener
	Kournikakis (2001)	*Bacillus atrophaeus*	2.5 – 10†	Mailroom	Dispersal from an anthrax letter	n/a
	Ganio	*Bacillus atrophaeus*	2.5†– 15	Open-air	Model outdoor dispersal in a non-urban area	Comparison to computerized dispersal model

Selected for further analysis. Excluded (Reason). * Based on Messelson, *et al.*[43] ** Based on scaled molecular diffusivity. † Based on Kournikakis, *et al.*[44] ‡ Based on Bergey's.[45] (References are provided in the table only for excluded literature. Included literature references can be found later in this chapter.)

[38] A Hathway, C Noakes, and A Sleigh, "CFD modelling of a hospital ward: Assessing risk from bacteria produced from respiratory and activity sources" (paper presented at the Indoor Air 2008, Copenhagen, Denmark, 17-22 August 2008).

[39] MD Sohn *et al.*, "Responding to sudden pollutant releases in office buildings: 1. Framework and analysis tools," *Indoor Air*, 13(3), 2003.

[40] LM Wein, DL Craft, and EH Kaplan, "Emergency response to an anthrax attack," *Proc Natl Acad Sci U S A*, 100(7), 2003.

[41] ML Fischer *et al.*, "Rapid measurements and mapping of tracer gas concentrations in a large indoor space," *Atmospheric Environment*, 35, 2001.

[42] S Duncan and J Ho, "Estimation of Viable Spores in *Bacillus atrophaeus* (BG) Particles of 1 to 9 µm Size Range," *Clean*, 36(7), 2008.

[43] M Meselson *et al.*, "The Sverdlovsk anthrax outbreak of 1979," *Science*, 266(5188), 1994.

[44] B Kournikakis *et al.* "Risk Assessment of Anthrax Threat Letters," Defence Research Establishment Suffield, (Suffield, Alberta, Canada: 2001).

[45] F Grimont and PAD Grimont, "Genus XXXIV. *Serratia* Bizio 1823, 288[AL]," in Bergey's Manual of Systematic Bacteriology, Ed. DJ Brenner, NR Krieg, and JT Staley (New York: Springer, 2005).

In order to focus the literature review on the research most relevant to *Bacillus* spore dispersal, the papers listed in Table 2-1 were evaluated according to two secondary criteria. The first criterion was a primary focus on particulates within the size range generally associated with aerosolized *Bacillus* spore particles. As noted in Chapter 1, individual spores of *B. anthracis* can range in size from approximately 0.5 – 2.5 μm. However, spores, like other small particles, also have a tendency to agglomerate through interparticulate forces such as van der Waal's forces, electrostatic forces, capillary forces, gravitational forces, and mechanical interlocking. Thus, spores can clump together and grow to a size that prevents them from efficiently reaching the alveoli. Instead, a greater fraction of larger particles are deposited in the larger airway passages as a function of their diameter. This relationship is clearly visible in the bottom part of Figure 2-1.[46] Because the particles of greatest concern to biodefense are those which can experience deposition in the alveoli or lower bronchioles, the second criterion applied to the reviewed literature was a primary focus on particulates in the size range of 0.01 – 10 μm, shown highlighted in Figure 2-1 in yellow. The particle sizes outside this range are shown in red in Figure 2-1.[47] As can be seen in Table 2-1, this resulted in the exclusion of three papers: Hathway, *et al*. (2008), Sohn, *et al*., and Fischer, *et al*. The second criterion was a primary focus on spore dispersal, a relevant component of dispersal (e.g., deposition or

[46] Figure adapted from US Departments of the Army, the Navy, and the Air Force, NATO Handbook on the Medical Aspects of NBC Defensive Operations, AMedP-6(B) (Washington, DC1996).

[47] Sohn, *et al*., which provides no particle size for the modeled particulate, is not included in Figure 2-1.

spore tracking), modeling/simulation, or creation of nonbiological simulants with biological properties. Because these papers discuss dispersal only in a manner tangential to the main focus of their research, three papers, shown in orange in Figure 2-1, were excluded from the data set: Sohn, *et al*. (already excluded by the size criterion), Wein, *et al*., and Duncan and Ho.

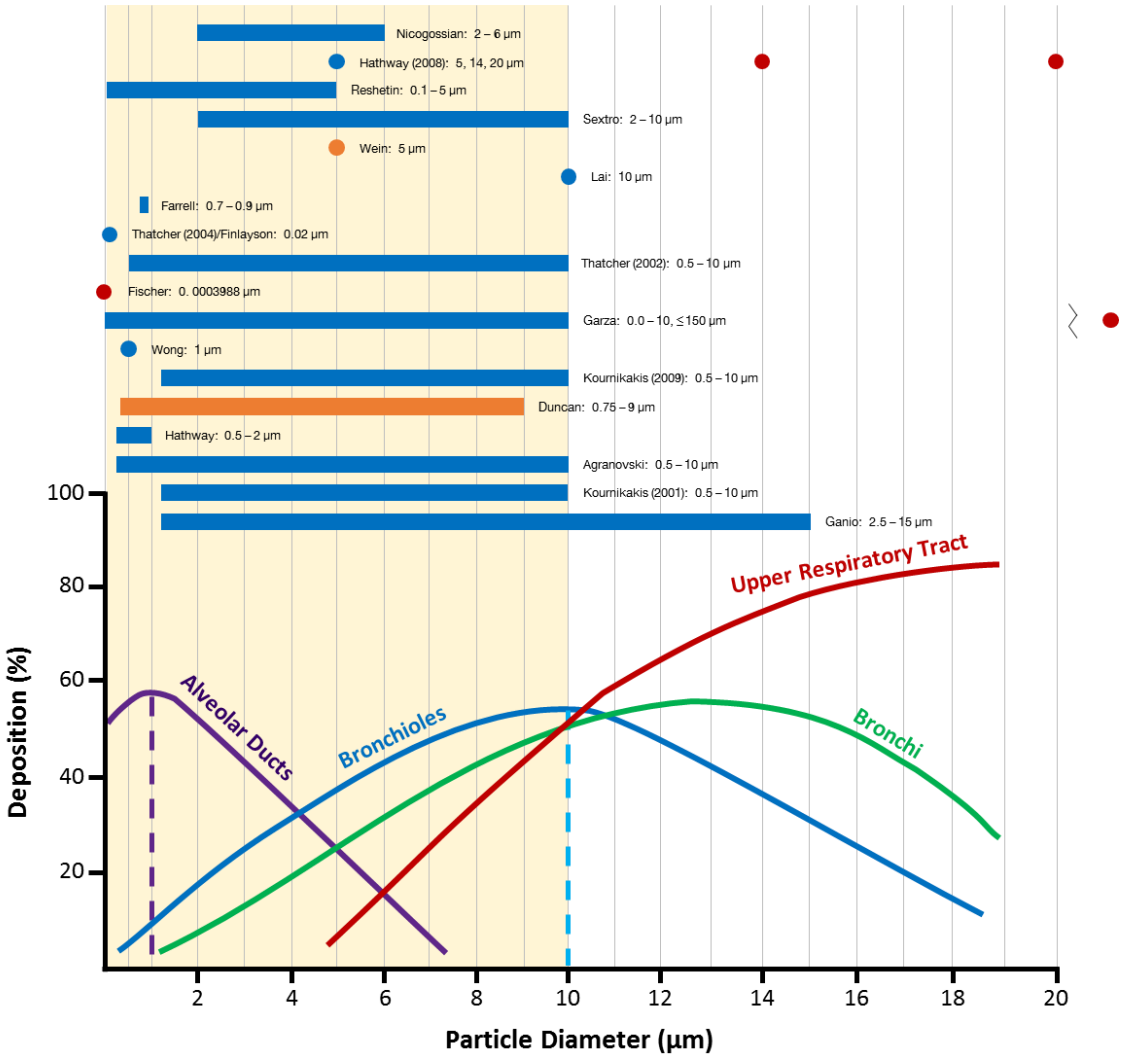

Figure 2-1: Respiratory deposition as a function of particle size and application to selection of literature

The remaining fourteen papers are reviewed in the remainder of this chapter.

2.2. Mathematical Models

One of the techniques used to study bioaerosol dispersal is the development of a mathematical simulation model. Mathematical models vary in scope and complexity from simple particle distribution models to advanced computational fluid dynamics (CFD) models of large buildings with complex air handling systems.

Such mathematical models are advantageous for simulating dispersal of airborne contaminants for many reasons. Foremost, because no system is needed for direct physical testing, a mathematical model can avoid necessitating the construction of intricate, unwieldy, and costly physical environmental models. Instead, quantitative parameters can be used to model relevant factors such as various characteristics of the environment (e.g., airflow, temperature, and humidity), the aerosol (e.g., particle diameter, aerodynamic properties, biological viability, and infectivity), and the dispersal system (e.g., mechanism and rate of dispersal). Additionally, such a model allows relevant data to be generated without the hazard associated with viable biological organisms. Further, because the relevant properties are given the form of quantitative parameters, these variables can be changed repeatedly to examine different scenarios with the model. As summarized by Reshetin and Regens (whose model is discussed in Chapter 2.2.2): “In the case of biological events, [mathematical] simulation has

predominated because of the inherent constraints (i.e., potential for human exposure, experiment cost) on releasing sufficient volumes of materials, even inert simulants, to meet detection criteria for micrometer size particles.[48]"

However, the use of computational models for the simulation of such an inherently dynamic and turbulent system presents numerous difficulties. The most serious drawback to this computational approach to simulation is the need to incorporate the mathematical properties of the entire environment in order to be completely accurate. Even the simplest commercial or residential building model must incorporate airflow, temperature, lighting, humidity, and surface properties; the values for some of these parameters can change during the course of the simulation, further complicating the model. Each element plays a role in determining the behavior and properties, both physical and biological, of a bioaerosol. The outdoor world is significantly more complex: as the environment is expanded from a closed indoor setting, local and regional meteorology, precipitation, pollutants, and other aerosols, must all be considered for accurate modeling. The number of variables quickly becomes computationally overwhelming. As a result, the design of many of these models chooses to focus on relatively simple environments, such empty rooms. Even models that incorporate more complex components of environments, such as furniture or floor carpeting, can examine only specifically chosen elements. Changing any of

[48] VP Reshetin and JL Regens, "Simulation modeling of anthrax spore dispersion in a bioterrorism incident," *Risk Anal*, 23(6), 2003.

these elements requires recalculation of the simulation or, worse, could invalidate the entire model.

A corollary to this serious drawback of mathematical modeling is that, because determination of the properties of all the elements in an environment can rarely, if ever, be achieved, these models rely heavily on assumptions and approximations. By assuming that certain environmental characteristics do not play a role in determining the dispersal of an aerosol, these criteria are ignored in the construction or implementation of a model. One example of such a common assumption is that an environment is isothermal. However, even across small distances, differences in temperature can cause airflow currents that can carry aerosol particles. This process is known as thermophoresis, and it is well-known to be an important factor in mass transfer in many systems. Thus, the existence of an isothermal environment is in most cases not a valid assumption. Occasionally the designers of such models will examine a parameter and determine that it has a negligible impact on the results of the computation; they then make an acceptable choice to simplify the model by disregarding the variable in their calculations due to its minimal contribution. However, more often, potentially important parameters are ignored, without explicitly described reasoning or regard to their effects on the results, simply for the ease of computation.

A second corollary follows from the first. Because of the difficulty in accurately reducing environmental conditions to predictive numbers and

equations, the estimation of a large number of parameters and the equations that describe their effects on and interactions with aerosol particles results in a computationally cumbersome model that generally has a poor accuracy or predictive value. Because the dispersal of an aerosol is an inherently chaotic and stochastic process, often various iterations of the exact same model with the exact same input parameters will results in drastically different results. (Often, these differing results between individual runs require performing hundreds or thousands of simulation runs in order to force the results into a consensus.) These results may be still different from the actual behavior of an aerosol in such an environment.

The end result is that mathematical models of biological aerosol dispersal tend to be either a) accurate, but severely oversimplified and applicable only to a very specific environment with a minimal set of contributing factors, or b) more realistic, but of very questionable accuracy and versatility. This section reviews and critically analyzes recent literature on mathematical models of aerosol and particle dispersal, highlighting both the benefits and the drawbacks to each of the models described. Although some of these models are not designed specifically for simulation of bioaerosols, many of the principles involved in the mechanics of small particles are also applicable to aerosolized biological agents.

2.2.1. Nicogossian, *et al*., 2011

The work of Nicogossian, *et al*.[49] models the release of weaponized *B. anthracis* spores from within an underground mass-transit rail system and their subsequent dispersal over the National Capital Region (NCR), i.e., Washington, DC, and the surrounding metropolitan areas. The model then evaluates the direct impact of the dispersal on the affected infrastructure.

Methods The work of Nicogossian, *et al*. uses an atmospheric simulation system called the Operational Multiscale Environment Model with Grid Adaptivity (OMEGA) with its embedded Atmospheric Dispersion Model (ADM) to model the dispersal of *B. anthracis* spores released into the atmosphere following a release within the DC Metro subway system. The spore particles in the simulation are 2 – 6 μm in size (representing very small agglomerations consistent with weapons-grade material). During the simulation, up to 10 kg of spores (at a concentration of 10^6 spores/mg) are released simultaneously from multiple locations within the DC Metro system. The transit of the subway trains pushes the spores throughout the tunnels and out of the subway stations via the stairs and escalator openings used to access the underground stations. Because the spore preparation is of such high quality, the aerosol is able to remain suspended essentially indefinitely (within the timeframe considered by the simulation). After the plume is modeled, the result is superimposed onto a Geographic Information System (GIS) map of the NCR, which provides detailed information including "business

[49] A Nicogossian, LA Schintler, and Z Boybeyi, "Modeling Urban Atmospheric Anthrax Spores Dispersion: Assessment of Health Impacts and Policy Implications," *World Medical & Health Policy*, 3(3), 2011.

establishments, demographics by census block group, location and type of medical care facilities, transportation infrastructure (subways, highways, industrial rail, and airports), and schools.[50]" This allows a detailed analysis of the spore dispersal's impact on the NCR to be evaluated.

Results The results of the simulations show that the highest impact scenario would be a release on an early summer morning. During the summer, the prevailing winds in the NCR blow toward the northeast. Using the parameters used in the models, clouds of the released spores would emerge from the Metro stations and coalesce into a large spore plume that would travel northeast from Washington, DC. The model estimates that nearly 175,000 Metro riders would be exposed to the released spores. This represents over 18% of the estimated daily Metro ridership throughout the NCR.[51] After 120 minutes, the plume predicted by the OMEGA model would impact approximately 19.4 square miles. Superimposing the plume onto the NCR GIS shows that less than 5% of the NCR's infrastructure would be directly affected.

Discussion The work of Nicogossian, *et al*. not only uses a CFD-based atmospheric simulation to model the spread of *B. anthracis* spores escaping into the environment after multiple bioterrorist releases within the DC Metro system, it also examines the potential impact of the resulting data in direct relation to the NCR infrastructure by means of GIS superimposition. Not surprisingly, an

[50] Ibid.
[51] American Public Transportation Association, "Public Transit Ridership Report, First Quarter 2011," (2011). Cited in: A Nicogossian, LA Schintler, and Z Boybeyi, "Modeling Urban Atmospheric Anthrax Spores Dispersion: Assessment of Health Impacts and Policy Implications."

underground release of *B. anthracis* spores that results in an airborne plume of spores would expose a great number of people and have significant effects in the NCR.

Nicogossian, *et al*. list numerous critical assumptions used as model inputs in the methods section of the paper. The first is that the "movement and speed of the subway cars... would facilitate the aerosol dissemination[52]" of the spores through the subway tunnels. This is a fairly safe assumption, as this "piston effect" of trains pushing aerosolized spores through tunnels has been demonstrated in studies at least dating back to 1966.[53] Another assumption is that the spores aerosolized within the tunnels will "escape through the ventilation system(s) and surface passenger access areas.[54]" US Army research showed that release of spores outside surface-level subway system ventilation intakes can result in the contamination of station interiors.[55] Because such HVAC systems would inherently contain intakes for fresh air and exhausts for expulsion of indoor air, this effect can likely be assumed to be bidirectional. Likewise, the aforementioned piston effect is capable of forcibly ejecting aerosolized

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[52] A Nicogossian, LA Schintler, and Z Boybeyi, "Modeling Urban Atmospheric Anthrax Spores Dispersion: Assessment of Health Impacts and Policy Implications."
[53] US Army. "Miscellaneous Publication 25: A Study of the Vulnerability of Subway Passengers in New York City to Covert Attack with Biological Agents," US Army Biological Laboratories, (Fort Detrick, Frederick, Maryland: 1968).
[54] A Nicogossian, LA Schintler, and Z Boybeyi, "Modeling Urban Atmospheric Anthrax Spores Dispersion: Assessment of Health Impacts and Policy Implications."
[55] US Army, Miscellaneous Publication 25: A Study of the Vulnerability of Subway Passengers in New York City to Covert Attack with Biological Agents.

contaminants out through ventilation shafts.[56] Additionally, it would be expected that the large openings to the surface that include stairs and escalators for passenger ingress and egress would prove a particularly easy escape for aerosolized spores. Thus, this assumption is most likely valid.

An additional assumption is that "multiple atmospheric releases would act as one point source, subject to the prevailing wind conditions.[57]" This might be a reasonable assumption for releases from Metro stations located close together. However, the furthest apart Metro stations are the Shady Grove station in Maryland (Red Line) and the Franconia-Springfield (Blue/Yellow Lines) station in Virginia, located a straight-line distance of 24.47 miles apart. Releases at these stations likely could not reasonably be considered to be a single source unless the overall scale of dispersal considered was so large as to render the distance between the individual sources negligible, e.g. tracking the resultant dispersal across the entire US East Coast. Based on the origin of the spore plume in Figure 2 of the paper, the assumption in the paper presumably refers to multiple releases in the area of the National Mall in Washington, DC. This area contains the highest density of Metro stations in the NCR: nine different stations are located within a one mile radius of the Washington Monument. These include six

[56] M Dybwad, G Skogan, and JM Blatny, "Temporal variability of the bioaerosol background at a subway station: concentration level, size distribution, and diversity of airborne bacteria," *Appl Environ Microbiol*, 80(1), 2014.
[57] A Nicogossian, LA Schintler, and Z Boybeyi, "Modeling Urban Atmospheric Anthrax Spores Dispersion: Assessment of Health Impacts and Policy Implications."

of the stations with the highest average weekday boardings[58] as well as two transfer stations where three different lines converge (Metro Center and Gallery Place-Chinatown) and one station where four lines converge (L'Enfant Plaza). In 2013, the average number of passengers boarding at these nine stations on each weekday was 168,049.[59] This would make the stations in this area ideal targets for a bioterrorist attack such as that posited in the paper. Therefore, it can be assumed that this particular assumption by Nicogossian, *et al*. was intended to refer to these central stations located close together. Based on the overall scale of the area over which the resultant spore plume is predicted, the assumption that multiple releases at these stations can be treated as a single source can likely be considered valid.

Another assumption made by Nicogossian, *et al*. is that "the number of spores in the aerosol will diminish with distance but will be sufficient enough to produce pulmonary infections at least 100 miles from the source.[60]" Dispersal of aerosolized *B. anthracis* spores over long distances has been previously observed: investigation of the Sverdlovsk *B. anthracis* release shows that some exposed sheep 50 km (31 miles) from the factory suffered lethal effects.[61] The mass of released *B. anthracis* spores in this accident was estimated to be "as

[58] Washington Metropolitan Area Transit Authority, "Metrorail Average Weekday Passenger Boardings," http://www.wmata.com/pdfs/planning/FY12_Historical_Ridership_By_Station.pdf.
[59] Ibid.
[60] A Nicogossian, LA Schintler, and Z Boybeyi, "Modeling Urban Atmospheric Anthrax Spores Dispersion: Assessment of Health Impacts and Policy Implications."
[61] M Meselson *et al*., "The Sverdlovsk anthrax outbreak of 1979."

little as a few milligrams or as much as nearly a gram.[62]" Assuming that one gram of spores released from the Sverdlovsk facility resulted in infectious concentrations 31 miles away, it seems reasonable that the 10 kg release – approximately 10,000× greater – posited in the scenario could extend the range of lethal concentration at least as far as 100 miles.

Overall, the assumptions made by Nicogossian, *et al*. are reasonable. However, the authors state quite clearly in the "Limitations" section of the abstract: "This study has not been validated by actual field test data and as such is hypothetical and subject to a significant bias.[63]" This differs significantly from other studies whose authors are less forthcoming about the limitations and relevance of their studies. Because the model proposed by Nicogossian, *et al*. has not been validated experimentally, there is significant uncertainty inherent in the conclusions reached. Without testing the scenario proposed, it is not clear whether the model is accurate. But dispersing 10 kg of a potentially infectious biological agent across at least 100 miles in the vicinity of Washington, DC, is clearly unrealistic and unwise. However, if the validation of this model could be performed using a biological simulant that has essentially zero chance of establishing undesired colonization, critical information regarding the dispersal of a primary threat agent in a real-world scenario without jeopardizing the safety of the public. Thus, the type of study performed by Nicogossian, *et al*. is the perfect

[62] Ibid.
[63] A Nicogossian, LA Schintler, and Z Boybeyi, "Modeling Urban Atmospheric Anthrax Spores Dispersion: Assessment of Health Impacts and Policy Implications."

candidate for validation by the novel simulant described in the latter half of this dissertation.

2.2.2. Reshetin and Regens, 2003

The work of Reshetin and Regens[64] developed a model to simulate the dispersal of *B. anthracis* spores after an intentional bioterrorist release inside a 50-story building. Reshetin and Regens claim that the model they present can be used as a tool for predicting the amount of time required for the dispersal of spores throughout a high-rise building after an intentional release.

Methods The building in the model consists of 50 floors each with a volume of 2,000 m^3 (total building volume: 100,000 m^3). All the rooms are interconnected by a single HVAC system, which operates such that five air changes occur every 24 hours. In the described model, a bioterrorist releases *B. anthracis* spores in a room located on the first floor of the building. The initial concentration is 1.6×10^8 spores/m^3, and a total of 3.2×10^{12} spores are released. The HVAC system propagates the spores and carries the contamination throughout the building.

As with the other mathematical models, the work of Reshetin and Regens is replete with abundant assumptions used "to minimize computational time.[65]" These assumptions include the following:

> "(1) particles are uniformly distributed over the control volume (building interior), with the exception of the boundary layer near the

[64] VP Reshetin and JL Regens, "Simulation modeling of anthrax spore dispersion in a bioterrorism incident."
[65] Ibid.

> walls; (2) no differences in particle composition exist among particles of the same size group; (3) particle characteristics are a function of particle size and particle density, which can vary in accordance with composition; (4) transfer coefficients (as well as form factor, boundary layers, etc.) do not depend on particle size; (5) internal mixing among particles of the same size group occurs due to gravitational and Brownian (thermal) coagulation; and (6) space uniformity in the control volume occurs due to atmospheric convection.[66]"

Unlike in many other models, however, most of the assumptions listed seem fairly realistic and justified. Particularly, as in other models, the authors choose to neglect thermophoresis for computational simplicity. However, unlike in other papers, Reshetin and Regens validate this assumption. The paper presents a proof showing mathematically that the rate of sedimentation from thermophoresis at the conditions of the simulation is negligible at the particle size ranges considered ($r \approx 0.1$ to 5 μm). Thus, the authors explicitly show that it is reasonable to simplify the model by eliminating this factor from consideration.

Results The work of Reshetin and Regens presents results for numerous aspects of the scenario constructed, including the particle size at which gravitational sedimentation becomes the predominant process driving the removal of airborne spores ($r \approx 0.3$ μm), the time required for removal (i.e., deposition or sedimentation) of airborne spores from a room of a specified size, the mean dose of spores inhaled on each floor, the total number of people

[66] Ibid.

exposed, the total number of exposed people infected, and an overall numerical model of the dispersal of *B. anthracis* spores throughout the simulated building.

By evaluating the distribution of particle sizes as a set of monodisperse fractions, a system of differential equations can be established:

Equation 2-1: Numerical model of *B. anthracis* spore dispersal

$$\frac{\partial c(r,t)}{\partial t} = S(r,t) - Q(r,t) - \left[\alpha_D(r) + \alpha_S(r)\right]c(r,t) + \int_0^r K\left(\left(r^3 - r'^3\right)^{1/3}, r'\right)c\left(\left(r^3 - r'^3\right)^{1/3}, t\right)c\left(r', t\right) \times \frac{r^2}{\left(r^3 - r'^3\right)^{2/3}}\,dr' - c(r,t)\int_0^\infty K\left(r, r'\right)c\left(r', t\right)dr' ,$$

where

Equation 2-2: α_D (for Equation 2-1)

$$\alpha_D = kT \cdot B(r) \cdot \frac{A_D}{\delta_D V} ,$$

Equation 2-3: α_S (for Equation 2-1)

$$\alpha_S = \frac{\frac{4}{3}\,\pi\rho r^3 g B(r) A_H}{V} ,$$

$S(r,t)$ is the source of the spores, $Q(r,t)$ is the sink for the spores, and "$c(r)$ is the concentration of suspended particles with radius from r to $r + dr$.[67]"

One of the advantages of the work of Reshetin and Regens over others in this section is that this work provides an estimate of the number of people infected by the intentional dispersal of *B. anthracis* spores:

Equation 2-4: Number of humans infected with anthrax

$$K \sim \sum_i K_i \left(1 - \frac{B(\alpha, \beta + i)}{B(\alpha,)} \right),$$

where K_i is the number of building occupants who have inhaled i spores and $B(\alpha,\beta)$ is the beta function.

Additionally, an equation for estimating the maximum spore dose inhaled by an exposed person during a specified time interval, τ, is provided:

Equation 2-5: Maximum spore dose inhaled by an exposed person

$$d_{max} = \frac{\gamma V_t \tau \tilde{Q}}{\alpha_\Sigma + \xi},$$

where γ is the coefficient of absorption, V_t is the volume of air inhaled into a person's lungs per unit of time, τ is the duration of exposure to the spores, "$\tilde{Q}$ is

[67] Ibid.

the power of the spore source (the number of new spores entering a given floor per second and per cubic meter), $(\alpha_{\Sigma})^{-1}$ is the typical sedimentation time defined by gravitational and Brownian sedimentation, and ξ^{-1} is the typical removal time due to active ventilation, which depends on the number of times for complete renewal of air through the ventilation system (normally 5 – 6 times per day) and the fraction of fresh air in each cycle (as a rule, 20 – 25%).[68]" Together, Equation 2-4 and Equation 2-5 provide an ability to assess the conditional relationship between dose and response for a hypothetical scenario such as the described intentional dispersal of *B. anthracis* spores within a high-rise office building.

Based on Equation 2-1 through Equation 2-5, the Reshetin and Regens model is able to describe the dispersal of *B. anthracis* spores released on the first floor throughout the building's fifty floors and its associated HVAC system. The paper presents a figure illustrating the concentration of spores as a function of time for both the first and fiftieth floors. The general patterns of the results are as would be expected. The concentration on the first floor begins at the highest level at the 0 hour timepoint when the spores are released (presumably instantaneously within the model) and decays as the spores undergo deposition, sedimentation, or transport to other floors. The graph of spore concentration on the fiftieth floor equals zero at 0 hours, increases as airborne spores are transported to the floor by the building's HVAC system, and decays as the spores undergo deposition, sedimentation, or transport to other floors. Although not

[68] Ibid.

described by the paper, the concentration graphs for the second through forty-ninth floors would be expected to exhibit the same general shape as that of the fiftieth floor, albeit with floor-specific concentrations and timepoints. The difference between the concentrations of spores on various floors can differ by greater than a factor of 1,000, with the highest concentration being observed on the floor of release (assuming a single-floor release).

Altogether, the model presented by Reshetin and Regens illustrates that even a minute quantity of *B. anthracis* spores (2.24 g) is capable of dispersal throughout a large multi-story building at concentrations high enough to cause infection in a significant fraction of the exposed population.

Discussion Unlike many other mathematical models, the work of Reshetin and Regens incorporates many important characteristics instead of ignoring them for convenience. Additionally, this work includes predictive calculations of total numbers exposed and infected, which are ultimately the most critical pieces of information gleaned from the analysis of such an attack scenario.

Although the model presented by Reshetin and Regens is generally good, it does have some flaws that render it questionable. In particular, one of the questionable input parameters is the frequency of air exchanges in the model. The parameter value stipulates that the ventilation system causes the air exchange on each floor to be renewed five times within each 24-hour period. In reality, air exchange frequencies for typical office buildings in the United States

are typically measured in full air changes *per hour* (ACH), rather than per day. Depending on the size of the office and its level of occupancy, a typical requirement is 4 – 10 ACH[69], approximately 20 – 48 times more frequently than the five air changes per 24 hours in the model. For example, the work of Hathway, *et al*.[70] (described in Chapter 2.4.4) uses airflow parameters that correspond to a mechanical ventilation rate of approximately 6 ACH. Thus, it is possible that five air changes per 24 hours parameter value in the Reshetin and Regens model may have been selected primarily for computational simplicity.

Secondly, the assumption of uniform particle distribution is unrealistic to say the least. The distribution of spores throughout the building would likely eventually approach a uniform distribution over a long period of time. However, the assumption that spores are instantaneously uniformly dispersed throughout the building interior is highly unrealistic.

Individually, many of the assumptions posited by Reshetin and Regens seem reasonable and, most importantly, justified within their paper. However, the most serious concern with respect to the significant number of assumptions in their paper is the propagation of uncertainty. Some of the assumptions and simplifications presented are said to be correct to within an order of magnitude (i.e., the actual value is 1/10 to 10 times the calculated value). Individually, an order of magnitude is a significant, but not unreasonable, uncertainty. However,

[69] American Society of Heating, Refrigerating, and Air-Conditioning Engineers (ASHRAE), "Ventilation for Acceptable Indoor Air Quality," (Atlanta, GA: ASHRAE, Inc., 2004).
[70] EA Hathway, A Sleigh, and CJ Noakes, "CFD Modelling of Transient Pathogen Release in Indoor Environments due to Human Activity" (paper presented at the Roomvent 2007).

the propagation of such large uncertainties can compound into dramatically greater uncertainty. For example, multiplication of two assumptions each with an uncertainty of an order of magnitude results in a compound uncertainty of two orders of magnitude, i.e., an actual value between 1/100 to 100 times the calculated value. Considering the liberal use of assumptions in the construction of the Reshetin and Regens model, the overall uncertainty of the model could be as high as several orders of magnitude, which would essentially invalidate its usefulness.

In beginning the section addressing the validation of their mathematical model, the authors make the following amusing quip: "While it is obviously neither desirable nor feasible from both a public health and economic standpoint to verify our modeling results by conducting a large-scale field experiment in a 50-story building given the possibility of residual contamination, the question of validation is nonetheless important.[71]" The point they raise is quite valid. While enacting the modeled scenario in the real world by releasing viable spores throughout the ventilation system of a 50-story building would certainly provide a basis of comparison for the otherwise hypothetical results of the mathematical model, the potential of unintended infections from the organisms released presents an unacceptable risk that prevents such a study from being undertaken. However, if such a study could be performed with a viable organism which possessed no risk of unintended growth, the main barrier to performing the

[71] VP Reshetin and JL Regens, "Simulation modeling of anthrax spore dispersion in a bioterrorism incident."

validation to which Reshetin and Regens allude would be removed. The design of such a system is the ultimate goal of this dissertation and will be addressed in greater detail in future chapters.

2.2.3. Sextro, *et al*., 2002

Similar in concept to the Reshetin and Regens model, Sextro, *et al*.[72] seeks to model the spread of *B. anthracis* throughout a building. Although the building model in this case is much smaller and less complex, the model also incorporates the effect of certain human activities such as tracking on spore dispersal.

Methods The model uses a simple multi-zone system to examine the dispersal of *B. anthracis* spores after a release from an envelope in one room of an office building with eight 80 m^3 rooms. Six hypothetical people are assigned locations and activity patterns. After an envelope containing 1 g of *B. anthracis* spores is opened in Room 4, 0.375 g deposits on the floor, and 0.125 g becomes airborne. The dispersal of spores throughout the building and the levels of exposure for the six people are tracked over a 48 hour period. Unfortunately, no actual equations are presented in the paper. Although Table 1 of the paper includes some of the aerosol parameters used, the authors provide no opportunity to evaluate the construction or validity of their model.

Results The results show that after the envelope is opened in Room 4, the concentration of airborne spores decreases rapidly; this occurs by a

[72] RG Sextro *et al.*, "Modelling the Spread of Anthrax in Buildings" (paper presented at the Proceedings of the Indoor Air 2002 Conference, Monterey, CA).

combination of air exchange to the other rooms in the office building, exhaust of indoor air to the outdoors, and deposition onto the floor of the room. As air flows from Room 4 into the other rooms, the spore concentration within those rooms increases. As the people move throughout the office, they increase the concentration of spores on the tracked surfaces. Using the people's locations throughout the 48-hour simulation and the concentration of airborne spores in the rooms at the time, the dosage of *B. anthracis* spores to which the people were exposed is estimated. Not surprisingly, the person occupying Room 4 received the highest dosage, while a person who enters the building two hours after the release and spends a limited amount of time being exposed receives a lower dosage. In the first 24 hours, all the people in the offices at the time of release were exposed to between 10 and 1,000 times the LD_{50} of *B. anthracis*.

Discussion The model presented by Sextro, *et al*. makes some unrealistic assumptions and neglects many important factors in favor of a simplistic model. One such assumption is that, upon opening the envelope, the mass of spores that falls on the floor "deposits **uniformly** on the floor surface.[73]" [emphasis in original] Similarly, the remaining fraction of spores "disperses immediately and uniformly into the room air.[74]" Neither of these events would occur instantly, and certainly not uniformly. The greatest concentration of spores would occur in the area immediately surrounding the location in which the envelope was opened. Because the current model differentiates between

[73] Ibid.
[74] Ibid.

tracked and untracked spaces within the rooms, the number of spores tracked from Room 4 into the remainder of the building would rely heavily on the specific location where the envelope was opened. If this occurred in an untracked area, fewer spores would be tracked, and the model overestimates the level of exposure. If the deposition from the letter occurs in a heavily tracked area, then more spores would be tracked and the people in the simulation would actually have dramatically higher exposure levels than predicted by the model. The authors themselves state that "[a]lthough tracking and resuspension account for only a small amount of mass transfer, the model results suggests they can have an important effect on subsequent exposures.[75]" Thus, the distribution of spores relative to the heavily and lightly tracked areas is admittedly more critical than is accounted for in the model. By using a uniform spore distribution across the entire room, Sextro, *et al*. simplifies the simulation but skews the results obtained from their model, with potentially lethal implications.

Of the three "other key assumptions" listed by the study authors, the first and second seem unlikely. (The third is that spore uptake onto shoes is equivalent to the deposition from spores falling off shoes. The authors state that these are "strictly estimates.[76]" There appears to be no literature regarding this specific topic, and since their guess is as good as any, there is no basis to question this assumption.) The first assumption is that "spores deposited on 'untracked' surfaces, in the ducts or on the HVAC filter remain in those

[75] Ibid.
[76] Ibid.

locations.[77]" The assumption that spores become trapped in HVAC filters is reasonable, given that that is precisely the design of such filters. The assumption that spores that settle on surfaces that are untracked do not become resuspended is also mostly reasonable, given that in the absence of human-induced tracking, the low-velocity airflow inside a typical office may not have enough energy to lift settled spores. However, spores deposited in active air ducts could easily be resuspended by the turbulent airflow. The typical velocity of airflow within HVAC air ducts is likely high enough to easily lift tiny spores off the inside surfaces of the ductwork. This would allow the previously deposited spores to reenter the in-duct airstream and travel out of the HVAC system back into the office, providing an additional source of spores that is not accounted for in the model.

The second assumption, "spores deposited on the floor do not change physically, that is, spores deposited with a given aerodynamic diameter maintain that diameter for purposes of resuspension,[78]" is not realistic. For the purposes of modeling aerodynamic properties such as dispersal and resuspension, a well-validated assumption is that agglomerations of particles behave aerodynamically as though they are a single object of the total size.[79,80] However, after such

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[77] Ibid.
[78] Ibid.
[79] W Stöber, "A Note on the Aerodynamic Diameter and the Mobility of Non-Spherical Aerosol Particles," *J Aerosol Sci*, 2, 1971.
[80] For example, a group of 1 µm spores that have clumped together into a particle with a total diameter of 5 µm is subject to physical forces (aerodynamic, gravitational, etc.) equivalent to those experienced by a single object of equivalent density with a diameter of 5 µm. Therefore, the clump of spores is said to have an aerodynamic diameter of 5 µm.

agglomerations have been subjected to the forces involved in deposition, such as impact with the ground, a large, loosely held-together clump of multiple spores that deposited onto a surface would likely crumble into smaller clumps and/or individual spores. This would result, for the purposes of resuspension, in a single, heavier clump with a larger aerodynamic diameter turning into numerous, lighter spores with smaller aerodynamic diameters; each of these would be easier to resuspend than would the original agglomeration. While incorporating such occurrences would provide the model with a greater level of realism and accuracy, it would require incorporation of the cohesive forces between a great number of microscopic particles of varying sizes and shapes, making the authors' model far more complex. No doubt this was the basis for neglecting the separation of such agglomerated spores in favor of a less realistic model.

Overall, the model presented by Sextro, *et al*. is an overly simplistic and unrealistic simulation. Because of the unrealistic assumptions made, the results could be wildly inaccurate, either underestimating or overestimating the levels of exposure. Furthermore, the results provide little information that could not have been gained from a simple intuitive examination of the scenario.

2.2.4. Lai and Nazaroff, 2000

The work of Lai and Nazaroff[81] was designed to yield a complex yet usably practical model capable of describing particle deposition from a system of turbulent airflow onto an indoor smooth vertical surface. The proposed model

[81] ACK Lai and WW Nazaroff, "Modeling Indoor Particle Deposition from Turbulent Flow onto Smooth Surfaces," *J Aerosol Sci*, 31(4), 2000.

attempts to reduce the complex process of particle deposition to a single independent parameter, the friction velocity.

Methods The vertical wall onto which the simulated particle deposition is occurring is "isolated, smooth, isothermal, and electrically neutral...[82]" The flux of particles is assumed to be steady, occurs in a single dimension, and is comprised of particles of a single diameter (10 μm). Turbulent and Brownian diffusion are assumed to be the primary drivers of particle transport and deposition to the vertical wall. A gradient in the concentration of particles exists only near the surface of the wall and not in the remainder of the room, which is assumed to be well-mixed. Once deposition occurs, particles are assumed to remain deposited, with no resuspension or rebounding.

Lai and Nazaroff present a model based on the following equation (in expanded form):

Equation 2-6: First-order loss coefficient for deposition

$$\beta = \frac{\left(\frac{u^*}{\left[3.64\left(\frac{\nu}{D}\right)^{2/3}(a-b)+39\right]}\right)A_v + \left(\frac{v_s}{1-\exp\left(-\frac{v_s\left[3.64\left(\frac{\nu}{D}\right)^{2/3}(a-b)+39\right]}{u^*}\right)}\right)A_u + \left(\frac{v_s}{\exp\left(\frac{v_s\left[3.64\left(\frac{\nu}{D}\right)^{2/3}(a-b)+39\right]}{u^*}\right)-1}\right)A_d}{V},$$

where

[82] Ibid.

Equation 2-7: Integral *a* (for Equation 2-6)

$$a = \frac{1}{2}\ln\left[\frac{\left(10.92\left(\frac{v}{D}\right)^{-1/3} + 4.3\right)^3}{\left(\frac{v}{D}\right)^{-1} + 0.0609}\right] + \sqrt{3}\tan^{-1}\left[\frac{8.6 - 10.92\left(\frac{v}{D}\right)^{-1/3}}{\sqrt{3}\left(10.92\left(\frac{v}{D}\right)^{-1/3}\right)}\right],$$

Equation 2-8: Integral *b* (for Equation 2-6)

$$b = \frac{1}{2}\ln\left[\frac{\left(10.92\left(\frac{v}{D}\right)^{-1/3} + \frac{d_p u^*}{2v}\right)^3}{\left(\frac{v}{D}\right)^{-1} + 7.669\times10^{-4}\left(\frac{d_p u^*}{2v}\right)^3}\right] + \sqrt{3}\tan^{-1}\left[\frac{2\left(\frac{d_p u^*}{2v}\right) - 10.92\left(\frac{v}{D}\right)^{-1/3}}{\sqrt{3}\left(10.92\left(\frac{v}{D}\right)^{-1/3}\right)}\right],$$

"*v* is the kinematic viscosity of air and *D* is the Brownian diffusivity of the particle... d_p is particle diameter; u^* is friction velocity; v_s = gravitational settling velocity of particle; A_v = area of vertical surfaces; A_u = area of upward-facing surfaces; A_d = area of downward-facing surfaces; [and] *V* = room volume.[83]"

Results Lai and Nazaroff apply their model to the description of particle deposition in typical office room with turbulent airflow supplied by a mechanical HVAC system. Their results plot the deposition velocity on vertical, downward-facing, and upward-facing surfaces as a function of particle diameter at three different friction velocity values. The composite particle loss rate, *β*, takes into account the deposition onto all three orientations of surfaces and is given by

[83] Ibid.

Equation 2-6 through Equation 2-8. The general patterns of the results are comparable to the previous research cited.[84]

Discussion The authors begin the construction of their model by presenting a long list of assumptions that are not validated and may be unrealistic. Inertial drift and electrostatic drift are neglected without explanation. The latter property is particularly non-negligible with respect to *Bacillus* spores: one well-known method of weaponizing spores of *Bacillus anthracis* is to impart upon them an electrostatic charge. The assumptions that both the particles and the surfaces upon which they deposit are electrically neutral ignores an important characteristic that could have been intentionally altered to maximize infectivity. Additionally, the authors state that a recent study has modified previously used equations by the addition of a term to account for the effects of turbophoresis, a phenomenon in which particles migrate from areas of higher turbulence to those with lower turbulence. Comparison of the modified equation with previous experimental data validated the cited paper's use of the modification. The currently reviewed paper's authors then proceed to state that their model will neglect to account for turbophoresis, offering only an unsubstantiated handwaving dismissal. No proof is presented validating their claim that the modification for turbophoresis can be disregarded. Although their contention that the particle sizes and air velocities considered render turbophoresis negligible

[84] J Corner and ED Pendlebury, "The Coagulation and Deposition of a Stirred Aerosol," *Proceedings of the Physical Society. Section B*, 64(8), 1951; JG Crump and JH Seinfeld, "Turbulent deposition and gravitational sedimentation of an aerosol in a vessel of arbitrary shape," *Journal of Aerosol Science*, 12(5), 1981.

may be correct, the lack of explicit justification leaves the reader to wonder whether this assumption is valid for the situation being modeled.

Graphing the turbulent viscosity:molecular viscosity ratio (ν_t/ν) as a function of y^+ requires three different equations rather than a single equation that is continuous across the distances considered. The forces in nature which govern the motion and deposition of particles are, of course, continuous across all distances (both microscopic and macroscopic). The fact that the authors were unable to reduce their model to a single continuous function indicates that the equations they have used to describe the deposition are to some degree incorrect.

Obviously, using equations as complicated as Equation 2-6 through Equation 2-8 is quite unwieldy. Worst of all, the only reason the model equations are in their current "simple" forms is because of all the assumptions made and factors neglected. The authors specifically admit that "the assumptions of smooth surfaces may not be fully met experimentally. Any irregularities in surface geometry, such as surface roughness, would tend to increase deposition… For some situations, it may be important to include other transport mechanisms, such as thermophoresis..., electrostatic drift..., and turbophoresis...[85]" Even the single independent parameter on which their model is built, the friction velocity, is not applicable in all situations: "it is only meaningful when the flow has a prevailing direction... for some situations, a prevailing flow

[85] ACK Lai and WW Nazaroff, "Modeling Indoor Particle Deposition from Turbulent Flow onto Smooth Surfaces."

direction may be weak or nonexistent. For such cases, another normalization parameter may be necessary to describe the air flow conditions.[86]"

The authors conclude their paper with a final paragraph that makes it clear the model as presented has little practical application without additional work conducted to account for simple everyday characteristics such as surface roughness, temperature fluctuations, or low air velocities. In describing the previous models whose shortcomings were the impetus for their work, Lai and Nazaroff state that the airflow and turbulence assumptions used mean that "[e]xisting formulations of such models… lack a thorough physical foundation.[87]" Unfortunately, the same appears to be true of the model proposed by Lai and Nazaroff.

2.3. Physical Simulant Models

It is clear from Chapter 2.2 that many mathematical models present significant questions regarding their accuracy and realism given their liberal use of assumptions and approximations for the sake of computational simplicity. Without real-world validation of a computational simulation model, it is impossible to verify whether the model is realistic or entirely unrealistic; its predictions remain entirely hypothetical. In order to determine the accuracy level of a given model, it should be directly compared to empirical results gathered from a similarly constructed physical simulation. This section examines studies which

[86] Ibid.
[87] Ibid.

have made such direct comparisons between the predictions of computational models and the experimental results from physical simulations.

2.3.1. Farrell, *et al*., 2005

The work by Farrell, *et al*.[88] introduces "BG Bugbeads" as an artificial model of a *Bacillus atrophaeus* (BG) spore. Proteins were extracted from intact *B. atrophaeus* spores and attached to carboxyl-coated polystyrene beads using carbodiimide coupling. The most important advantage of the BG Bugbead system is that it introduces the capability to evaluate some of the biological properties of the *B. atrophaeus* spore surface into a nonviable simulant system. By attaching spore surface proteins onto the surface of a polystyrene bead approximately the size of the desired biological agent, it creates an artificial spore model that eliminates the hazard typically associated with the use of a naturally-occurring biologically viable simulant.

Methods Proteins from *B. atrophaeus* spores were extracted by two different methods, mechanical grinding via shaking in the presence of glass beads and a chemical decoating process. In order to approximately match the mean size of a *B. atrophaeus* spore (1.1 ± 0.6 μm according to the paper), spherical particles of polystyrene 0.8 ± 0.1 μm in diameter were used as the core of the Bugbeads. The proteins extracted from the *B. atrophaeus* spores were then covalently attached to the surface of the polystyrene spheres via carbodiimide coupling, forming the BG Bugbead. The detectability of the BG

[88] S Farrell, HB Halsall, and WR Heineman, "*Bacillus globigii* bugbeads: a model simulant of a bacterial spore," *Anal Chem*, 77(2), 2005.

Bugbeads was determined by sandwich immunoassay using rabbit anti-BG immunoglobulin G (IgG) antibodies.

Results The protein concentration assay results show that significantly more protein was extracted with the mechanical grinding method. Furthermore, the proteins extracted by the chemical decoating were generally of low molecular weight, whereas those extracted mechanically consisted of a wider range of molecular weights which more likely represent the protein makeup of actual *B. atrophaeus* spores. The BG Bugbeads were detectable by anti-BG IgG sandwich immunoassay, and the detection limit was similar for the BG Bugbeads and previous results for *B. atrophaeus* spores.

Discussion The most significant advantage to the BG Bugbead system developed by Farrell, *et al*. is that it is a physical (as opposed to biological) particle that retains some biological properties while essentially eliminating the hazard posed by using viable microorganisms. Coupling the proteins extracted from *B. atrophaeus* spores to polystyrene beads theoretically gives the BG Bugbead some antigenic characteristics of the original spore surface. However, the same lack of viability that provides an important safety margin to the BG Bugbeads provides limitations on their applications. Because the BG Bugbeads are not alive, this system does not allow testing for reduction in viability, as might be necessary for validation of decontamination treatments or inactivation countermeasures.

Based on the information provided by Farrell, *et al*., the BG Bugbeads would also be expected to have different mass, density, and aerodynamic properties than actual *B. atrophaeus* spores. The dry density of *Bacillus atrophaeus* spores is 1.45 g/cm^3; therefore, an individual spore of the mean volume 0.273 μm^3 has a mass of 328 fg. Because the polystyrene that forms the core of the BG Bugbeads has a density of 1.05 g/cm^3, a bead 0.8 μm in diameter has a mass of 537.6 fg. This makes the simulant beads 64% more massive than the spores simulated, possibly making them more prone to gravitational deposition. However, the aerodynamic diameter (D_a) of *B. atrophaeus* spores is 0.710 μm, while the BG Bugbeads have a D_a of 0.820 μm. Thus, the differences in size and density would not be expected to significantly affect the aerodynamics of BG Bugbead dispersal relative to actual *B. atrophaeus* spores, particularly in small-scale experiments. This suggests that, physically, the BG Bugbeads might serve as a reasonable simulant for *B. atrophaeus* spores. This, however, was not demonstrated in the current paper by Farrell, *et al*.

However, despite the results in the paper showing some level of immunodetection, there is cause to question whether the BG Bugbeads actually provide a reasonable biological simulant for *B. atrophaeus* spores. First, the protein extract is claimed to be from the spore coat, but no evidence is presented indicating that the proteins present are from the spore coat exclusively. Excreted proteins and internal proteins from lysed cells could be present. Without purification of the proteins specific to the spore coat (which was not

demonstrated in the paper), any additional proteins present would also be carbodiimide-coupled to the surface of the BG Bugbead. Furthermore, the coupling of the proteins to the polystyrene bead probably occurs without respect to orientation. Therefore, many of the proteins bound will not be presenting the proper antigenic surface for binding.

Farrell, *et al*. state in the introduction to their paper: "the ideal simulant should have a surface that morphologically and antigenically resembles the surface of a pathogen and be nonhazardous at the same time.[89]" While the BG Bugbeads they describe meet the latter criterion, it is questionable to what degree the former criterion is met. Thus, an aerosol dispersal of BG Bugbeads could have significantly different properties, both physical and biological, than would a dispersal of actual *B. atrophaeus* spores. Even so, the simulant particles' lack of viability likely limits their application to physical dispersal studies only. These drawbacks to the use of artificial spores such as BG Bugbeads would be eliminated by using actual spores, which would of course have identical physical properties and protein profiles (surface and otherwise). The remaining reluctance is the potential for viability in undesired environments. If this remaining obstruction were remedied, the resulting system would be an ideal simulant.

[89] Ibid.

2.3.2. Thatcher, *et al*., 2004 and Finlayson, *et al*., 2004

Unlike the other sections in this chapter which each examine a single paper, this section examines two related papers that were published by the same group (the Indoor Environment Department at Lawrence Berkeley National Laboratory) in the same journal issue. The simulation conducted by Thatcher, *et al*. (2004)[90] physically releases uranine dye (sodium fluorescein) into a water-filled 30:1 scale model of a large atrium to simulate the dispersal of a gaseous pollutant in the full-scale room. The research of Finlayson, *et al*.[91] uses computational fluid dynamics (CFD) to simulate the dispersal of a pollutant into a computer model of the same large atrium. One of the stated specific goals of the latter paper was to compare the CFD model predictions with the results from the physical dispersal model in the former paper. They are, therefore, considered as a single comparative research work for the purposes of this section.

Methods The work of Thatcher, *et al*. (2004) uses laser-induced fluorescence (LIF) to examine the simulated dispersal of a gaseous pollutant from a point source throughout a water tank (simulating a large room with simulated HVAC airflow). In a later stage of the simulations, scale-size obstacles meant to simulate tables and people are added to assess their influence on pollutant dispersal. According to Thatcher, *et al*. (2004), the reason for investigating the use of a scale model is that the collection of high-resolution data

[90] TL Thatcher *et al.*, "Pollutant dispersion in a large indoor space: Part 1 -- Scaled experiments using a water-filled model with occupants and furniture," *Indoor Air*, 14(4), 2004.
[91] EU Finlayson *et al.*, "Pollutant dispersion in a large indoor space. Part 2: Computational fluid dynamics predictions and comparison with a scale model experiment for isothermal flow," *Indoor Air*, 14(4), 2004.

regarding concentration and flow from such a model is far easier to accomplish than a similar collection from a full-scale space. Naturally, the properties of air and water are quite different, so the use of a water-filled scale model to simulate particle dispersal in a room filled with air requires some validation. Unlike many simulations, the work of Thatcher, *et al*. (2004) directly addresses this need and explicitly presents the results their comparative analysis. The parameter most critical to development of an accurate comparative simulation is the Reynolds number (Re), a unitless measure of turbulence that is calculated from the viscosity of the medium and the particle velocity through the medium; turbulent flow results in large Reynolds numbers, while small Reynolds numbers represent laminar flow.[92] The paper provides a comparison of the Reynolds number calculated for the full-scale room filled with air, three water-filled scale models (4:1, 10:1, and 30:1), and two air-filled scale models (10:1, and 30:1). These properties are summarized in Table 2-2. Additionally, the ratio of the Reynolds number for each scale model to the Reynolds number for the full-scale air model was calculated and is provided in Table 2-2 as a percentage; this allows for direct comparison of the turbulence inherent in the systems tested by Thatcher, *et al*. (2004).

[92] AA Lushnikov, "Introduction to Aerosols," in Aerosols – Science and Technology, Ed. IE Agranovski (Weinheim, Gerrmany: WILEY-VCH Verlag GmbH & Co. KGaA, 2010); TL Thatcher *et al.*, "Pollutant dispersion in a large indoor space: Part 1 -- Scaled experiments using a water-filled model with occupants and furniture."

Table 2-2: Physical properties of scale models in Thatcher, *et al*. (2004)

Medium	Full-scale: Model Ratio	Inlet Velocity (m/s)	Reynolds Number	Percent of Full-scale Reynolds Number
Full-scale Air	–	0.44	2861	100%
Water	4:1	1.65	**2683**	**93.8%**
	10:1	0.66	5007	175%
	30:1	0.22	**556**	**19.4%**
Air	10:1	0.66	429	15.0%
	30:1	0.22	48	1.68%

Results The paper's results show that any of the air-filled scale models has a Reynolds number much further away from the full-scale model than do any of the water-filled models. Indeed, this is specifically highlighted in the paper: "Due to the differences between physical properties of water and air, a small water-filled model can match the turbulence characteristics of the full-scale room more closely than an air-filled model operated at the same flow rates.[93]" However, the point the authors fail to address is the basis for their selection of the 30:1 scale water-filled model. As is clearly evident from Table 2-2, the Reynolds number of the 4:1 scale water-filled model (shaded in blue) is much more representative of the full-scale system than is the Reynolds number of the 30:1 scale water-filled model (shaded in red) that was used in the simulation. Presumably, the basis of their selection was the increased ease of constructing a physical model one-thirtieth the size of the full-scale atrium versus a model one-fourth the size. However, because Thatcher, *et al*. (2004) do not provide any

[93] ———, "Pollutant dispersion in a large indoor space: Part 1 -- Scaled experiments using a water-filled model with occupants and furniture."

type of justification for their selection, the reader is left to wonder why the researchers choose to use a model that they demonstrated is a far less accurate representation of the atrium. The 4:1 model would have been a far more realistic and relevant model.

Putting aside the question of the relevance of their 30:1 scale model, the work of Thatcher, *et al*. (2004) shows two important results regarding the collection and interpretation of data from their model. The first is that "[e]ven after the concentration distribution becomes fully established, the stochastic nature of the flow leads to large changes in the instantaneous concentration distribution in the measurement plane over time.[94]" In other words, the dispersal of dye into the model is a dynamic and highly unpredictable process that results in very different patterns of dispersal between many of the individual runs. However, this leads to the second important result of the paper: this variability was compensated for using a time-average approach. By conducting one thousand individual runs of their simulation and averaging the collected data together, they were able to eliminate much of the variability in the results. The paper's results show that such an approach has the net effect of converging the dynamic stochastic results into a consensus result that is far more reproducible. To further demonstrate the consistency introduced by this approach, the researchers compared the averaged results from the initial 1,000 simulation runs to the similarly averaged results from a second set of 1,000 simulation runs

[94] Ibid.

conducted at the same conditions. Their analysis showed an extremely consistent pattern of dispersal between the two data sets.

As with many similar models of particle dispersal, a great number of variables are ignored for the sake of computational ease. According to Thatcher, *et al*. (2004), "In this purely isothermal situation, **we do not consider the important complications that arise** from positive or negative buoyancy in the supply air, in the source gas or aerosol, from heat generation by occupants, or from heating by temperature differences between the room surfaces and the room air.[95]" [emphasis added] The criteria ignored by the model are unrealistically, but necessarily, quite numerous. Although the researchers explicitly explored the difference in Reynolds numbers between the full-scale system and their scale model (their less representative choice notwithstanding), there are likely other parameters of the air-to-water fluid switch which were not compensated for. Thus, the overall accuracy and relevance of the model to the environment simulated is questionable.

The data-averaging required to obtain meaningful results requires running a time- and labor-intensive simulation ≈1,000 times, followed by computationally-intensive analysis of large amounts of data. Even so, the end result is merely an oversimplified approximation of a very specific and artificial scenario. As a result, the applicability of the model to simulating a pollutant dispersal in the desired environment is questionable.

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[95] Ibid.

Another complication is introduced by the use of a simulant within a simulated (e.g., scaled-down) environment. The same change in scale presumably applies to the simulated pollutant as to the 30:1 scale room. Does this imply that the actual pollutant dispersed in the full-size room would be thirty times more massive? The molecular mass of sodium fluorescein ($C_{20}H_{10}Na_2O_5$) is 376.27 g/mol. Extrapolating this from a 30:1-scaled model provides the full-size pollutant with a molecular mass of 11,288.1 g/mol. Such a mass is far larger than most chemical toxins, but too small for most complete protein toxins. For example, the toxic nerve agents sarin, tabun, soman, and VX have molecular masses of 140.09, 162.13, 182.17, and 267.37 g/mol, respectively. On the other hand, the anthrax toxins protective antigen (PA), lethal factor (LF), and edema factor (EF) have molecular masses of approximately 83,000, 90,000, and 89,000 g/mol, respectively.[96] Other bacterial toxins include botulinum and tetanus toxins, which both have molecular masses of approximately 150,000 g/mol. Thus, the realism of the physical properties for this theoretical scaled-up pollutant is questionable at best. This critical issue is conveniently left unaddressed by Thatcher, *et al*. (2004).

The work presented in Finlayson, *et al*. created a computational model of the atrium described in Thatcher, *et al*. (2004). The model is composed of a computational mesh totaling 900,000 nodes, with a core resolution of 0.3 cm and a resolution of 0.06 cm at distances ≤ 0.3 cm from the walls of the simulated

[96] One kilodalton (kDa) is equal to 1 kg/mol (i.e., 1,000 g/mol).

room. Scaled up to the full-size atrium, this corresponds to a core resolution of 10 cm and 2 cm at the walls.

Results The results of the simulation are presented in two different sections, one for the finalized steady-state concentration field and one for the developing concentration field. For the fully-developed scenario, plotting the predicted concentrations against the experimentally measured concentrations results in a slope of 1.1 and an R^2 value of 0.66. The CFD model, however, tended to overpredict the results in high concentration areas and underpredict the results in low concentration areas. Generally, the CFD simulation provides a good predictive model of the water/uranine dye experiments.

Discussion The authors note that an important error was introduced during the creation of the computer model of the room. The internal dimensions of the computational mesh were inadvertently defined to match the external dimensions of the water tank described in Thatcher, *et al*. (2004). An explanation is provided as to why the error is unlikely to affect the desired correlation to the experimental observations. This explanation, while reasonable and probably correct, is not validated and is left only as an assumption: "we assume that this increase will not result in a significantly different flow field... we concluded that this discrepancy is negligible.[97]" The error made in the construction of the computational mesh highlights the inherent difficulties in constructing such a virtual model. An important parameter was input incorrectly, resulting in a

[97] EU Finlayson *et al.*, "Pollutant dispersion in a large indoor space. Part 2: Computational fluid dynamics predictions and comparison with a scale model experiment for isothermal flow."

discrepancy between the mathematical model and the physical environment it was intended to represent. Rather than recreate the mesh at the proper dimensions, the authors presumably found that recreating such a complex model and rerunning the simulations would prove too complicated and/or time-consuming and instead decided to rationalize and explain away the difference, despite a linear dimensional discrepancy of 2.5%. This is intended not as a critique of the authors or the creation or implementation of their computational model as much as it is intended to emphasize the inherent challenges in creating a computational model that is useful, accurate, and widely applicable. If the model of Finlayson, *et al*. is so inflexible as to be incapable of handling a minor 2.5% adjustment, it is extremely likely that it is not applicable to any other minor variations. This would likely include changes in room geometry, pollutant source, pollutant type, etc. Thus, it appears that the model created by Finlayson, *et al*. is, in fact, applicable *only* to the specific environment it was designed to represent (i.e., the water tank of Thatcher, *et al*. (2004)) and not any other scenarios.

2.3.3. Thatcher, *et al*., 2002

The research of Thatcher, *et al*. (2002)[98] investigates the effects of room furnishings and air velocity on the rate of particle deposition to surfaces. These results were compared to a dispersal model that calculates the time-dependent particle concentration using a mass-balance differential equation.

[98] TL Thatcher *et al.*, "Effects of room furnishings and air speed on particle deposition rates indoors," *Atmospheric Environment*, 36(11), 2002.

Methods The work of Thatcher, *et al*. (2002) used an atomizing nozzle to generate a spray of a mixture of 10% olive oil in isopropanol; approximately 7 mL were sprayed within 3 seconds. The isopropanol in the droplets generated by the atomizer is calculated to evaporate in less than one second, leaving behind only aerosolized olive oil droplets of 0.5 – 20 μm in diameter. A room with a volume of 14.2 m^3 was used for the dispersal. The level of furnishing was varied into three different configurations: unfurnished (empty room with a metal floor), carpeted (empty room with carpet), and furnished (carpet, a bookcase, a table, two large chairs, and curtains). The increase in the area of projected surfaces was 34% greater for the furnished room than for the bare room. The air velocity within the room was varied for the different trials using fans operating at different speeds and orientations. The particle properties (e.g., size, shape, density) and the properties of the environment in which the particles are depositing (e.g., temperature, airflow, electrostatics) determine β, the first-order deposition loss rate coefficient. The deposition loss rate coefficient is "the number of particles depositing on the total surface available per unit time...[99]" A mass-balance equation can be used to describe the concentration of aerosolized particles in the room:

[99] L Morawska and T Salthammer, "Fundamentals of Indoor Particles and Settled Dust," in Indoor Environment: Airborne Particles and Settled Dust (Wiley-VCH Verlag GmbH & Co. KGaA, 2003).

Equation 2-9: Concentration of particles inside a room

$$\frac{\mathrm{d}C_{\mathrm{i,dp}}}{\mathrm{d}t} = \lambda_{\mathrm{v}} P_{\mathrm{dp}} C_{\mathrm{o,dp}} - \lambda_{\mathrm{v}} C_{\mathrm{i,dp}} - \beta_{\mathrm{dp}} C_{\mathrm{i,dp}},$$

"where the subscript 'dp' denotes the particle diameter of interest, t is time (h), C_i is the indoor particle concentration (# m^{-3}) at time t; λ_v is the air exchange rate (h^{-1}), P is the fraction of infiltrating particles which penetrate the room shell, C_o is the outdoor concentration at time t (# m^{-3}), and β is the particle deposition loss-rate coefficient (h^{-1}).[100]" With the assumptions that particle infiltration into the room can be neglected and that the values of λ_v and β are constant, the time-dependent solution is:

Equation 2-10: Time-dependent solution to Equation 2-9

$$C_{\mathrm{i,dp}}(t) = C_{\mathrm{i,dp}}(0)\exp\left[-\left(\lambda_{\mathrm{v}} + \beta_{\mathrm{dp}}\right)t\right],$$

"where $C_i(0)$ is the indoor concentration at t = 0.[101]" The value for β can be determined empirically by plotting the experimental data.

Results The experimental data show that after the aerosol spray was generated, the particles quickly (≈3 minutes) become well mixed within the room and begin to undergo deposition in a size-dependent manner. For all particle

[100] TL Thatcher *et al.*, "Effects of room furnishings and air speed on particle deposition rates indoors."
[101] Ibid.

sizes studied, increased deposition loss rate coefficients were calculated as the experimental fan speed (and thus in air velocity within the room) increased. The calculated increase in β was greater (≈2.0×) for larger particle sizes than for submicron particles (≈1.5×). The orientation of the fans had little effect on the deposition rates for submicron particles, and no effect was observed for larger particles. The level of furnishing in the room also had a size-dependent impact on the particles of aerosolized olive oil. Submicron particles showed 2.1× greater deposition to surfaces in the furnished room than in the bare room; particles with a diameter <1.0 μm only showed 1.2× greater deposition. The greater relative effect for submicron particles is attributed to gravitational deposition being the primary source of deposition for larger particles. Because particles in this size range do not effectively deposit onto vertical and downward-facing surfaces, the addition of such surfaces in the form of furniture affects their rate of deposition to a lesser degree. Because diffusional deposition is dominant for particles <1.0 μm, furnishing the room provides additional surface area onto which these smaller particles can effectively deposit. The paper also provides a summary of deposition loss-rate coefficients as a function of particle size gathered from a variety of published experimental studies and predictive models.

Discussion The work of Thatcher, *et al*. (2002) is based on an experimental setup that is more obviously realistic than the Thatcher, *et al*. (2004) experiments in the water-filled room model described in Chapter 2.3.2. Because the 30:1 scale model of the room was also used as the basis for the

CFD model constructed by Finlayson, *et al*., it can only be assumed that this was the primary reason for the adoption of the room model in the 2004 paper. Otherwise, it seems highly illogical that the intuitively more applicable full-size air-filled room would be abandoned for a scaled-down water-filled model. The paper discussed in the current chapter examines the impact of various levels of furnishing (unfurnished, minimally furnished, and fully furnished) on the deposition of aerosolized particles. The authors use a more realistic furnishing setup (in the most complicated scenario tested) than some other studies that have investigated dispersals within furnished rooms.

In the Introduction of the paper, the indoor particle contaminants mentioned include cooking fumes, tobacco smoke, and pet dander. However, the choice of aerosolized simulant appears significantly different than these pollutants. For example, tobacco smoke has a density of 1.12 g/mL.[102] The figure reviewing various models uses a range of specific gravity values from 1.0 – 2.5, stated as representative of the particulate composition of typical indoor environments. Olive oil is composed primarily of a mixture of triglyceride esters (including oleic, linoleic, palmitic, stearic, and α-linolenic acids) and has a specific gravity of approximately 0.920.[103] Thus, the rationale behind the selection of olive oil as the simulant for dispersal is neither immediately clear nor described in the paper.

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[102] PJ Lipowicz, "Determination of cigarette smoke particle density from mass and mobility measurements in a millikan cell," *Journal of Aerosol Science*, 19(5), 1988.

[103] The Olive Oil Source, "Chemical Characteristics," http://www.oliveoilsource.com/page/chemical-characteristics.

The model presented for predicting the indoor concentration of particles (i.e., Equation 2-10) reduces the multitude of factors influencing particle deposition to only three: indoor concentration ($C_i(0)$), air exchange rate (λ_v), and particle deposition loss-rate coefficient (β). While avoiding the computational complication inherent to more detailed models, it is unlikely that such a simplified model will provide a very accurate prediction of aerosol particle concentrations or deposition rates. As the authors themselves state, "It is difficult even in the best of conditions to isolate deposition from the many competing factors that can influence airborne particle concentrations.[104]"

In plotting the results of their experiments together with the results gathered from the various literature sources, it becomes quite apparent that there is significant discrepancy between the different models. Although the general trend of β as a function of particle sizes is somewhat similar for each model, comparing the data between models for a given particle size shows a degree of variability in the loss-rate between models as high as ≈100× for some sizes. Further, the two sets of parameters (based on expected values for common indoor environments) used for calculation via Lai and Nazaroff[105] result in calculated bounds that include only 43.8% of the cumulative data points. Only two data sets (including the minimum calculated β value from the current paper) fall entirely within the bounds calculated using the method of Lai and Nazaroff.

[104] TL Thatcher *et al.*, "Effects of room furnishings and air speed on particle deposition rates indoors."
[105] ACK Lai and WW Nazaroff, "Modeling Indoor Particle Deposition from Turbulent Flow onto Smooth Surfaces."

The model of Lai and Nazaroff underpredicts the experimentally-determined deposition loss-rate coefficients significantly, with an error of an order of magnitude for many of the data points. The frequency and magnitude of the discrepancy is greatest at particle sizes <0.5 μm. This provides further evidence that the mathematical model of Lai and Nazaroff does not accurately predict the deposition loss-rate coefficient of particles.

2.4. Biological Simulant Models

The mathematical and computational models discussed in Chapter 2.2 all have significant flaws inherent to this type of simulation. First, the necessary reliance on assumptions results in significant uncertainty. Second, the profligate use of assumptions means that the simulations may bear little similarity to the environment or scenario being simulated. Third, the assumptions and specific parameter values selected often render the model valid only within the specifically constructed artificial scenario. The models are often not generally applicable to scenarios beyond the preselected artificial constraints.

The next best method for simulation is the actual dispersal of a physical simulant, such as those discussed in Chapter 2.3. Such simulations allow many of the problems with mathematical models to be avoided. By actually creating an aerosol (or simulated aerosol, e.g., within a water chamber) and tracking its dispersal, a model can be created based on the experimental observations or a predictive model can be tested and adjusted to more accurately predict the observed dispersal characteristics. However, the physical models have their own

disadvantages, the most critical of which is how accurately the chosen physical simulant simulates that which is being simulated (known as the fidelity of the simulant). For example, models which use methane (e.g., Fischer, *et al*.[106]) as a simulant are likely better suited to simulating dispersals of toxic chemicals than bioaerosols composed of microorganisms such as bacteria.

Thus, it would seem intuitively obvious that the most accurate simulant for microorganisms would be microorganisms. One of the primary advantages to using a biological simulant is that the uncertainty of whether the simulant appropriately simulates that which is being simulated can be reduced significantly, even almost entirely.[107] An additional advantage to the direct testing of viable bacteria is that it allows the use of bacterial growth as the parameter of detection. Thus, the experiments could be designed to investigate the effects of filtration or viability countermeasures (e.g., UV or gamma irradiation) in reducing the numbers of detected viable bioaerosol particles.

However, the most significant and concerning disadvantage is that the aerosol dispersal of viable microorganisms inherently carries the risk of potential pathogenesis. When considering the simulation of pathogenic *Bacillus anthracis* specifically, the most accurate ostensibly safe simulant would be a nonvirulent strain of *B. anthracis*, such as the Sterne strain. However, despite such strains

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[106] ML Fischer *et al.*, "Rapid measurements and mapping of tracer gas concentrations in a large indoor space."

[107] Naturally, this assumes an appropriately selected simulant from the wide variety of microorganisms. For example, in simulating the dispersal of *B. anthracis* spores, selecting similarly-sized spores of another Gram-positive bacterium would result in greater simulant accuracy than would selecting the Gram-negative *S. marcescens* or a bacteriophage.

being nonvirulent, using such a strain to analyze *B. anthracis* bioaerosol dispersal remains risky. One reason is because such strains run the risk of acquiring plasmids that could confer virulence through horizontal gene transfer. The risk that a dispersed nonvirulent strain could become pathogenic is simply too great. Thus, a similar organism, such as *Bacillus atrophaeus*, must be used as a simulant instead. Although the spores of *B. atrophaeus* differ slightly from those of *B. anthracis*[108], the slight discrepancy between the two is vastly outweighed by the increased safety in using the former over any strain of the latter.

This section reviews and critically analyzes recent literature on biological simulants used to model aerosol and particle dispersal, highlighting both the benefits and the drawbacks to each of the models described.

2.4.1. Garza, *et al*., 2014

In both 2005 and 2009, the Pentagon Force Protection Agency (PFPA) conducted open-air releases of a biological simulant outside the Pentagon in Arlington, Virginia. The results of this study have only recently (April 2014) been published in the open literature.[109] The work published by Garza, *et al*. examined the dispersal and post-release detectability of an aerosolized *Bacillus anthracis* simulant in an urban outdoor setting.

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[108] M Carrera *et al.*, "Difference between the spore sizes of *Bacillus anthracis* and other *Bacillus* species."

[109] AG Garza *et al.*, "Detection of the Urban Release of a *Bacillus anthracis* Simulant by Air Sampling," *Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science*, 12(2), 2014.

Methods The material dispersed in both tests was a commercial organic pesticide with *Bacillus amyloliquefaciens* as its active ingredient. *B. amyloliquefaciens* is very closely related to both *B. subtilis* and *B. atrophaeus*.[110] In the 2005 experiment, the particles that comprised the simulant powder had diameters of up to 150 μm, while the majority of the particle sizes during the 2009 dispersal were between 0 – 10 μm. The simulant material was dispersed from a truck-mounted blower. In 2005, the truck was driven along South Washington Blvd., which runs roughly north-south along the west side of the Pentagon. In 2009, the truck was driven along North Rotary Road, which runs roughly east-west along the south side of the Pentagon. Aerosol sampling was performed using high-volume air samplers operating at 100 L/min. To preferentially collect particles in the 0 – 10 μm range, the samplers were outfitted with a size-selective inlet that excluded >50% of particles with a diameter greater than 10 μm. Environmental sampling was also conducted by collection of swipe, soil, and water samples. A set of five real-time PCR assays was used to determine the presence of dispersed *B. amyloliquefaciens* in the collected samples.

The dispersal of the simulant was modeled using the Quick Urban and Industrial Complex (QUIC) system, which calculates three-dimensional airflow around buildings. This allows the high-resolution modeling of particulate dispersal in complex urban settings. The parameters used for the postrelease

[110] V Bhandari *et al.*, "Molecular signatures for *Bacillus species*: demarcation of the *Bacillus subtilis* and *Bacillus cereus* clades in molecular terms and proposal to limit the placement of new species into the genus *Bacillus*," *Int J Syst Evol Microbiol*, 63(Pt 7), 2013.

simulation were a total dispersed simulant mass of 30 g, the wind measurements recorded during the experimental dispersals, and a log-normal particle-size distribution with mass median diameters of 177 μm (2005) or 2.5 μm (2009), with a standard deviation of 2.5 for both.

Results For the 2005 experiment, the QUIC model shows a large plume of simulant material traveling eastward across the Pentagon and across the Potomac River into Washington, DC. The area displayed in the figure in Garza, *et al*. extends approximately two miles east from the point of release. However, the figure still shows a significant concentration of simulant material at the edge of the figure. Thus, it must be assumed that the calculated area that would be affected by the dispersal extended further, into southwest and probably southeast DC. Seven of thirteen (54%) of the postrelease environmental samples collected from around the Pentagon tested positive for *Bacillus amyloliquefaciens*. This includes an aerosol sampler located in the adjacent Pentagon Metro Station which showed that the simulant released above ground penetrated into the underground station.

For the 2009 experiment, the calculated simulant plume dispersed northward across the Pentagon and into Washington, DC. The concentrations throughout the plume were far greater than in the 2005 test, and the overall affected area was much larger. Both of these results would be expected from the much smaller particulate diameter of the simulant material used during the 2009 dispersal. Again, the sampler located inside the adjacent Pentagon Metro

Station showed penetration into the underground station. More alarmingly, a sampler located inside the Foggy Bottom Metro Station approximately 3.5 km away also showed penetration into the underground station. Indeed, even locations several kilometers east of the predicted plume tested positive at 24 hours via both PCR and culturing.

Discussion The experiments conducted by the Pentagon Force Protection Agency and described by Garza, *et al*. dispersed spores of a viable biological agent outside the Pentagon. The simulant clouds traveled across parts of Arlington, Virginia and Washington, DC, potentially exposing thousands of US citizens to the aerosolized bacteria.

Of all the biological simulants discussed throughout Chapter 2.4, however, *Bacillus amyloliquefaciens* may possibly be the safest. There does not appear to be any literature attributing human infection to *B. amyloliquefaciens* specifically. However, it is possible that any infections caused by what is now known as *B. amyloliquefaciens* could have been incorrectly attributed to a different species, such as *Bacillus subtilis*.[111] Clearly, *B. amyloliquefaciens* is viable, which means it can colonize any environments that it finds survivable.

It is not known whether *B. amyloliquefaciens* is more pathogenic to immunocompromised individuals who are at impaired ability for fighting off infections. Therefore, the true level of *B. amyloliquefaciens*' human pathogenicity cannot be known based on currently available data. Further, the potential for

[111] See Chapter 1.3 for a discussion of changes in bacterial nomenclature and the implications of incomplete identification on the certainty of bacterial species.

transfer of dangerous plasmids into *B. amyloliquefaciens* means that even this safe simulant is not safe enough. Thus, the only obvious technical flaw in this study is the potential for pathogenicity by the chosen simulant. Because the pathogenicity of *B. amyloliquefaciens* cannot be definitively discounted, it would be highly preferable to use a biologically viable simulant without any realistic probability of pathogenicity.

2.4.2. Wong, *et al*., 2010

Wong, *et al*.[112] uses the bioaerosol dispersal of two common indoor spherical bacteria to study the spatial deposition resulting from various air mixing conditions and compares the observations with their predictive CFD model based on the equation of motion for a small spherical aerosol particle that considers drag, gravity, and Brownian forces acting upon the particle.

Methods The work of Wong, *et al*. uses compressed air to aerosolize a suspension of spherical bacterial cells into a ventilated 70 L chamber inside a Class II biological safety cabinet (BSC). Two different types of Gram-positive cocci were aerosolized: *Staphylococcus aureus* subsp. *aureus* (ATCC® 6538™) and *Micrococcus luteus* (ATCC® 4698™). Inside the chamber, the deposition of the bioaerosol particles was recorded using 28 open plates of tryptic soy agar (TSA) arranged into a 7 column × 4 row array.

The predictive CFD deposition model was modeled using steady-state conditions and an assumed spherical particle (corresponding to the bacterial cell)

[112] LT Wong *et al.*, "An Experimental and Numerical Study on Deposition of Bioaerosols in a Scaled Chamber," *Aerosol Science and Technology*, 44, 2010.

with a 1 μm diameter. Turbulent flow was assumed to be negligible, and resuspension of particles after deposition was assumed not to occur. By incorporating the gravity, drag, and Brownian forces acting on the bioaerosol particle, the equation governing the motion can be written (in its full form) as:

Equation 2-11: Motion of a small aerosol particle

$$\frac{du_p}{dt} = \left[\left(\frac{3\nu\rho d_p|u_p - u|}{4d_p^2\rho\mu}\right)\left(a_1 + \frac{a_2}{Re} + \frac{a_3}{Re^2}\right)\right](u - u_p) + \frac{g(\rho_p - \rho)}{\rho_p} + \xi_i\sqrt{\frac{216\pi\nu K_B T}{\Delta t\rho d_p^5\left(\frac{\rho_p}{\rho}\right)^2 C_C}},$$

"where u_p and u are the particle and fluid parcel velocities, ρ_p and ρ are the particle (bacteria) and carrier phase densities, respectively... v is the kinematic viscosity of the carrier phase, d_p is the particle diameter... a_1, a_2, and a_3 are the constants given by Morsi and Alexander[113]... ξ_i are zero-mean, unit-variance independent Gaussian random numbers, Δt is the time step, T is the absolute temperature of the fluid, k_B is the Boltzmann constant, and C_c is the Cunningham slip correction, respectively.[114]"

Results The experimental results of Wong, *et al*. show that the fractional deposition along the length of the chamber agrees reasonably well with the prediction of the CFD model, though some discrepancies were noted (p = 0.04 – 0.06). As might be intuitively expected, a greater fraction of bacterial particles

[113] SA Morsi and AJ Alexander, "An investigation of particle trajectories in two-phase flow systems," *Journal of Fluid Mechanics*, 55(02), 1972.
[114] LT Wong *et al.*, "An Experimental and Numerical Study on Deposition of Bioaerosols in a Scaled Chamber."

were deposited at the far end of the chamber (opposite the air inlet introducing the bioaerosol) than at the near end. Including the operation of an air mixing fan during the experiment resulted in a more uniform distribution along the chamber length. Additionally, the observed results show that the ratio of deposition is directly proportional to the rate of ventilation ($p < 0.05$). However, the CFD-predicted deposition ratios were overestimated by approximately 2 – 3×. The authors attribute this significant discrepancy to "the counting of the bioaerosols,... [the] repeatability level of experiments,..." and the fact that the "[d]epositions were measured on circular TSA plates in the experiments whereas they were predicted within rectangular floor sections in the mathematical model.[115]"

Discussion The research presented in Wong, *et al*. investigates the dispersal and deposition of aerosolized spherical bacteria. Unlike some of the strictly mathematical models described in Chapter 2.2, Wong, *et al*. use the actual dispersal of bacteria similar to those modeled to determine the validity of their model. The direct testing of viable microorganisms allows the use of bacterial growth as the measured parameter. Thus, the scenario could be adapted (within the confines of the otherwise same experimental setup) to investigate the effects of filtration or viability countermeasures in reducing the deposition of viable bioaerosol particles. The construction and operation of the experiment within a BSC is a wise choice given the organisms selected for aerosolization and dispersal: both *Staphylococcus aureus* subsp. *aureus* and

[115] Ibid.

Micrococcus luteus are known to have some human pathogenicity. Although both species are commonly found as part of the normal human skin flora, the possibility of gene transfer between the dispersed strains and more pathogenic strains – for example, methicillin-resistant *Staphylococcus aureus* (MRSA) – supports this prudent approach to the biosafety of the experiment.

However, because the physical design of the experiment by Wong, *et al*. is restricted to a small (70 L), artificially-constructed scenario inside a BSC, it is not generally applicable to larger scenarios. Furthermore, in the Conclusions section of their paper, the authors note that their work is limited in applicability to the deposition of 1 μm-diameter spherical microorganisms. Adjusting the CFD model to accommodate particle sizes smaller or larger than 1 μm and/or non-spherical particle shapes would no doubt complicate Equation 2-11 significantly.

2.4.3. Kournikakis, *et al*., 2009

The work of Kournikakis, *et al*. (2009)[116] uses spores of *Bacillus atrophaeus* as a simulant to study the dispersal of *B. anthracis* spores throughout a building after aerosolization from an "anthrax letter". Also investigated were the impact of various different responses by the letter opener and the impact of various different potential mitigation procedures.

Methods The experiments by Kournikakis, *et al*. (2009) were performed inside a 36 × 9.4 m (338.4 m^2, 1015.2 m^3) building that was divided into a central

[116] B Kournikakis, J Ho, and S Duncan, "Anthrax Letters: Personal Exposure, Building Contamination, and Effectiveness of Immediate Mitigation Measures," *Journal of Occupational and Environmental Hygiene*, 7(2), 2009.

hallway, fifteen offices of various sizes, and two washrooms.[117] The spore powder used in the experiments consisted of dry *B. atrophaeus* spores (which were not milled or fluidized) at a concentration of 1×10^{11} CFU/g. In five scenarios, 0.1 g of spore powder was placed inside of a tri-folded sheet of standard letter paper, which was then placed inside a standard business envelope. The sampling of the aerosolized powder was performed by slit-to-agar samplers stationed in various locations throughout the building. The design of the slit-to-agar samplers allows the time of plate exposure to be determined. Thus, the spatiotemporal dispersal of the aerosol cloud can be tracked. Additionally, personal contamination to the person opening the letter was sampled using microfiber filters were placed on the coverall worn by the individual. Opening the letter began the experiment (t = 0), and the subsequent dispersal of spore powder was tracked through the building.

Results The results of the study demonstrate that the concentration of aerosolized spores increases rapidly shortly after the letter is opened. In the room in which the letter was opened, the concentration increased over 100-fold within the first minute, but quickly began to decline after approximately three minutes. An aerosol particle sizer in the room in which the letter was opened showed that the majority of the particles were detected between 24 and 36 seconds after opening the letter. At approximately 30 seconds, 84% of the detected particles were between 3 and 10 μm in size, with a peak particle size of

[117] Ibid.

8.3 μm. The cloud of aerosolized spores spread throughout the building very rapidly. The times of detection ranged from 1.93 to 4.45 min. The distribution of spores across the person opening the letters showed personal contamination was generally greatest (among the sampled locations) at the hips, forearms, and chest. None of the mitigation techniques tested were effective; surprisingly, they were generally counterproductive. In particular, the level of exposure for the person opening the letter was significantly greater – between 3.6× and 6.8× – than the control, where the person remained seated after opening the letter. Based on an estimated human LD_{50} for *B. anthracis* of 10,000 spores, these results correspond to between 39× and 160× the LD_{50}.

Discussion The research presented in Kournikakis, *et al*. (2009) investigates numerous aspects of dispersal of aerosolized *B. anthracis* spores. The study is well designed and avoids many of the drawbacks discussed in the previous sections. Attempting to predict dispersal using a mathematical model inherently necessitates an extreme reliance on assumptions and can result in significant uncertainty. This study is able to avoid these issues by actually dispersing a biological simulant and determining its dispersal based on actual detection rather than attempted prediction. Unlike the various physical simulants discussed, the uncertainty regarding the appropriateness of the simulant dispersed is avoided by using a very closely related spore-forming bacterium. Any differences between *B. anthracis* spores and *B. atrophaeus* spores (relative to other simulants) are outweighed by the far more numerous similarities.

However, the one major flaw in this study is the potential for pathogenicity by the chosen simulant, *Bacillus atrophaeus*. Although there does not appear to be literature attributing human infection to *B. atrophaeus* specifically, the name "*Bacillus atrophaeus*" was only proposed in 1989.[118] Previously, it was considered to be *Bacillus subtilis*. Additionally, clinical *Bacillus* isolates were often not further identified to the species level. (These topics are addressed in greater detail in Chapter 1.3.) Thus, it is possible that any infections caused by what is now known as *B. atrophaeus* might have been improperly attributed to a different incorrect species. Therefore, the true level of *B. atrophaeus*' human pathogenicity cannot be known based on currently available data. Because the pathogenicity of *B. atrophaeus* cannot be definitively discounted, it would be highly preferable to use a biologically viable simulant without any realistic probability of pathogenicity.

2.4.4. Hathway, *et al*., 2007

The research of Hathway, *et al*.[119] sought to use computational fluid dynamics to model the dispersal of an infectious bioaerosol generated by distributed (i.e., non-point) sources such as bedmaking in a hospital ward and the hazard it poses to hospital patients and staff. The authors introduce the concept of a "zonal" bioaerosol source according to the following rationale:

> "A number of published works using CFD to model bio-aerosol spread in hospitals have considered respiratory infections such as

[118] LK Nakamura, "Taxonomic Relationship of Black-Pigmented *Bacillus subtilis* Strains and a Proposal for *Bacillus atrophaeus* sp. nov.."
[119] EA Hathway, A Sleigh, and CJ Noakes, "CFD Modelling of Transient Pathogen Release in Indoor Environments due to Human Activity".

> SARS and Tuberculosis. In all of these cases a directed point source is used to represent the dispersal of particles from a cough, which is appropriate where the patient is primarily bed bound... Certain routine activities in hospital wards can also cause a number of large particles, e.g. skin particles, to be dispersed into the environment... For instance it is well recognised that activities such as walking, undressing/dressing and bedmaking all disperse large numbers of bacteria into the air... Since this type of dispersal will vary with space and time a single point source would not provide adequate information to represent these sources... Activity related dispersal may occur over a large area varying in position and rate with time as people move about the hospital, carrying out different tasks... The study introduces the concept of using a 'zonal' source that time averages the dispersion over the area in which the activity occurs.[120]"

In this work, a CFD model was developed and compared to the results of a dispersal of *Serratia marcescens* in a bioaerosol test chamber.

Methods *S. marcescens* was aerosolized using a Collison nebulizer within a 32.25 m^3 (3.35 m × 4.26 m × 2.26 m) HEPA-filtered room designed for aerobiological tests. A zonal bioaerosol source was created by introducing the bacteria into the room through a pipe with 36 holes (eight sets of four) with a spacing of 15 cm. Particles < 2 μm were sampled using an Anderson sampler.

For the CFD simulation, two three-dimensional models of the room, each under different ventilation parameters, were created. The first ventilation arrangement used an air inlet at the bottom corner of the long wall and an outlet at the top opposite corner of the opposite long wall, while the second scenario used a ceiling-mounted inlet and outlet. An air exchange rate of 6 ACH was

[120] Ibid.

used in both cases. Because the air temperature was regulated and no additional sources of heat were present in the room, an isothermal model was constructed. The zonal bioaerosol source was defined as a 0.1 m × 0.1 m × 1.2 m, corresponding to the dimensions of the pipe introducing the aerosolized bacteria into the room. The aerosol particles "were assumed to remain airborne for long periods of time which is suitable for small microorganisms 2 μm in diameter or less.[121]" For numerical validation of the zonal model, three types of bioaerosol sources were evaluated: zonal sources, a stationary point source, and a moving point source. Two zonal source scenarios were tested, one centered in the room oriented along the length of the room, and one similarly centered along the width. The stationary point source was centered in the room, and the transient point source moved through the room along the same path represented by the zonal sources. All the sources injected the same scalar quantity (representing the aerosolized bacteria) into the room.

Results The experimental results showed considerable variance, but were judged to be within expectations given the nature of turbulence within the room and the potential impact on viability of the aerosolization and dispersal processes. Comparison of the experimental results with the predictions of the CFD model show a general agreement, though some differences appear to the result of the aforementioned experimental variance. Comparison of the three bioaerosol models shows that the zonal source is a better representation of the

[121] Ibid.

time-averaged dispersal caused by the transient point source than is the stationary point source. However, a dramatic underestimation of the maximum scalar (i.e., the greatest number of aerosolized bacteria at any point in the room) is produced using the zonal source, which predicts only 4 – 9% the maximum scalar predicted by the transient point source. Thus, while a steady-state zonal source provides an overall better representation of the dispersal caused by a moving bioaerosol source, the maximum degree of contamination is greater than this method would indicate.

Discussion Proceeding from the assumption that the usual point source is not a good model for a distributed bacterial bioaerosol source, this paper introduces a zonal source and attempts to validate it. As with all the models discussed in Chapter 2.4, the use of a viable microorganism as a biological simulant by Hathway, *et al*. has the advantage of collecting real data (as opposed to hypothetical predictions) regarding the dispersal of a biological aerosol. Again, however, this also presents a number of potential problems, particular with regard to the specific microorganism selected. *Serratia marcescens*, the bacterium used for the experimental dispersal, has been commonly used for such aerosol dispersal studies. However, it is well-known as a low-level, opportunistic pathogen with considerable history of clinical infection dating back as far as 1913.[122]

[122] SD Mahlen, "*Serratia* Infections: from Military Experiments to Current Practice," *Clinical Microbiology Reviews*, 24(4), 2011.

Additionally, the results of the experimental dispersal show considerable variance that was attributed to a potential loss of viability resulting from the aerosolization process. Because *Serratia marcescens* is a Gram-negative rod, it is particularly susceptible to desiccation and osmotic shock[123], and its viability is severely reduced (≈100×) within 20 minutes of aerosolization.[124] Thus, *S. marcescens* is a poor choice because it is more likely to be affected by the dispersal than a heartier organism, such as a Gram-positive bacterium or spores. (Furthermore, *S. marcescens* seems a strange choice for a simulant organism given that the majority of normal skin flora consists of Gram-positive bacteria, including various species from the *Staphylococcus*, *Streptococcus*, *Micrococcus*, *Corynebacterium*, and *Propionibacterium* genera.[125])

Compared to the results of the experimental dispersal, the CFD model used by Hathway, *et al*. "greatly underestimates[126]" the maximum level of bacterial contamination caused by bedmaking activity. While this suggests some flaw in the construction or execution of the CFD model, this discrepancy would not have been apparent without the comparison to the biological dispersal performed in conjunction. The results gleaned from the latter allow the modification of the CFD model to ensure it properly predicts the dispersal.

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[123] AJ Mohr, "Aerosol (Aerobiology, Aerosols, Bioaerosols, Microbial Aerosols)," in Encyclopedia of Bioterrorism Defense, Ed. RF Pilch and RA Zilinskas (Hoboken, New Jersey: John Wiley & Sons, Inc., 2005).
[124] JF Heidelberg *et al.*, "Effect of aerosolization on culturability and viability of gram-negative bacteria," *Appl Environ Microbiol*, 63(9), 1997.
[125] CP Davis, "Normal Flora," in Medical Microbiology, Ed. S Baron (Galveston, TX: University of Texas Medical Branch at Galveston, 1996).
[126] EA Hathway, A Sleigh, and CJ Noakes, "CFD Modelling of Transient Pathogen Release in Indoor Environments due to Human Activity".

2.4.5. Agranovski, *et al*., 2005

The work of Agranovski, *et al*.[127] investigates the cause of spore dispersal from an "anthrax letter", the velocity with which the spores disperse, and the effect of reactionary movement by the person opening the letter. A corollary experiment conducted was whether reaction and movement by the person opening the letter could occur rapidly enough to avoid (i.e., "outrun") the bioaerosol cloud front before it reached the individual's location.

Methods The research of Agranovski, *et al*. utilized spores of *Bacillus thuringiensis*, a member of the same closely-related *Bacillus cereus* group as *B. anthracis* and which essentially the same genotypic and phenotypic characteristics. A folded letter was loaded with 50 mg of spores and placed in an envelope. Bioaerosol particle counters, a laser diffraction particle sizer, and monitors located on the individual opening the envelope and various distances (0.1, 0.5, 1.0, and 1.5 m) were activated immediately prior to the opening of the envelope. Two different scenarios were tested: (1) the still air scenario, in which the individual remains stationary after opening the envelope and all airflow is assumed to originate from the individual opening the letter, and (2) the disturbed air scenario, in which the individual, "after observing a dust cloud appearing as the result of the opening of the envelope, recoils in fright creating additional air flows significantly disturbing the aerosol propagation...[128]" The authors' suggestion that this is a more realistic scenario upon a person's discovery of a

[127] IE Agranovski, OV Pyankov, and IS Altman, "Bioaerosol Contamination of Ambient Air as the Result of Opening Envelopes Containing Microbial Materials," *Aerosol Science and Technology*, 39, 2005.
[128] Ibid.

potentially deadly substance is intuitively valid. The velocity of the person's recoil movement was determined by analysis of the video recording of the experiment.

Results The work of Agranovski, *et al*. shows that the opening of the envelope creates a dispersal of the spore powder located within the letter. The *B. thuringiensis* spore cloud is detected by the samplers at 0.5, 1.0, and 1.5 m at approximately 6, 25, and 55 seconds, respectively. Compared to the spore concentration at the source, the concentration at these distances was approximately 33%, 10%, and 5%, respectively. Although some deposition of the bioaerosol particles can be expected, the likely primary factor in the reduction of concentration with distance is simple diffusion of a fixed number of spores across an increasing radius. The velocity of the spore front was calculated at approximately 20 cm/s, which was consistent with the 20 – 30 cm/s measured velocity of the unfolding paper. Because the individual remained stationary after opening the letter, the particle dispersal is attributed to the air convection caused by the opening of the letter itself rather than natural diffusion of the spore particles; the velocity of the dispersal is a result of the velocity at which the letter is opened. In the disturbed air scenario, the individual opened the letter, moved one meter away from the letter, and stopped; the aerosol samplers located on the person operated for 20 seconds after movement ceased. (Because the results from the previous scenario show that the spore front reaches 1.0 m at approximately 25 seconds, any spores captured by the samplers can be attributed to the person's movement causing the cloud to move toward the

person.) The results from the samplers show that the number of spores captured increased as the person's recoil velocity increased. This indicates that the air currents created by the individual's movement altered the dispersal of the spore cloud, causing greater numbers of spores to move in the direction of the person. The mathematical model proposed by the authors to predict the efficiency of bioaerosol capturing as a function of recoil velocity fits the observed experimental data well. (However, no measure of statistical significance is provided in the paper.) In the last experiment, the individual moved away slowly in an attempt to avoid the dispersing spores; the results show that no spores were captured within the 20-second sampling time. Thus, moving away slowly, rather than quickly, can allow a person who opens such an anthrax letter to escape exposure. The main conclusion of this research is that "avoiding fast movement during the opening of a letter and, especially, after the observation of microbial material, significantly increases the time to exposure and, respectively, decreases the amount of particles inhaled by an envelope recipient.[129,130]"

Discussion The work of Agranovski, *et al*. shows that (1) the initial velocity of aerosolized spores dispersed from an anthrax letter is determined by the manner in which the letter is opened, and (2) reactionary movements by the individual opening the letter can increase or decrease the level of personal exposure. In the case of a real anthrax letter, the spore powder could have

[129] Ibid.

[130] Because it is not explicitly stated within the paper, it must be assumed that the tests were conducted in a room with no artificial ventilation. Otherwise, the cause of the aerosol dispersal cannot be conclusively attributed to the airflow created by the opening of the letter.

additional properties which aid dispersal. A variety of methods exist for the weaponization or fluidization of spores, such as application of electrostatic charge or silica.[131] The material collected from the 2001 anthrax letters was initially described as having such properties. Dr. Peter Jahrling from USAMRIID described the difficulty of weighing it during his analysis: "It literally jumped off the spatula and was repelled by the weighing paper; it was like nothing I had ever seen before.[132]" The research of Agranovski, *et al*. does not address diffusion mediated by repulsion or fluidization.

As with the other models discussed in Chapter 2.4, this work has the advantage of using a biological simulant whose dispersal properties can be directly observed, as well as the disadvantage of aerosolizing bacterial spores of a potentially pathogenic species. *Bacillus thuringiensis* is closely related to *B. anthracis*, so much so that some have suggested they are actually the same species.[133] Although few human infections have been attributed to *B. thuringiensis*[134], the possibility that spores of this species could acquire dangerous genetic features suggests that use of viable *B. thuringiensis* spores should be replaced by an organism with a greater margin of safety.

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[131] G Matsumoto, "Anthrax powder: state of the art?," *Science*, 302(5650), 2003.
[132] A Dance, "Silicon highlights remaining questions over anthrax investigation," Nature Publishing Group, http://www.nature.com/news/2008/080929/full/news.2008.1137.html.
[133] E Helgason *et al.*, "*Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* - One Species on the Basis of Genetic Evidence," *Appl Environ Microbiol*, 66(6), 2000.
[134] J Han, Y Cheng, and MS Wang, "*Bacillus thuringiensis* poisoning related acute transverse myelitis," *Can J Neurol Sci*, 40(3), 2013; JP Siegel, "The Mammalian Safety of *Bacillus thuringiensis*-Based Insecticides," *J Invertebr Pathol*, 77(1), 2001.

2.4.6. Kournikakis, *et al*., 2001

On January 30, 2001, a letter was received at Citizenship and Immigration Canada in Ottawa, Ontario. The letter purported to contain *B. anthracis*, although it did not. Citing a lack of experimental studies which could form the basis of a realistic assessment, the Defence Research Establishment Suffield (DRES), located in Suffield, Alberta, conducted a study to determine what the risks were from such an anthrax letter. The experiments described by Kournikakis, *et al*.[135] were conducted from February to April 2001.

Methods An 18 ft × 10 ft × 10 ft (1,800 ft^3) aerosol test chamber was set up to simulate a mail room or office. The DRES researchers placed a single sheet of tri-folded copier paper into a standard envelope; the paper was folded around spores of *Bacillus atrophaeus* (BG). They determined that 1.0 grams of *B. atrophaeus* spore powder could not be felt though the envelope, thus avoiding alerting a person holding the letter to the presence of a suspicious substance inside. A person seated at a desk in the room opened an envelope containing a letter loaded with either 1.0 g or 0.1 g (three trials each) of *Bacillus atrophaeus* spores at a concentration of approximately 1×10^{11} CFU/g. In addition to both high- and low-resolution slit-to-agar samplers used to detect and quantify aerosolized spores, the person opening the letter wore a respirator equipped with a collection filter for measuring personal inhalation exposure. The sampling period extended 10 minutes from the time the first letter was opened.

[135] B Kournikakis *et al.*, Risk Assessment of Anthrax Threat Letters.

Results After the letter was opened in a typical manner inside the aerosol chamber, the results showed extensive spore dispersal and contamination of the entire testing area, including on the front and back of the clothing worn by the subject who opened the letter. In the three trials which used letters loaded with 1.0 g of spores, the number of colonies formed on the agar plates was so great that discrete colonies were not visible, overwhelmed the detection resolution and prevented an accurate reading from being obtained. However, the general pattern of the results showed a significant spike in the concentration of aerosolized spores followed by a gradual decline. The cloud of aerosolized spores spread throughout the room, with calculated concentrations on the far side of the room nearly as high as those at the desk. Based on the subject's level of exposure, if the powder had been anthrax, the subject would have received between 122 – 2,680 LD_{50}s from the 1.0 grams contained within the envelope. The trials with 0.1 g showed the same general patterns of dispersal. However, the lower initial number of spores allowed the bacterial growth from the slit-to-agar samplers to be resolved into discrete colonies, allowing accurate scanning and estimation of concentration. The concentrations measured by the respirator filter were approximately 10× greater for the 1.0 g trials as for the 0.1 g trials, as would be expected from 10× the initial spore load in the envelope. However, the results from the slit-to-agar samplers estimated the spore concentration for the 1.0 g trials at only 4.375× the concentration of the 0.1 g trials. This strongly suggests that the overgrown agar plates had a

detrimental effect on the colony scanning accuracy. The fraction of particles 2.5 – 10 μm collected was greater than 99%, corresponding to agglomerations of spores. Because each of these particles is recorded as a single colony-forming unit, the number of viable spores to which the individual opening the letter was exposed was likely greater than estimated using the CFU-based method.

Discussion Because the work of Kournikakis, *et al*. (2001) used viable spores of *Bacillus atrophaeus* as a simulant for *B. anthracis*, their study avoids the assumptions inherent to mathematical simulations and the uncertainty associated with dissimilar physical simulants. However, the use of viable bacteria poses a biosafety risk, even though *B. atrophaeus* is ostensibly nonpathogenic. Although the experimenter in the study who opened the simulated anthrax letter was wearing personal protective equipment (PPE) and a respirator, the individual still could have been exposed to a significant number of spores upon removal of the PPE. This type of spore dispersal study would be an ideal candidate for the use of a simulant composed of biologically viable bacterial spores incapable of colonization of undesired environments.

The work of Kournikakis, *et al*. (2001) was designed to address the lack of empirical data regarding the threat from an anthrax letter. They point out that such an anthrax letter had been previously considered to be a “passive” dissemination method which would be of little threat. A 1999 article in the CDC journal *Emerging Infectious Diseases* reviews the epidemiology, clinical characteristics, and disease management of anthrax. Addressing the threat from

the various anthrax hoaxes in the previous year, the authors make the following unfortunate risk assessment:

> "When evaluating a threatened release of anthrax, the lack of volatility of the disease, as well as its inability to penetrate intact skin, should be taken into account. These factors make it unlikely, in most cases, that persons coming in contact with letters, packages, and other devices purported to contain anthrax will be at risk for aerosol exposure. Moreover, because energy is required to aerosolize anthrax spores, opening a letter, even if it contained anthrax, would be unlikely to place a person at substantial risk.[136]"

The authors of the DRES report indicate that their experimental results were quite unexpected. Their findings show that "dissemination of anthrax spores from an envelope presents a far more serious threat than had previously been assumed.[137]" Rather ominously, and in retrospect quite presciently, the DRES report ends with a warning: "It is only a matter of time until a real 'anthrax letter' arrives in some mail room.[138]" The date of publication for this report was September 1, 2001. Ten days later, a coordinated attack on the United States by the terrorist group al-Qaeda shocked the nation. Shortly afterward, sometime between September 17 – 18, 2001, the first batch of anthrax letters was mailed.[139] On October 5, 2001, the first of eleven victims died from anthrax as a result of an intentional bioterrorist attack using the US Postal Service.

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[136] TJ Cieslak and EM Eitzen, Jr., "Clinical and epidemiologic principles of anthrax," *Emerg Infect Dis*, 5(4), 1999.
[137] B Kournikakis *et al.*, Risk Assessment of Anthrax Threat Letters.
[138] Ibid.
[139] US Department of Justice. "Amerithrax Investigative Summary," (Washington, DC: 2010).

2.4.7. Ganio, *et al*., 1995

Ganio, *et al*.[140] uses a computer-simulated model of bioaerosol particle dispersal coded in FORTRAN and compares the results generated to the deposition pattern observed from a dispersal of *Bacillus atrophaeus* (ATCC® 6537™) spores across a 10^5 m^2 grid.

Methods The dispersal tests were conducted at the US Army's Dugway Proving Ground in Utah. An aerosolizing nozzle positioned 2 m above ground level dispersed approximately 2.5×10^{11} *B. atrophaeus* spores during the 5 minute spray event. Air sampling was effected by 49 Andersen samplers arranged 15 m apart in a 7 × 7 grid, followed by incubation of the exposed sample plates at 37˚C for 18 hours. Samplers placed upwind from the spray nozzle provided background counts. Sixteen sedimentation plates were positioned around the sampler closest to the nozzle.

The computational grid was programmed to be comprised of 7,381 cells with an area of 1 m^2 each (61 m wide × 121 m long). The FORTRAN program conducted a simulated spray event by creating 360,000 droplets of randomly-distributed size filled with a randomly-distributed number of bacteria.

Results Comparison of the bacterial growth on plates with the number of spores released suggests that less than 0.0001% (2.98×10^4 CFU) of the airborne spores were captured by one of the samplers. The viable bacteria recovered by the aerosol samplers is shown in Figure 2-2. The *x*- and *y*-axes

[140] LM Ganio, AJ Mohr, and B Lighthart, "A comparison between computer modeled bioaerosol dispersion and a bioaerosol field spray event," *Aerobiologia*, 11, 1995.

represent the spatial distribution of the samplers (e.g., Sampler A1, A2, A3,... B1, B2,... G6, G7) and the *z*-axis displays the number of CFU recorded by the specific sampler. The spray nozzle was located approximately 5 m from Sampler A4. The direction of the spore dispersal is clear from the graph, proceeding in the direction of the wind at the time of the experiment. The samplers recording the highest bacterial counts were Samplers C6, D6, and E6, which provided values between 6,219 and 9,581 CFU.

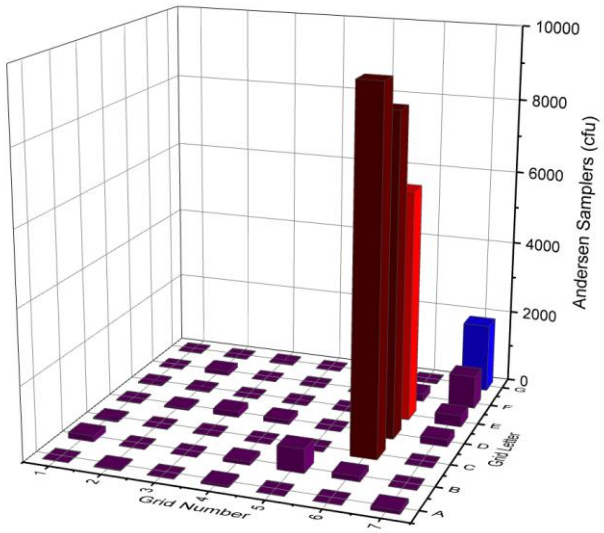

Figure 2-2: Spatial detection of dispersed simulant in Ganio, *et al*.

Based on the direction in which the experimentally dispersed spore plume actually travelled, the simulation grid had to be reoriented by approximately 30˚ in order for the simulated bioaerosol plume to line up with the experimental results. The highest concentration (CFU/m^2) of spores was predicted to be in Grid A4, the

location of the spray nozzle. The values predicted per grid are shown in Figure 2-3. As with the previous figure, the direction in which the simulated spore plume travelled is evident from the graph. The sample grids with the highest predicted bacterial counts were Grids A4, B4, C5, and D5, which provided values between 1,753 and 136,850 CFU.

Discussion Even a cursory glance at the experimental and simulated results (Table 1 in the paper) shows clear differences between the two data sets. In many cases, the computer simulation overpredicted the number of viable spores relative to those that were actually enumerable via experimental sampling and plating. However, in even more cases, the simulation greatly underpredicted the experimental results.

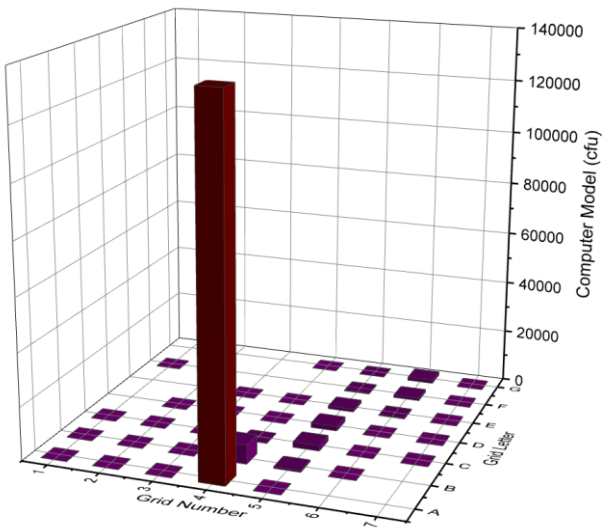

Figure 2-3: Predicted dispersal of simulant in Ganio, *et al*.

The levels of overprediction and underprediction for each grid cell were determined by dividing the experimentally observed value by the computationally predicted value. (Simulation grid cells with values of zero were changed to have values equal to one in order to avoid shattering the space-time continuum by dividing by zero.) These quotients were then plotted in the same manner (*z*-axis) as the results shown in the previous two figures. The overprediction/ underprediction results can be seen in Figure 2-4, which shows the fold increase ($z > 0$) or decrease ($z < 0$) of the simulation results relative to the experimental results.

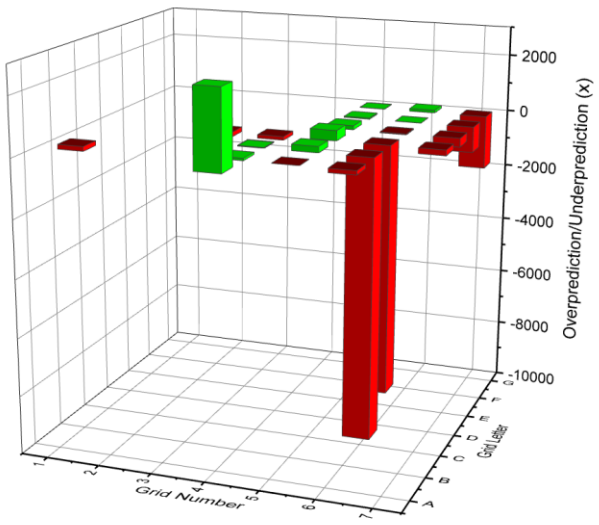

Figure 2-4: Observed versus predicted simulant dispersal in Ganio, *et al*.

As can clearly be seen in the graph, the simulation predicted results that in many cases were dramatically different than the observed results. The greatest

underpredictions occurred in Grids C6, D6, and G7. The Andersen samplers for these grids showed between 1,860 – 9,581× greater *B. atrophaeus* CFU than were predicted by the computer model. With the exception of Grid A4, the greatest overpredictions were in Grids C5, D5, and E5, which predicted between 136.5 – 350.6× more *B. atrophaeus* CFU than were actually observed in the experimental results. The results from Grid A4 appear to represent the single largest overprediction by the computer model: the simulation predicted 136,850 CFU, while only 51 CFU were experimentally observed. This appears to represent an overprediction of 2,683.33×. However, based on the explanation provided by Ganio, *et al*. for this discrepancy, it is more likely due to an undersampling of the spray droplets due to the threshold sampling size of the Andersen samplers.[141] Due to the inherently unreliable experimental result at this grid cell, this cell is excluded from further comparison with the predicted results from the computer simulation.

The computer model presented by Ganio, *et al*. for the simulation of *B. atrophaeus* spore dispersal shows marked differences from the results observed during the experimental spray dispersal. As was noted in previous discussions, performing an actual bioaerosol dispersal in conjunction with the computational model allows the later modification of the model to correct the discovered discrepancies and more accurately model the actual dispersal. Nonetheless, *Bacillus atrophaeus* remains a potentially pathogenic simulant. This introduces a

[141] See Ganio, *et al*. for a more complete discussion of this discrepancy.

certain level of risk into experimental aerosolizations, particularly outdoor releases such as the one described by Ganio, *et al*.

2.5. Conclusions

Clearly each of the types of models discussed in this chapter has both advantages and significant disadvantages. Mathematical modeling eliminates the need for conducting actual dispersals of chemical or biological simulants and can provide predictive analysis. It does so, however, at the expense of creating models which are replete with assumptions and uncertainties, inextricably constructed upon predetermined parameters, not flexible enough to be generally applicable to different scenarios, and can be dependent on substantial computing power to process repetitive iterations of the same scenario and yet still have a significant level of inaccuracy.

Physical simulation provides a more realistic model by actually releasing then analyzing the dispersal of a simulant, thus eliminating many of the uncertainties introduced by the assumptions inherent to mathematical models. Additionally, when computational models are constructed together with physical models, the data gained from the latter can provide validation and allow the modification of the former. However, physical simulation can suffer from a questionable appropriateness to the systems they are designed to simulate (particularly in the use of nonbiological simulants to simulate biological particles) as well as discrepancies between the properties (e.g., airflow velocity,

turbulence, fluid density) of the simulation versus the environment simulated, particularly when model scaling is not properly considered.

Biological simulation provides a more directly comparable simulant in terms of both properties and behavior. As with physical simulants, the collection of real bioaerosol dispersal data allows the progressive refinement of associated computational models. Furthermore, the use of viable organisms allows the introduction of biological viability as a dependent variable in the experimental design, enabling investigation of techniques for reducing the numbers of viable bioaerosol particles. Thus, for simulating the dispersal of pathogenic bioaerosols, it would seem that the dispersal of viable biological simulants is far superior to either physical simulants or mathematical modeling. However, a significant risk introduced by the use of viable microorganisms is the potential for inoculation and colonization of undesired environments and potential pathogenesis, particularly in exposed immunocompromised populations. Because such pathogenicity cannot be discounted, it would be preferable to use a biologically viable simulant without any realistic probability of pathogenicity. The ideal biological simulant would be a nonpathogenic microorganism which is incapable of growth in undesired environments. However, such a microorganism does not occur naturally.

Up to now, the best simulant of the release of a dangerous biological agent has been considered to be a similar, yet nonpathogenic, agent. This is no doubt one of the reasons why the US military chose to use various different types

of bacteria and viruses in many of its biological weapons simulations and biodefense research. However, no naturally-occurring microorganism can be definitively considered entirely nonpathogenic. In its quest to obtain accurate data for both offensive and defensive purposes, the US government dispersed potentially pathogenic microorganisms and showed a blatant disregard for the safety of the American public in many of its tests. Some of these experiments, which allegedly resulted in the death of at least one person, are discussed in Chapter 3 and illustrate the various risks involved in releasing aerosolized microorganisms that are potentially pathogenic.

3. HISTORICAL CHRONOLOGY OF US OPEN-AIR TESTING

The United States has in the past conducted many experiments in which biological agents or their simulants were released into the environment, exposing human subjects (either knowingly or unknowingly) to these agents or simulants. Most of these tests were performed during the United States' offensive biological weapons program, which was discontinued in 1969. Although some of these tests were primarily geared toward gaining information which would help uncover US vulnerabilities to biological attack, due to the nature of the experiments, most of these tests simultaneously developed methods and generated results which could easily be applied toward improving US offensive BW capabilities. In some cases, development of offensively valuable information was officially listed as a secondary research goal, but often this remained unstated. However, it is generally agreed that the line between biodefense research and offensive BW research can be extremely fine and unclear. It is nearly unfathomable that those conducting these experiments could have been unaware of the offensive implications of their work.

This chapter provides a historical chronology of numerous open-air tests conducted by the US government during the era of the American BW program where people were exposed – often without their knowledge – to BW agents or

their simulants. For the purposes of this work, “open-air testing” is defined as the intentional non-warfare, open-environment (i.e., not in a controlled chamber), aerosol release of biological weapons agents or their simulants resulting in the exposure of humans to the released material. After a short description of the rationale for performing such open-air testing, each section in this chapter examines a single test or series of tests. Where sufficient information is available, the analysis of each test aims to answer the following questions for each:

- Who conducted this test, when, and where?
- What was the purpose of this test?
- What agents were released into the environment?
- Who was or may have been exposed to these agents?
- At what level was this test or testing program approved?

Limitations on Access to Information Due to the highly sensitive nature of the information, it is not surprising and certainly understandable that many of the documents describing the proper dispersal of biological agents (or their simulants) in order to achieve efficient exposure of human populations are still classified and/or are unavailable to the general public. Indeed, one of the categories of information specifically exempted from release under the Freedom of Information Act (FOIA, 5 USC §552) is information regarding weapons of mass destruction. Section (b)(1) of FOIA exempts properly classified material from

release[142], while Section 3.3(b)(2) of Executive Order 13526 allows for the classification of material which could "reveal information that would assist in the development, production, or use of weapons of mass destruction.[143]" Many of the documents describing open-air testing no doubt detail dispersal methods of biological agents and reveal data on how these bioaerosols behave (e.g., dispersal patterns, concentrations, lethal doses) under certain conditions. It is clear that if such detailed protocols and test results were made available to someone with access to pathogenic microorganisms (of which there are a multitude), the ability to grow such germs in large scale (which, as mentioned previously, is not particularly difficult), and the intent to harm the United States and its citizens (of which, again, there is no shortage), the consequences to our national security could be disastrous.

Although our national security is best served by restricting access to the potentially dangerous information in these documents, from the standpoint of academic research into the topic, this has resulted in an unfortunate paucity of primary sources for information regarding the topics covered in this chapter. Only a few documents have been declassified in their entirety, many have been released with heavy redaction, and many more still are unavailable or likely entirely unknown to the public. Therefore, much of this chapter relies out of necessity on information published in various secondary sources. Understandably, the academic threshold for citation from secondary sources

[142] 5 USC §552, *The Freedom of Information Act (FOIA)*.
[143] BH Obama, "Classified National Security Information," (2010).

should be higher than for citation from primary documents. Therefore, where possible, an attempt has been made to ensure that any information cited from a secondary source has been referenced, in a corroborating or complimentary manner, in multiple reputable secondary sources. While this is clearly not the ideal academic situation, the nature of the topic has required some flexibility in this regard.

3.1. The Baldwin Report, 1948

In 1948, the Research and Development Board of the National Military Establishment (NME) within the Department of Defense established the Committee on Biological Warfare. Serving as the committee's chairman was Dr. Ira L. Baldwin, previously the president of the University of Wisconsin. The committee was charged with evaluating whether biological agents could be used for covert operations such as sabotage and, if so, whether the United States was vulnerable to such an attack. On October 5, 1948, the Committee on Biological Warfare published a memorandum entitled "Report on Special BW Operations", which detailed its findings and recommendations. Often referred to as the "Baldwin Report", the memorandum described the threat:

> "Biological agents would appear to be well adapted to subversive use since very small amounts of such agents can be effective. A significant portion of the human population within selected target areas may be killed or incapacitated. The food supply of the nation could be depleted to an extent which materially would reduce the nation's capacity to defend itself and to wage war. Serious

> outbreaks of disease of man, animals or plants also would result in profound psychological disturbances.[144]"

The report's additional findings were that:

> "The US is particularly susceptible to attack by 'special BW operations' (meaning subversive or covert actions involving the use of biological agents)... The subversive use of biological agents by a potential enemy prior to a declaration of war presents a grave danger to the US; and... The BW R&D program is not now authorized to meet the requirements necessary to prepare defensive measures against special BW operations.[145]"

The report's recommendations for addressing the threat from subversive biological weapons use included 1) the development of systems for the detection and identification of BW agents, 2) developing ways to protect against, treat, and decontaminate BW agents, and 3) assess methods for disseminating BW agents, with particular emphasis on "special operations" (e.g., clandestine use).[146] The report also suggested some specific actions in order to accomplish the latter recommendation, proposing that clandestine teams "test ventilating systems, subway systems, and water supply systems with innocuous organisms to determine quantitatively the extent to which such subversive dissemination of pathogenic biological agents is possible.[147]" Additional research was

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[144] Committee on Biological Warfare, "Report on Special BW Operations," Ed. IL Baldwin (1948). Quoted in: US Army, US Army Activity in the US Biological Warfare Programs.
[145] Committee on Biological Warfare, "Report on Special BW Operations." Quoted in: US Army, US Army Activity in the US Biological Warfare Programs.
[146] DR Franz, CD Parrott, and ET Takafuji, "The U.S. Biological Warfare and Biological Defense Programs," in Medical Aspects of Chemical and Biological Warfare, Ed. FR Sidell, ET Takafuji, and DR Franz, *Textbook of Military Medicine* (Washington, DC: Office of The Surgeon General, Department of the Army, United States of America, 1997).
[147] Committee on Biological Warfare, "Report on Special BW Operations." Quoted in: US Army, US Army Activity in the US Biological Warfare Programs.

recommended into determining "quantitatively the extent to which contamination of intimately used objects such as stamps, envelopes, money, and cosmetics as a means of subversively disseminating biological agents is possible.[148]" Perhaps the most large-scale research recommended was into whether the naturally-occurring atmospheric shifts could be used to infect the nation with dangerous pathogens: "Large air masses are constantly moving from the polar region over certain key areas of the United States. Possibly, these air masses could be utilized for the dispersion of BW agents.[149]" The recommendations issued by the Committee on Biological Warfare in 1948 would provide direction for the US offensive and defensive biological weapons programs until President Nixon's 1969 decision to outlaw all such weapons. In many ways, the Baldwin Report is the direct progenitor to the biological weapons simulant tests described throughout this chapter.

In May 1949, Camp Detrick, the US Army's biological weapons research facility, established a new unit to execute the new mission outlined in the "Report on Special BW Operations": the Special Operations Division (SOD), with Dr. John L. Schwab as the first director of the division. The SOD's focus was to "to carry out research on potential methods of enemy covert BW attack and also to assess the BW implications of the growing concern about sabotage in the cold war.[150]" They provided other parts of the government and military what were

[148] E Regis, The Biology of Doom: The History of America's Secret Germ Warfare Project (New York: Henry Holt and Company, 1999).
[149] Ibid.
[150] US Army, US Army Activity in the US Biological Warfare Programs.

essentially “contract services” regarding Camp Detrick’s fairly unique area of expertise.[151] In particular, the SOD worked closely with the Central Intelligence Agency (CIA) on “developing special applications for BW agents and toxins,” including “the development of both suitable agents and delivery mechanisms for use in paramilitary situations.[152]” Now Camp Detrick would concern itself with more than just the biological, such as how to grow these dangerous pathogens and how to make them more deadly. Now they would be actively involved in researching their applications, their dissemination, and in determining the best way to infect large numbers of people in clandestine and covert manners.

3.2. Pentagon Ventilation System, 1949 – 1950

One of the highest priorities for the SOD was to study the dispersal of a biological agent through the ventilation system of a building. Echoing the concerns raised in the Baldwin Report, they felt that this was a likely method by which the US could be covertly attacked using biological weapons. For their first ventilation dispersal test, they chose an office building; it just happened to be one of the largest and most strategic in the world.

Beginning in 1949, the SOD conducted a series of four dispersals designed to test the ventilation system of the Pentagon, the headquarters of the US military, located in Arlington, Virginia.[153] Dr. John Schwab, the SOD director had “convinced the relevant Pentagon chiefs to permit a realistic test of the

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[151] Ibid.
[152] *Hearings Before the Select Committee to Study Governmental Operation with Respect to Intelligence Activities*, 94th Congress, First Session, 1975.
[153] US Army, US Army Activity in the US Biological Warfare Programs.

building's physical security" at an unknown time and in and unknown manner.[154] Using false documentation identifying them as employees of a fictitious air-quality testing company, teams of SOD operatives surreptitiously sprayed suspensions of the bacterium *Serratia marcescens* into the air handling system of the Pentagon.[155] The reason that *S. marcescens* was selected for this test, as well as a multitude of future simulant disseminations, is that it produces a red pigment that makes it easy to track in dispersal studies. Although it was considered at the time to be nonpathogenic, we have since learned that this may not be the case (see Chapter 3.4). The first test took place on August 18, 1949. Subsequent tests occurred on August 26 and December 12-13, 1949, and March 11, 1950.[156]

The results of the test showed bad news: the Pentagon was, in fact, vulnerable to such a covert attack. The *S. marcescens* spread throughout the building. In his book The Biology of Doom, Ed Regis summarizes the results of the mock attack and their stark implications: "The Pentagon's rudimentary air filtration systems proved to be no use against the bacteria, and if the organisms had been anthrax spores instead of harmless simulants, they would have knocked out half the country's top military ranks.[157]" The bad news, however, came with some corresponding good news for the SOD: if the Pentagon was vulnerable, so would be many or most other office buildings with a ventilation system. In researching our own vulnerabilities, the SOD also gained (most

[154] E Regis, The Biology of Doom: The History of America's Secret Germ Warfare Project.
[155] Ibid.
[156] US Army, US Army Activity in the US Biological Warfare Programs.
[157] E Regis, The Biology of Doom: The History of America's Secret Germ Warfare Project.

certainly intentionally) valuable information that could be applied offensively. If the United States were to decide to use biological weapons against a similar enemy target, Camp Detrick's Special Operations Division had just developed the beginnings of a blueprint for such a clandestine attack.

Documentation regarding this particular series of vulnerability tests is particularly scant; it is only mentioned in a handful of secondary sources and, even then, generally in passing, without much detail. Although only a single secondary source has been found that suggests there was some semblance of approval by the Pentagon's top brass[158], no documents are available which can provide a more detailed look into the processes of approval and consent for the intentional covert dispersal of live bacteria into a building filled with thousands of military and civilian workers. Certainly informed consent by any modern standard was not obtained from all the thousands of employees exposed. Fortunately, as with most of the tests, there are no documented cases of negative effects from this dispersal of *Serratia marcescens*. However, there may be undocumented cases in which mysterious illnesses or deaths could have occurred; doctors, patients, and their families would have had no reason to think that the cause may have been the inhalation of pathogenic bacteria intentionally released by their own government as part of a budding biological weapons program.

[158] Ibid.

3.3. Virginia Naval Tests, 1950

Beginning on April 1, 1950, the SOD conducted a series of tests that released both *Serratia marcescens* and *Bacillus atrophaeus* from naval vessels off the coast of Virginia. Similar to the use of *S. marcescens*, *B. atrophaeus* is a bacterium that is considered to be essentially nonpathogenic and has been used in a multitude of simulations. The destroyer USS *Kenneth D. Bailey* (DD-713) and the *Midway*-class aircraft carrier USS *Coral Sea* (CVB-43) participated in a total of 17 open-air dispersals conducted over 21 days.[159] The USS *Coral Sea*, presumably the target of the simulated attack, was docked in Hampton Roads, Virginia. The bacterial aerosols released by the USS *Kenneth D. Bailey* were carried inland by the ocean winds over the cities of Hampton, Newport News, and Norfolk.[160] The stated goal of these tests was to assess the vulnerability of the naval vessels to attack with biological agents, as well to test the performance of prototype electronic systems for detection of biological warfare agents.[161]

As with the previously described dispersal in the Pentagon, there is little documentation available which can allow for the clarification of any approval or consent processes that may or may not have occurred. Unlike some of the other tests described in this chapter, the specific goal of this test does not appear to have included tracking the dispersal of the aerosol clouds across the inland cities; the exposure of these three cities and the surrounding populated areas to the bacterial clouds seems to have been incidental to this test. However, given

[159] US Army, US Army Activity in the US Biological Warfare Programs.
[160] E Regis, The Biology of Doom: The History of America's Secret Germ Warfare Project.
[161] US Army, US Army Activity in the US Biological Warfare Programs.

that the target vessel for the test was docked within a large naval harbor, it is clear that exposure of the cities would necessarily occur. No effort appears to have been made to monitor the health of the unwittingly exposed populations, or even to conduct proper epidemiological studies to ensure that no statistically significant increases in conditions possibly attributable to the released organisms occurred.

3.4. Operation Sea Spray, 1950

The simulant dispersals into the Pentagon ventilation system represented the first tests designed to perform the sort of critical vulnerability studies recommended by the Baldwin Report. These initial tests examined the applicability of covert use of biological weapons at the large-scale tactical level, e.g., a specific building or installation. A test conducted during the following year took the scale of experimentation to the next level, a simulated attack of a small- to medium-scale strategic target, e.g., a city.

Such an aerosol dispersal test was conducted off the San Francisco Bay five months after the test series off the Virginia coast. From September 20-27, 1950, naval vessels conducted Operation Sea Spray, which consisted of six different releases of bacterial aerosols. These experiments are described in the Army publication “Special Report 142: Biological Warfare Trials at San Francisco, California, 20-27 September 1950.” This document is one of the few that has been declassified in its entirety, resulting in a much more detailed picture of the San Francisco tests than most of the others reviewed in this chapter. The stated

objectives for Operation Sea Spray were: "a) To study the offensive possibilities of attacking a seaport city with a BW aerosol generated from a ship or other source located some distance offshore; b) To attempt to measure the magnitude of the defensive problem presented by (a) above; [and] c) To gain additional data on the behavior of a BW aerosol as it is borne downwind.[162]" (It should be noted that the objectives of this experiment are listed as first offensive, then defensive.) Presumably, in seeking research into the offensive potential of aerosolized bacteria against coastal cities, the US military had Soviet seaports such as Leningrad, Vladivostok, and Murmansk in mind. "Special Report 142" indicates that the study was authorized by the Chief Chemical Officer and the Chief of Naval Operations. Furthermore, Appendix A of the report states: "Only personnel connected with the operation were aware that the tests were being conducted.[163]"

The bacterial aerosols were sprayed by the USS *ACM 13*, a Navy minelayer which sailed between 2 and 10 miles offshore releasing a bacterial aerosol, creating a line source of between 2 and 6 miles in length. Four of these tests used *Bacillus atrophaeus*, while the other two released *Serratia marcescens*. In addition to these bacteria, the ship simultaneously sprayed particles of zinc cadmium sulfide (ZnCdS), an inert powder which fluoresces under ultraviolet light, enabling its spread to be analyzed easily. The description

[162] US Army, Special Report 142: Biological Warfare Trials at San Francisco, California, 20-27 September 1950.
[163] Ibid.

provided for *B. atrophaeus* in the General Methods section of the report cites "its complete lack of pathogenicity" as one of the major reasons for its selection as a test organism in such dispersal studies.[164] However, this claim is contradicted by the medical literature. It had been well established by the time of this test that species related to *Bacillus atrophaeus* (see Chapter 1.3 for a discussion of the potential nomenclatural complexities) are opportunistically pathogenic bacteria. A review published on October 1, 1950 (coincidentally just four days after the completion of Operation Sea Spray) cites a plethora of earlier cases of *Bacillus atrophaeus*-related ocular, pulmonary, and genitourinary infections, as well as food poisoning and meningitis. Of particular note are the four cases of pulmonary infections, three of which were fatal. The papers describing these cases were published in 1912, 1924, 1927, and 1928.[165] This indicates that, at the time when Operation Sea Spray was conducted, there was already sufficient existing evidence to question the wisdom of intentionally dispersing an aerosol containing viable cells of a pathogenic bacterium over a populated American city. Interestingly, the report's description of *Serratia marcescens* notes that it is a bacterium that is "nonpathogenic in all but extremely large doses.[166]" Clearly, it was known at the time that this experiment occurred that the potential for pathogenesis did exist, and this passage from the report confirms that fact. A

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[164] Ibid.
[165] L Weinstein and CG Colburn, "*Bacillus subtilis* Meningitis and Bacteremia: Report of a Case and Review of the Literature on 'Subtilis' Infections in Man," AMA Archives of Internal Medicine, 86(4), 1950.
[166] US Army, Special Report 142: Biological Warfare Trials at San Francisco, California, 20-27 September 1950.

recent review of *Serratia marcescens* infections cites four published papers describing cases that predated Operation Sea Spray: pulmonary infections in 1913 and 1936, meningitis in 1942, and a urinary tract infection in 1948.[167] Although none of these cases were fatal, it seems that the deliberate exposure of a major metropolitan center to a cloud of an established bacterial pathogen should have been considered unwise.

The USS *ACM 13* conducted a total of six aerosol releases, sequentially referred to as Tests A through F. *B. atrophaeus* was dispersed in Tests A, B, C, and E, while Tests D and F released *S. marcescens*. The mean number of bacteria released during the *B. atrophaeus* tests was 2.06×10^{15} organisms (over 2 quadrillion bacteria), while the mean number of *S. marcescens* cells dispersed was 4.91×10^{15} organisms.[168] The geographical area exposed to these simulants covers most of the San Francisco Bay Area, including the surrounding cities of Oakland, Richmond, Alameda, and San Leandro. Although 43 sampling stations were set up, the tables showing the numerical results of respiratory exposure indicates that no results (as opposed to negative results, i.e., no organisms detected) were obtained from Station #11 in any of the tests. Presumably this station suffered some type of technical or mechanical difficulties which prevented sampling. Eight other sampling stations reported no results during a single test (three in Test A, two in Test B, one in Test C, and two in Test

[167] SD Mahlen, "*Serratia* Infections: from Military Experiments to Current Practice."
[168] US Army, Special Report 142: Biological Warfare Trials at San Francisco, California, 20-27 September 1950.

E), but reported results during all other tests. As such, data on the dispersal of the bioaerosol clouds were obtained from a total of 42 stations reporting results; Station #11 is not indicated on the report's map of sampling stations, and its location cannot be determined from the information in the report.[169]

The results of the six tests are summarized in Table 3-1. As with the tests off the coast of Virginia, the sea breeze carried the clouds of aerosolized bacteria inland over a population that was unknowingly breathing in these organisms.

Table 3-1: Summary of tests in Operation Sea Spray

	Test A	Test B	Test C	Test D	Test E	Test F
Distance Offshore	2 miles	2 miles	2 miles	10 miles	9 – 10 miles	2 miles
Distance Travelled	5 miles	6 miles	2 miles	2 miles	2 miles	2 miles
Organism Released	*Bacillus atrophaeus*	*Bacillus atrophaeus*	*Bacillus atrophaeus*	*Serratia marcescens*	*Bacillus atrophaeus*	*Serratia marcescens*
Concentration (cells/mL)	7.81×10^9	7.81×10^9	7.2×10^9	6.0×10^9	2.6×10^9	1.46×10^9
Total Organisms	1.92×10^{15}	1.48×10^{15}	3.54×10^{15}	2.65×10^{15}	1.28×10^{15}	7.17×10^{15}
Furthest Detection	Station #43 (23 miles)	17 miles	Station #43 (23 miles)	–	Station #37 (26 miles)	Station #43 (23 miles)

The tests that dispersed *B. atrophaeus* were the most technically successful. However, the tests that released *S. marcescens* were unsuccessful: the first test was declared a failure, while the second was deemed inconclusive. These experimental failures were "considered to be a result of the test agent

[169] Ibid.

failing to survive, at least in its usual form... the colonies of bacteria did not present the usual appearance of *S. marcescens* and could not be positively identified as such; therefore, no conclusive data were obtained.[170]" The report suggests that the "exposure of the organism to the elements caused it to lose its ability to pigment to its usual color.[171]" The long distance travelled by the bacteria between aerosolization and sample capture subjected the organisms to light that could have altered the results. In a 1929 paper, Gorbach states that "since prodigiosin [the characteristic red pigment produced by *S. marcescens*] as such is very photoliable, exposure tests may not be conducted with extended periods of irradiation. Long periods of exposure lead to the bleaching of the pigment, simulating an absence of pigment production.[172]" Another possibility is that the actual cause (or a further cause, in addition to the light exposure) of the lack of pigmentation may be the result of the conditions in which the collected aerosol samples were cultivated. Gorbach references an even earlier paper from 1903 describing the production of prodigiosin: the bacterium "shows the ability to produce pigment only at low temperatures below 33°C, while this ability is already completely eliminated by temperatures of around 37°C.[173]" After Test D, the samples collected were incubated at 37°C for 20 hours, at which point no colonies could be positively identified as *S. marcescens* based on pigmentation. Even after an additional 24-hour incubation at room temperature, only four

[170] Ibid.
[171] Ibid.
[172] G Gorbach, "Knowledge about the pigment of *Bacillus prodigiosus*. First report: On pigment production," *Zentralblatt f. Bakter. II. Abt.*, 79, 1929.
[173] Ibid.

pigmented colonies were observed. Once again, Gorbach cites previous research, this time from 1887, showing that nearly irreversible damage to prodigiosin production can occur as a result of incubation at 37°C: "Upon constant cultivation at this temperature, favorable to reproduction, chronic damage to the ability to form pigment ensues, which is not easily removed even by subsequent cultivation at room temperature and at 28°C.[174]" Thus, between exposure to light and an incubation temperature not conducive to prodigiosin production, it may be that the *S. marcescens* trials of Operation Sea Spray were doomed from the start. The dispersal patterns of the fluorescent ZnCdS tracer were similar to those of the bacteria, but the particles were detected in concentrations approximately ten times greater. The furthest sampler from the dispersal source was 38.5 miles away, and this sampler recorded significant levels of ZnCdS particles during at least one of the tests, suggesting that the cloud of tracer particles travelled even further inland for some unknown distance.

Although the trials with *S. marcescens* were unsuccessful, the *Bacillus atrophaeus* dispersals showed that "a successful BW attack on this area can be launched from the sea, and that effective dosages can be produced over relatively large areas for distances up to 20 miles inland provided the attack is timed to coincide with suitable meteorological conditions.[175]" However, as with nearly all the research into offensive use of biological weapons, it quickly

[174] Ibid.
[175] US Army, Special Report 142: Biological Warfare Trials at San Francisco, California, 20-27 September 1950.

becomes clear that the double-edged sword cuts both ways: "...the complexity and magnitude of the defensive problem involved in such an attack are almost inestimable. There seem to be no practical means presently at hand to provide suitable protection for either military or civilian personnel who might be exposed to such an attack... It is strongly recommended that the Civilian Defense Agency be made aware of the tremendous defensive problems that can arise from a BW attack similar to those described herein.[176]"

It turns out, however, that these experiments with ostensibly harmless simulants may have not been entirely safe. Beginning on September 29, 1950, two days after the completion of Operation Sea Spray, a hospital in Stanford began to notice an increase patients infected with nosocomial cases of *Serratia marcescens*. A total of eleven patients, all of which had catheters placed, became infected with *S. marcescens* which was isolated in their urine samples. In a hospital where no such infections had previously been reported, this outbreak was considered unusual and noteworthy enough to publish an article in a medical journal: "Infection Due to Chromobacteria" [*Serratia*]. Ultimately, one of the eleven patients, Edward J. Nevin, developed a bacterial endocarditis and died.[177] After learning in 1976 that the military intentionally sprayed *Serratia marcescens* over San Francisco just days before the unexplained outbreak, the surviving family of Mr. Nevin sued the US government "for the untimely death of

[176] Ibid.
[177] RP Wheat, A Zuckerman, and LA Rantz, "Infection Due to Chromobacteria: A Report of Eleven Cases," *AMA Archives of Internal Medicine*, 88(4), 1951.

Edward Nevin, for the willful failure of the United States Army to obtain informed consent of any persons being exposed, and for the inadequate pretest investigation of the potentials for disease by that organism...[178]" The trial proceedings of *Nevin v. United States of America* have been extensively reviewed previously and will not be detailed here.[179] The outcome of the trial, in part, was that the US government was cleared of causing Nevin's death because it could not be established with certainty that the *S. marcescens* (strain 8UK) used during the tests was the same strain with which he became infected.

Although the strain that infected Nevin was not archived (and thus could not be directly compared to the 8UK strain), an extensive study by the CDC showed that the 8UK strain has a very specific antigenic signature which was extremely rare and not seen in any previous human *Serratia marcescens* infections.[180] Additionally, the isolated strain was resistant to the antibiotics polymyxin B, terramycin, chloramphenicol, streptomycin, and penicillin[181], whereas the 8UK stain is not.[182] This suggested that the Stanford outbreak of *S. marcescens* infections and the aerosol dispersal of the same species by the US Army were not causally linked. This is further supported by the fact that the paper describing the hospital outbreak specifically notes the isolated strain's

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[178] *Mabel Nevin, et al., Plaintiffs, v. United States of America, Defendant*,(1981). Quoted in: LA Cole, Clouds of Secrecy: The Army's Germ Warfare Tests over Populated Areas (Lanham, Maryland: Rowman & Littlefield, 1988).
[179] LA Cole, Clouds of Secrecy: The Army's Germ Warfare Tests over Populated Areas.
[180] SD Mahlen, "*Serratia* Infections: from Military Experiments to Current Practice."
[181] RP Wheat, A Zuckerman, and LA Rantz, "Infection Due to Chromobacteria: A Report of Eleven Cases."
[182] *Mabel Nevin, et al., Plaintiffs, v. United States of America, Defendant*. Quoted in: LA Cole, Clouds of Secrecy: The Army's Germ Warfare Tests over Populated Areas.

ability to produce a red pigment.[183] In light of a) the difficulties described in "Special Report 142" of positively identifying bacterial colonies that were non-pigmented as *S. marcescens*[184] and b) the known difficulty in causing reversion of prodigiosin production to wild-type in strains that have lost the ability to produce the pigment[185], it seems unlikely that the infection that led to the death of Edward Nevin was caused by the same strain that was dispersed by the US Army during Operation Sea Spray. These nosocomial *Serratia marcescens* infections and Mr. Nevin's death are most likely the result of an unfortunate (and admittedly highly curious) coincidence.

3.5. Project St. Jo, 1953

Project St. Jo (otherwise known as the St. Jo Assessment Program) was a program designed to evaluate the potential of the E61R4 cluster bomblet filled with *Bacillus anthracis* ("Agent N") for use as an antipersonnel biological warfare munition. The E133 cluster bomb was meant to be the delivery vehicle for 536 individual E61R4 bomblets, each containing 35 mL of *B. anthracis* slurry at a concentration of approximately 5×10^{10} organisms/mL, which would be aerosolized by a small explosive charge upon impact.[186] Thus, each E61R4

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[183] RP Wheat, A Zuckerman, and LA Rantz, "Infection Due to Chromobacteria: A Report of Eleven Cases."

[184] US Army, Special Report 142: Biological Warfare Trials at San Francisco, California, 20-27 September 1950.

[185] G Gorbach, "Knowledge about the pigment of *Bacillus prodigiosus*. First report: On pigment production."

[186] J Cirincione, JB Wolfsthal, and M Rajkumar, Deadly Arsenals: Nuclear, Biological, and Chemical Threats, 2nd ed. (Washington, DC: Carnegie Endowment for International Peace, 2005); US Army. "Preliminary Discussion of Methods for Calculating Munition Expenditures, with Special Reference to the St. Jo Program," Munition Expenditure Panel St. Jo Program, (Camp Detrick, Frederick, Maryland: 1954).

bomblet would contain approximately 1.75×10^{12} (nearly 2 trillion) organisms. This means that the full payload of an E133 cluster bomb would be 9.38×10^{14} (938 trillion) organisms of a bacterium with an estimated respiratory LD_{50} of 2,500 – 55,000 spores.[187] Assuming the higher LD_{50} and just a 2% aerosolization efficiency (a value based on both "8-Ball" simulations and field tests[188]), if properly dispersed the biological material of a single E133 cluster bomb could have the potential to kill over 170 million people. Even assuming that the actual effectiveness was far less, it is clear that the E133 cluster bomb had the potential to be a horrifically disastrous weapon.

The basis of the St. Jo Assessment Program was to "enable an estimate of expenditure rates to be made for the antipersonnel attack of cities.[189]" A major variable in achieving this estimate of how many bombs would be required to kill how many people was a detailed understanding of how aerosol clouds moved through cities. A major part of the St. Jo program was geared toward the empirical determination of aerosol behavior under various conditions. This experimentation took the form of approximately 173 aerosol dispersals of zinc cadmium sulfide (ZnCdS) "in and near Minneapolis, Minnesota; St. Louis, Missouri; and Winnipeg, Canada," which were selected for these tests because they were "considered to include the range of conditions as regards climatology, urban and industrial development, and topographic features likely to be

[187] TV Inglesby *et al.*, "Anthrax as a Biological Weapon: Updated Recommendations for Management."
[188] US Army, Preliminary Discussion of Methods for Calculating Munition Expenditures, with Special Reference to the St. Jo Program.
[189] Ibid.

encountered on the more important potential target areas of the USSR.[190]" Specifically, these three cities were felt to be representative of the Soviet cities of Kiev, Leningrad, and Moscow.[191]

Although only ZnCdS was used and there was no release of biological material, Project St. Jo provides valuable information regarding the equipment and methods used to disperse biological warfare simulants, the strategic considerations of clandestinely releasing such materials in populated areas, and the relative disregard the military had for the safety of the public with regard to these tests. There appears to be little or no public information detailing the dispersal experiments conducted in Winnipeg; as a result, only the tests in Minneapolis and St. Louis will be addressed in this work.

The initial stages of the testing process in both Minneapolis and St. Louis involved performing extensive meteorological observations and developing detailed street-level weather maps of the cities, including temperature distribution maps with a resolution of 2 meters.[192] After the initial surveys were completed, the next steps involved the actual dispersal of simulant throughout each of the cities. In Minneapolis, a city containing 522,000 people at the time of Project St. Jo, a total of 124 individual releases of ZnCdS aerosol clouds were conducted in

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[190] Ibid.

[191] J Miller, S Engelberg, and W Broad, Germs: Biological Weapons and America's Secret War (New York: Simon & Schuster, 2001).

[192] US Army. "Joint Quarterly Report No. 2 (October – December 1952): Behavior of Aerosol Clouds within Cities," US Army Chemical Corps, (Camp Detrick, Frederick, Maryland: 1952); US Army. "Joint Quarterly Report No. 3 (January – March 1953): Behavior of Aerosol Clouds within Cities," US Army Chemical Corps, (Camp Detrick, Frederick, Maryland: 1953).

the course of 47 tests between January 19 and April 28, 1953.[193] In St. Louis, which had a population of 857,000 during these experiments, at least 35 releases of ZnCdS were conducted in at least 17 tests beginning on May 18, 1953.[194]

Similar to the way that the bacterial dispersal into the Pentagon's ventilation system was conducted, Project St. Jo included deliberate attempts to deceive the public being exposed to the dispersed simulant. Additionally, the city officials being asked for approval to conduct these tests within their cities were not notified as to their true intent. The cover story for the dispersal experiments of Project St. Jo was that the Army was testing the dispersal of a smoke screen that could be used to hide American cities from Soviet bombers. A local newspaper article published in the Minneapolis Tribune suggested that the whirring mechanical aerosol sampler boxes observed throughout the city by curious onlookers "are being used in a series of tests intended to help the Army learn to throw smoke screens over American cities... Government research has shown that even in an age of radar-bombing, it may be desirable to hide cities with smoke screens in even of atomic attack... Several other cities also are involved in the tests.[195]" In Minneapolis, letters were written by the mayor, the Chief of Civil Defense, and the Air Pollution Control Engineer asking for the cooperation of city employees and the public. These letters were presented to

[193] US Army, Joint Quarterly Report No. 3 (January – March 1953): Behavior of Aerosol Clouds within Cities; US Army. "Joint Quarterly Report No. 4 (April – June 1953): Behavior of Aerosol Clouds within Cities," US Army Chemical Corps, (Camp Detrick, Frederick, Maryland: 1953).
[194] US Army, Joint Quarterly Report No. 4 (April – June 1953): Behavior of Aerosol Clouds within Cities.
[195] US Army, Joint Quarterly Report No. 3 (January – March 1953): Behavior of Aerosol Clouds within Cities.

individuals and were "of great help to personnel in the process of securing use of private homes, buildings, and land for equipment locations. Thus 'official sanction' was given to otherwise questionable requests.[196]" Although the specific deceptive methods employed in St. Louis are not detailed, meetings with city officials and local businesses were held to describe the aerosol testing that would be occurring; presumably the same cover story as to the purported nature of the experiments was presented as was in Minneapolis. The Army's report notes that much lower levels of public curiosity were encountered in St. Louis versus the tests in Minneapolis (and implies that at least one of the testing locations being located in "a densely populated slum district" may have played a role), which resulted in fewer local news articles being published about the St. Louis tests.[197]

The reports detailing the methodology and the results of the ZnCdS dispersal experiments in Minneapolis and St. Louis make no mention of any assessments or monitoring conducted on the health of the exposed populations or on the environmental impact of the chemical.

3.6. Operation CD-22/Project Whitecoat, 1954 – 1973

In one of the longest-running test series of the biological warfare era, the military conducted Operation CD-22 ("Camp Detrick-22"[198]) and its successor, Project Whitecoat, from 1954 – 1973. Among the information required to

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[196] Ibid.
[197] US Army, Joint Quarterly Report No. 4 (April – June 1953): Behavior of Aerosol Clouds within Cities.
[198] JE Stephenson and AO Anderson, "Ethical and Legal Dilemmas in Biodefense Research," in Medical Aspects of Biological Warfare, Ed. ZF Dembek, *Textbook of Military Medicine* (Washington, DC: Office of The Surgeon General, Department of the Army, United States of America, 2007).

accurately assess biological vulnerability, data was lacking on some very critical parameters, such as the minimum infectious doses of various organisms, the clinical manifestations of different doses of infectious organisms, and the size range of inhaled BW agent aerosol droplets that could infect humans.[199] Additionally, information was missing on preventing and treating BW casualties and the effectiveness of various prophylactic and therapeutic measures.[200] Naturally, in order to obtain reliable data regarding human infectivity, exposing human subjects to infectious biological agents was necessary. However, conducting such experiments would require specific ethical safeguards, foremost the voluntary participation of the experimental subjects. The war crimes trials of German scientists who conducted heinous experiments on prisoners of war resulted in what became known as the Nuremberg Code, a set of very restrictive guidelines under which experiments involving human subjects can be considered ethical. (The Nuremberg Code is discussed further in Chapter 4.2.) The very first principle of the Nuremberg Code states in no uncertain terms: "The voluntary consent of the human subject is absolutely essential.[201]"

Operation CD-22 and Project Whitecoat were designed to obtain such data relating to human exposure to biological weapons agents. In order to accomplish this research, the US Army needed a pool of healthy military

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[199] RL Mole and DM Mole, For God and Country: Operation Whitecoat: 1954 – 1973 (New York: TEACH Services, Inc., 1998).
[200] US Army, US Army Activity in the US Biological Warfare Programs.
[201] "*United States v. Karl Brandt et al*., The Medical Case, Trials of War Criminals Before the Nuremberg Military Tribunals Under Control Council Law No. 10." US Government Printing Office, (Washington, DC: 1949).

volunteers willing to be exposed to infectious organisms in the name of biomedical research. For this, the Army turned to the Seventh-day Adventist (SDA) Church. The doctrine of the Seventh-day Adventist Church teaches the holistic nature of man, who is "an indivisible unity of body, mind, and spirit.[202]" As such, the SDA Church also teaches the importance of "adequate exercise and rest," "the most healthful diet possible," and abstinence from "unclean foods... alcoholic beverages, tobacco, and the irresponsible use of drugs and narcotics.[203]" Furthermore, one of the key tenets in SDA doctrine is that of noncombatancy. Although the Church has at various times considered varying opinions regarding the apparent conflict between its religious beliefs and the wartime obligations of its members, it has generally been in favor of patriotic support of national defense, so long as Church members were not required to bear arms.[204] Their belief of conscientious objection to combat (on a personal level, not a national level) stems from adherence to "Thou shalt not kill" and prevents SDA members from performing in combat roles. However, Church support of auxiliary military roles dates back to the Civil War.[205] Volunteering for Operation CD-22 and Project Whitecoat allowed Seventh-day Adventist soldiers to serve their country by helping generate data relevant to military medicine while staying true to their beliefs. Additionally, the participating volunteers were

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[202] Seventh-day Adventist Church, "28 Fundamental Beliefs," General Conference of Seventh-day Adventists, http://www.adventist.org/fileadmin/adventist.org/files/articles/official-statements/28Beliefs-Web.pdf.
[203] Ibid.
[204] RL Mole and DM Mole, For God and Country: Operation Whitecoat: 1954 – 1973.
[205] Ibid.

considered full-time soldiers serving on the home front. Thus, they were able to meet the obligations of the military draft while avoiding deployment to Korea or Vietnam.

Operation CD-22 began preliminary field tests in March 1955 and culminated in the exposure of thirty human volunteers on July 12, 1955.[206] Among the goals of the CD-22 program were the determination of vulnerability of humans in realistic biological weapons scenarios. *Coxiella burnetii* (which causes Q fever) and *Francisella tularensis* (which causes tularemia) were the agents of choice, since they have a low lethality and can be treated effectively once the symptoms of infection arise. After the conclusion of the Operation CD-22 tests, Project Whitecoat was instituted to continue gaining valuable human data. Project Whitecoat continued until the end of the military draft in 1973.[207] Throughout the programs, the human research subjects were voluntarily exposed to pathogens such as "the causative agents of Q fever, tularemia, sandfly fever, typhoid fever, Eastern, Western, and Venezuelan equine encephalitides, Rocky Mountain spotted fever, and Rift Valley fever.[208]" The information collected from these tests included the infectious dose of various organisms, dispersal patterns, the use of various dispersal devices, and vaccine data.

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[206] WA Blodgett, FI John, and JF Schindler. "Response of Man in a BW Field Test, Operation 'CD-22', BW 3-55," US Army, (Dugway Proving Ground, UT: 1956); E Regis, The Biology of Doom: The History of America's Secret Germ Warfare Project.

[207] JW Martin, GW Christopher, and EM Eitzen, Jr., "History of Biological Weapons: From Poisoned Darts to Intentional Epidemics," in Medical Aspects of Biological Warfare, Ed. ZF Dembek, *Textbook of Military Medicine* (Washington, DC: Office of The Surgeon General, Department of the Army, United States of America, 2007).

[208] E Regis, The Biology of Doom: The History of America's Secret Germ Warfare Project.

In one of the major differences between Project Whitecoat and most of the other biological releases discussed in this chapter, great care was given to ensure the full and informed consent of the volunteers in the experiments. Twice annually, medical recruits undergoing training at Fort Sam Houston, Texas who were listed as 1-A-O (conscientious objector) status and who had indicated a religious preference for the Seventh-day Adventist Church were interviewed. Project Whitecoat was explained to them, and they were able to ask additional questions. After being fully informed, those recruits who chose to volunteer for the program and were deemed medically qualified were assigned to the Medical Unit, where they underwent further medical and laboratory tests. When a specific research study was recruiting volunteers, the Project Whitecoat personnel were assembled and briefed as to what the study entailed, what their role would be, and what risks were involved. After the general briefing, each person was interviewed and asked whether he wished to participate in the specific study described. Those who chose to volunteer for a given study were asked to sign a consent form detailing the nature of the experiment, the risks involved, the subject's understanding of all the information presented, and, most importantly, the subject's statement that he was volunteering of his own free will without any type of coercion. Those who chose not to participate in a given study resumed their normal duties without any consequences and were given future volunteer opportunities when additional studies arose.[209]

[209] RL Mole and DM Mole, For God and Country: Operation Whitecoat: 1954 – 1973.

In studying the way in which Project Whitecoat was conducted, it quickly becomes clear that it serves as an important contrast – a stark exception to those studies that came before it and after it. It is evident that, from the very outset, the entire study was designed to be conducted in an ethical manner. This brings up an interesting question: why was this done for these tests but not for previous or later tests?

3.7. Operation Large Area Coverage (LAC), 1957 – 1958

If the simulant dispersals into the Pentagon ventilation system represented a study of the covert use of biological weapons at the large-scale tactical level and Operation Sea Spray studied an attack at the small- to medium-scale strategic level, the remaining question was whether the use of biological weapons was feasible at the large-scale strategic level, e.g. entire nations or large subnational regions. The question had been raised in the 1948 "Report on Special BW Operations" by Ira Baldwin's Committee on Biological Warfare: "Large air masses are constantly moving from the polar region over certain key areas of the United States. Possibly, these air masses could be utilized for the dispersion of BW agents.[210]" In search of answers, the US Army established the appropriately-named Operation Large Area Coverage (LAC).

The goal of Operation LAC was to determine the feasibility of and the logistics involved in dispersing microscopic particles across a large geographical region. The test program involved multiple dispersals of zinc cadmium sulfide

[210] E Regis, The Biology of Doom: The History of America's Secret Germ Warfare Project.

(ZnCdS) fluorescent particles across various large swaths of the United States. In the first test, conducted on December 2, 1957, the Army dispersed the ZnCdS simulant from a cargo plane flying from South Dakota to International Falls, Minnesota. The goal of the test was to track the dispersal of the particles as a large mass of cold air descending from Canada carried them into the southern states. However, an unexpected shift in the direction of the movement of the air mass resulted in most of the simulant particles being carried into Canada. However, the fluorescent particles were detected in New York State, 1,200 miles away. A similar test was conducted in February of 1958, in which an aerial dispersal resulted in a ZnCdS line source of 200 miles. This time, the Canadian cold air mass travelled down to the Gulf of Mexico as expected. The results from the sampling stations showed that the cold front had broadened, and the line of fluorescent particles detected at the Gulf had widened to 600 miles.[211]

After determining that large air masses could indeed carry suspended microscopic particles great distances, the Army sought to investigate the dispersal of such particles under different meteorological conditions. Rather than a single large air mass with a specific overall movement direction, two additional tests were conducted in early 1958 under conditions of winds blowing in different directions along the flight paths. During the first test, a plane departed Toledo, Ohio flying south before turning west and landing in Abilene, Texas. In the

[211] US Army. "Summary of Major Events and Problems, United States Army Chemical Corps, Fiscal Year 1958," US Army Chemical Corps Historical Office, (Army Chemical Center, MD: 1959).

second test, the plane took off from Detroit, Michigan, flew to Springfield, Illinois, and landed in Goodland, Kansas. In both cases, the ZnCdS simulant particles were detected on either side of the flight path, "proving that random flight over a target area would disperse small particles widely.[212]"

In the end, Operation LAC uncovered the truth about US vulnerability to a strategic-level attack using biological weapons. If a Soviet bomber carrying biological weapons agents were to disperse its payload in conjunction with the movement of a large air mass, millions of Americans could be exposed. And while the goals of these tests were ostensibly to determine the vulnerability of the United States to a massive biological attack conducted in this manner, the data gathered clearly has significant offensive value as well.

3.8. Project 112/Project SHAD, 1963 – 1973

In terms of scope, complexity, geographic area, and the number of experiments conducted, no known series of open-air tests comes close to Project 112. In the early days of the Kennedy administration, a review of the US military authorized by Secretary of Defense Robert S. McNamara resulted in 150 different projects for study. One of these, Project 112, was for "research, testing, and development for chemical and biological weapons.[213]" Specifically, McNamara's charge to the military's Joint Chiefs of Staff was to "consider all possible applications, including use as an alternative to nuclear weapons. Prepare a plan for the development of an adequate biological and chemical

[212] Ibid.
[213] Quoted in: M Wheelis, M Dando, and L Rózsa, Deadly Cultures: Biological Weapons since 1945 (Cambridge, MA: Harvard University Press, 2006).

deterrent capability, to include cost estimates, and appraisal of domestic and international political consequences.[214]"

The operational research conducted under the auspices of Project 112 consisted of over 50 different tests, mainly in Alaska and in the Pacific. While Project 112 consisted of experiments over both land and sea, probably the most elaborate component of the research endeavor were the sea trials, which were collectively known as Project Shipboard Hazard and Defense (SHAD).[215] Project SHAD's goal was "to identify US warships' vulnerabilities to attacks with chemical or biological weapons and to develop procedures to respond to such attacks while maintaining a war-fighting capability.[216]" The US Navy conducted tests to assess the vulnerability of naval vessels by exposing them to various biological and chemical agents. Most of these were simulants, but others were actual dangerous pathogens and toxic chemicals. This section discusses only the subset of tests involving BW agents or simulants.

A series of fact sheets was published by DOD in response to requests from the Department of Veterans' Affairs (VA) regarding potential exposures of soldiers to harmful biological and chemical substances during Project SHAD. The information in these fact sheets is aggregated in Appendix 1. Based on the information released by DOD, approximately 25 tests (most involving numerous

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[214] Quoted in: ibid.
[215] J Guillemin, Biological Weapons: From the Invention of State-Sponsored Programs to Contemporary Bioterrorism (New York: Columbia University Press, 2005).
[216] Quoted in: M Wheelis, M Dando, and L Rózsa, Deadly Cultures: Biological Weapons since 1945.

individual trials) occurred in which pathogenic biological agents or simulants meant to simulate pathogens were released.

A summary of the agents and simulants used during these tests is presented in Table 3-2. Five offensive tests dispersed biological pathogenic BW agents or biologically-derived toxins. Shady Grove dispersed both *Coxiella burnetii* and *Francisella tularensis*. Red Cloud and Watch Dog dispersed both wet and dry forms of *Francisella tularensis*. These three tests included infectivity tests on monkeys. Speckled Start dispersed *Staphylococcus* enterotoxin, type B (SEB). DTC 69-75 dispersed *Puccinia graminis* var. *tritici*, an agriculturally pathogenic fungus that is the causative agent of wheat stem rust. The other twenty tests released ostensibly nonpathogenic biological simulants. The simulants used during many of the dispersal experiments during Project SHAD included: *Bacillus atrophaeus* (BG), *Escherichia coli* (*E. coli*), *Serratia marcescens* (SM), and T-3 coliphage (P). As can be seen in Table 3-2, *B. atrophaeus* was the preferred simulant and was dispersed in all but a single one of the tests. Five tests dispersed *E. coli*, and seven tests dispersed *S. marcescens*. A single test dispersed T-3 coliphage, a bacteriophage that infects *E. coli*.

Table 3-2: Summary of agents and simulants used in Project SHAD

Test #	Test Name	Year	Location	Pathogenic/Toxic Agents						Biological Simulants				Chemical Tracers			
				Coxiella burnetti (OU)	*Francisella tularensis* (UL)	*Francisella tularensis* (wet) (TT)	*Francisella tularensis* (dry) (ZZ)	Staphylococcal enterotoxin, Type B (SEB) (PG2)	*Puccinia graminis var. tritici* (TX)	*Bacillus atrophaeus* (BG)	*Escherichia coli* (*E. coli*)	*Serratia marcescens* (SM)	T-3 coliphage (P)	Zinc cadmium sulfide (FP)	Tiara	Calcofluor	Uranine dye
63-1	Eager Belle, Phase I	1963	Pacific Ocean							•							
63-1	Eager Belle, Phase II	1963	Pacific Ocean							•							
63-4	Big Jack, Phase A	1963	Ft. Sherman, Panama Canal Zone							•				•			
63-2	Autumn Gold	1963	Pacific Ocean							•							
64-1	Errand Boy	1963	Oahu, HI							•							
64-5	Night Train	1963-1964	Ft. Greely, AK							•				•			
64-6	Yellow Leaf	1964-1966	Various							•					•		
64-4	Shady Grove	1964-1965	Various	•	•					•				•			
66-8	West Side, Phase II	1965	Canada							•				•			
65-3	West Side, Phase I	1965	Ft. Greely, AK							•				•			
65-1	Copper Head	1965	Atlantic							•				•			
65-6	Big Tom	1965	Pacific Ocean							•				•			
66-6	Scarlet Sage	1966	Pacific Ocean							•							
66-13	Half Note	1966	Pacific Ocean							•	•	•		•		•	
67-7	Red Cloud	1966-1967	Ft. Greely, AK			•	•			•	•	•					
67-8	Watch Dog	1967	Ft. Greely, AK			•	•			•	•	•					
67-6	Blue Tango	1967	Ft. Greely, AK							•	•	•		•			
68-71	Folded Arrow	1968	Oahu, HI							•							
69-31	--	1968	Pacific Ocean							•							
68-50	Speckled Start	1968	Eniwetok Atoll					•		•							•
69-75	--	1968	Florida						•								
69-32	--	1969	Pacific Ocean							•	•	•				•	
70-73	--	1970	Dugway Proving Ground, UT							•				•			
70-74	--	1972-1973	Dugway Proving Ground, UT							•		•					
73-30	--	1973	Dugway Proving Ground, UT							•		•	•				
				1	1	2	2	1	1	24	5	7	1	10	1	2	1
				OU	UL	TT	ZZ	PG2	TX	BG	*E. coli*	SM	P	FP	Tiara	Calcofluor	Uranine dye

One of the most noteworthy aspects of Project SHAD was the meticulous attention paid to the environmental impact of the testing. In 1963, shortly after the first tests had been conducted, President John F. Kennedy issued a National Security Action Memorandum outlining a new approval process for "the conduct of large-scale scientific or technological experiments that might have significant or protracted effects on the physical or biological environment.[217]" The new policies stipulated that such experiments could not be conducted without prior presidential approval.

However, in order to determine if the planned Project SHAD tests would have any protracted environmental impacts, it was first necessary to establish a baseline, which would involve conducting a full biological and environmental survey. Of particular concern to the US military was the contamination of migratory birds with pathogenic agents. The fear was that the birds could then track the agents into populated areas. Because the scientific experience required to conduct such a biological and environmental survey would require expertise in niche areas (particularly ornithology) that are not typically within the purview of military, the work would have to be performed by a contractor.

In order to have the research conducted while maintaining secrecy regarding the biological weapons motivations behind the survey, the military reached out to the Smithsonian Institution. Known primarily for its various

[217] JF Kennedy. "National Security Action Memorandum No. 235: Large-Scale Scientific or Technological Experiments with Possible Adverse Environmental Effects," The White House, (Washington, DC: 1963).

museums, the Smithsonian Institution is also a scientific research institution. Because the entire Smithsonian Institution is funded by a trust administered by the US government, it would be the best choice for conducting the research with respect to the security concerns. The Smithsonian Institution and the military reached an agreement: the research findings would be completely unclassified and freely publishable. The only secret that had to be kept was the program's connection to the military, particularly the US Army's biological warfare program. Thus, the Pacific Ocean Biological Survey Program began in 1963 and provided the military with the biological and environmental information it needed to plan and perform its BW and simulant dispersals throughout the Pacific Ocean.[218] The apparent discrepancy between the environmental precautions taken during Project SHAD and other tests conducted in cities has been noted: "Ironically, given the rather cavalier approach to testing simulants in urban areas, a good deal of sensitivity was shown in the Pacific Ocean operations.[219]"

Although the dual-use nature of such experiments inevitably produces data that is both offensively and defensively valuable, the primary purpose of the tests can be extrapolated based on the information presented in the "Test Operations" sections of the Project SHAD fact sheets. It appears that six of these tests were mostly or entirely defensive in nature, fifteen were mostly or entirely offensive in nature, and four were ambiguous or could not be determined.

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[218] E Regis, The Biology of Doom: The History of America's Secret Germ Warfare Project.
[219] M Wheelis, M Dando, and L Rózsa, Deadly Cultures: Biological Weapons since 1945.

The six tests that were primarily defensive in nature were Eager Belle (Phase I), Autumn Gold, Errand Boy, Scarlet Sage, DTC Test 69-31, and DTC Test 70-73. The Eager Belle (Phase I) and Autumn Gold tests were designed to measure the vulnerability of US naval vessels by measuring the penetration of bioaerosols into ships as well as testing two different types of protective masks. An additional component of Autumn Gold, as well as the major focus of Errand Boy, Scarlet Sage, and DTC 69-31, was the testing of various washdown methods to decontaminate the exterior of ships after exposure to bioaerosols. DTC 70-73 investigated the hazards posed to friendly troops by secondary aerosolization after an attack with biological weapons.

However, the majority of the tests conducted as part of Project SHAD appear to have been primarily offensive in nature. Eight of the primarily offensive tests – Eager Belle (Phase II), Night Train, Shady Grove, Half Note, Red Cloud, Watch Dog, Blue Tango, and Folded Arrow – had the study of downwind travel or the biological decay rates of bioaerosols as their major experimental component. Four of the tests – Big Jack (Phase A), Yellow Leaf, Big Tom, and Folded Arrow – were designed to develop tactics for conducting BW attacks in specific environments. These included jungle climates, island complexes, and naval port facilities. Five of the tests – Shady Grove, West Side (Phase I), West Side (Phase II), Folded Arrow, and DTC 69-75 – were designed to determine the offensive performance characteristics of various BW dispersal systems. These

included both airborne dissemination systems and a submarine-deployed biological disseminator installed on the USS *Carbonero* (SS-337).

Another of the most critical components of most of the Project SHAD tests is the known or potential exposure of US military service members to a multitude of pathogenic agents, toxic chemicals, and not thoroughly characterized simulants. In most cases, the ships involved in the tests were fully staffed by their standard crew complement. Because the primary responsibility of military service members is to follow orders, even if that entails placing themselves in harm's way, their ability to decline participation in Project SHAD was likely nonexistent. It is not known whether the crews were even informed of the agents to which they would be exposed or of the potential risks to their health. The bioethics of this situation, particularly with regard to military service members, is discussed further in Chapter 4.3.

3.9. Washington, DC Bus and Airport Terminals, 1965

In an effort to investigate the vulnerability of the US population to covert attack with variola virus (the etiologic agent of smallpox), the US Army initiated Study US65SP. The results of Study US65SP are published in a report entitled "Miscellaneous Publication 7". Parts of the report detail characteristics of the variola virus which make it an ideal choice for covert deployment, epidemiological descriptions of confirmed or suspected smallpox outbreaks in England and Washington, DC, and travel statistics for passengers in selected American cities. However, in order to properly calculate the susceptibility to induction of a

smallpox outbreak amongst the traveling public, it would be necessary to determine the level of exposure which passengers would receive as a result of such an attack. Thus, part of Study US65SP was dedicated to conducting open-air dispersals inside busy passenger terminals. Fort Detrick's Special Operations Division selected the Greyhound bus terminal in Washington, DC, and National Airport (now known as Ronald Reagan Washington National Airport) in nearby Arlington County, Virginia, as the targets for its mock attacks.

In May 1965, SOD operatives visited the Greyhound bus terminal, carrying briefcases that concealed spray generators filled with 50 mg of dried *Bacillus atrophaeus* spores at a concentration of 7.0×10^{11} spores per gram. Five of these devices were placed at different points in the main waiting room of the terminal and used to covertly aerosolize the simulant. Other agents posing as travelers operated sampling devices similarly hidden in briefcases at various locations throughout the terminal.[220]

Shortly after the mock attack at the Greyhound bus terminal, SOD agents conducted a similar mock attack in the north terminal of National Airport. Once again, suitcase-concealed sprayers were used to aerosolize dried *B. atrophaeus* spores throughout the terminal for a total of 30 minutes. In addition to one operator who sampled the air in the center of the terminal's waiting room, several other members of the attack team with concealed air sampling device each "selected a passenger at random at the entrance to the North Terminal and

[220] US Army. "Miscellaneous Publication 7: Study US65SP," Special Operations Division, (Fort Detrick, Frederick, Maryland: 1965).

covertly collected air samples in close proximity to the passenger during his stay in the Terminal.[221]"

In all the tests, each of the samplers was operated at a rate of 15 liters per minute (which was the assumed breathing rate for the targeted passengers). In a 10-minute sampling time, all of the samplers collected between 100,000 and 1,000,000 spores, with most collecting greater than 200,000 spores. Based on the time interval during which they were inside the affected terminal, passengers would have inhaled between 12,000 and 870,000 spores. Conversion of the *B. atrophaeus* exposure levels recorded in the trials to calculated inhaled smallpox virus shows that passengers could be exposed to several ED_{50}s of smallpox.[222] Thus, Study US65SP showed that an outbreak of smallpox could be created by clandestinely dispersing variola virus amongst unsuspecting travelers within commercial passenger terminals.

3.10. New York City Subway System, 1966

A year after the tests in Washington, DC, the SO Division conducted a series of dispersal trials to determine whether American subway systems were vulnerable to a clandestine attack with a biological agent. Rumors had existed for decades that Germany successfully tested a mock attack (using the simulant *Serratia marcescens*) on underground subway stations in France and the UK. Wickham Steed, a British journalist, published an article claiming that German agents dispersed the bacteria into the air outside various subway stations,

[221] Ibid.
[222] Ibid.

including the Place de la Concorde station of the Paris Metro and the Piccadilly Circus Underground station in London. The results, according to Steed's article, showed significant enough penetration into the stations that such a method could prove a highly successful BW attack. Although the claims Steed published remain unsubstantiated (and are considered by some to be entirely false[223]), they drew significant attention to a purported vulnerability and played a large part in launching the British BW program.[224]

However, the question remained: was a BW attack on subway systems feasible? The US Army suspected that "covert attacks with a pathogenic agent during peak traffic periods could be expected to expose large numbers of people to infection and subsequent illness or death.[225]" So Fort Detrick's SOD devised a study to provide a definitive answer. The designated target for the experiments was the subway system of New York City. As in the previous tests in Washington, DC, *Bacillus atrophaeus* was the dispersed simulant. The stated objectives of the study were "to provide information on (i) agent distribution and concentration in order to assess threat of infection to subway passengers, (ii) ease of agent dissemination in the system, and (iii) methods of delivery that could be used offensively.[226]"

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[223] M Hugh-Jones, "Wickham Steed and German biological warfare research," *Intelligence and National Security*, 7, 1992.
[224] J Guillemin, Biological Weapons: From the Invention of State-Sponsored Programs to Contemporary Bioterrorism; E Regis, The Biology of Doom: The History of America's Secret Germ Warfare Project.
[225] US Army, Miscellaneous Publication 25: A Study of the Vulnerability of Subway Passengers in New York City to Covert Attack with Biological Agents.
[226] Ibid.

From June 6 – 10, 1966, the SOD dispersed *B. atrophaeus* into several stations along three major subway lines in midtown Manhattan: the Seventh Ave., Eighth Ave., and Lexington Ave. lines. These particular lines were chosen for the tests "because of the heavy traffic and the number of lines available for tests.[227]" Two different types of tests were conducted. Two of the tests used E40 aerosol generators to produce a cloud of *B. atrophaeus* spores outside the subway stations. The cloud was drawn into the underground stations through sidewalk gratings by the pressure differential caused by trains departing the stations. The other set of three tests used a more ingenious and clandestine method of generating and dispersing the spore aerosol cloud. The SOD developed a device that consisted of a light bulb filled with 175 grams of dried *B. atrophaeus* spores (at a concentration of 5.0×10^{11} spores per gram) and 30 grams of charcoal. (The charcoal was added to darken the powder, making the mixture it less noticeable on the subway roadbed.) The agents dropped the light bulbs directly onto the tracks, which shattered them and released the powder. The movement of the trains over the deposited spore powder aerosolized it, and the pressure differentials caused by the arriving and departing trains pushed and sucked the clouds down the tunnels into stations progressively further down the subway lines in both directions.[228]

In all three of the light bulb-delivered trials, the agent was detected within 10 minutes in stations throughout the testing area. The aerosolized spores

[227] Ibid.
[228] Ibid.

reached their highest concentrations within the first 15 – 30 minutes for each station and persisted for at least between 1.5 – 2 hours. The results also showed that the aerosol clouds penetrated into the train cars, exposing the passengers. However, "the operative who dropped the agent package from the train received little exposure after its release[229]", demonstrating that such a technique could be used to covertly contaminate subway systems with little risk to the saboteur. These dispersals of *B. atrophaeus* spores "were conducted as completely independent operations without the knowledge or cooperation of the New York City Transit Authority or Police Department.[230]" In the event that the SOD agents conducting the tests were question regarding their activities, they carried cover letters which identified them as industrial research organization members conducting tests. However, the agents' dissemination and air sampling occurred without attracting attention or questions.

The conclusions of the study were as clear as they are stark in their implications:

> "Dropping an agent device onto the subway roadbed from a rapidly moving train proved an easy and effective method for the covert contamination of portions of subway lines. Agent delivered in this manner was aerosolized and dispersed rapidly by the movement of trains, penetrating stations and trains in the area and persisting there for one hour or more... Test results show that a large portion of the working population in downtown New York City would be exposed to disease if one or more pathogenic agents were

[229] Ibid.
[230] Ibid.

disseminated covertly in several subway lines at a period of peak traffic.[231]"

As with most of the tests discussed in this chapter, the little information that has been declassified shows no indication that any measures were taken to monitor for any effects on either the environment or human health that may have occurred as a result of intentionally dispersing trillions of bacterial spores into heavily populated areas.

3.11. Boston MBTA, 2012

The majority of open-air experiments such as the ones that have been described in previous sections of this chapter took place decades ago during the era of the US biological weapons program. However, in response to the increased threat from bioterrorism, the US government has once again begun limited open-air tests. This section concludes this chapter's descriptions of open-air tests with a description of a recent series of tests conducted in the greater Boston, Massachusetts area. However, the way these tests were conducted provides a stark contrast to the ones described in previous sections.

This series of tests was conducted in the greater Boston subway system, known as the Massachusetts Bay Transportation Authority (MBTA), or colloquially as the "T". Beginning on August 29, 2012, the Science and Technology Directorate (S&T) of the Department of Homeland Security (DHS) conducted aerosol dispersals of *Bacillus subtilis* spores in order to test newly-installed sensor systems designed to detect biological weapons agents. It was

[231] Ibid.

proposed that actual releases of spores into several T stations occur in order to properly challenge the detectors under realistic scenarios. Three stations along the MBTA's Red Line were selected for the series of tests: the Davis station in Somerville and the Porter and Harvard stations in Cambridge.

In January 2012, DHS S&T published an environmental assessment of potential test options and their impacts. The assessment detailed four potential action alternatives as to how the experiments could be conducted. In Alternative 1, a dry air pump would be used to aerosolize a powdered formulation containing between 7.5×10^{10} and 1.5×10^{12} spores of viable *Bacillus subtilis* "at peak operational capacity for trains and passengers... to most closely simulate the conditions that would likely exist in the event of a true bio-terrorist attack.[232]" After the initial aerosolization from the pump, the spore cloud would be further dispersed by the movement of the trains in and out of the stations. The second option would occur in the same operational conditions (peak operational capacity with passengers) as in Alternative 1, but the *B. subtilis* spores would be killed by gamma-irradiation prior to release. Thus, the dispersed material would be noninfectious and more akin to a particulate nuisance material such as dust. This would allow the testing of the system in a "real use" setting, but would avoid exposing passengers to biologically active material. In the third option, a similarly killed spore preparation would be dispersed but would occur after-hours

[232] US Department of Homeland Security. "Environmental Assessment for *Bacillus subtilis* Particles to Challenge Bio-Detection Sensors in Subway Stations," Science and Technology Directorate, (Washington, DC: 2012).

with no passengers in the station. Although the trains would be run on a schedule designed to simulate peak-hour activity, the absence of passengers arriving and departing from the stations would alter the test conditions slightly. The fourth alternative involved aerosolizing the live *B. subtilis* spore preparation directly into the air intake of one of the biological sensors without dispersal into the station.[233]

After detailing each of the alternatives, the environmental assessment provides an analysis of the potential consequences on human health and safety for each of the alternatives. The entirety of the section dedicated to the analysis of Alternative 1 consists of only two sentences that indicate that the risks to a small number of people are unacceptable and seem to imply that this option was never a serious consideration:

> "The presence of riders from sensitive populations groups during testing presents additional health factors that must be considered for a safe and effective test for all subway patrons. While the probability that an infection of a vulnerable subway rider may occur is very low due to the small number of spores proposed to be released in the station during testing, **the consequences of any infection caused by the proposed testing are not acceptable** and, as such, the use of viable spores in open air challenge testing of the biosensor system is not recommended.[234]" [emphasis added]

The assessment notes that killing the bacterial spores, as proposed in Alternatives 2 and 3, results in an organism that is biologically nonviable, eliminating "the potential for the spore to act as an opportunistic bacterium and

[233] Ibid.
[234] Ibid.

be the causative agent of a bacterial infection.[235]" It further indicates that because the effect that passenger movement has on aerosol dispersal is negligible relative to the effect from movement of trains, "Alternative 3 will not substantively change the performance evaluation but will reduce the risk of high dermal or inhalation exposure of the public to the particulate material.[236]" The assessment concludes that the option that best balances the risk to the public with the benefit of testing the detection sensors with the most realistic situation is Alternative 3, the dispersal of killed spores during after-hours periods when the MBTA system is closed to the public.

In stark contrast to many of the other tests described in this chapter, significant efforts were made in order to provide the residents of the Boston area with sufficient information ***before*** the tests were begun. The environmental assessment was posted online with an opportunity for the public to provide input via E-mail. Additionally, a public forum was held in Cambridge in May 2012. The story was covered extensively in local and national news media in late April and early May 2012. A press release dated August 27, 2012 announced: "These tests will begin on August 29, 2012 when the MBTA stations are closed to the public, and will continue periodically over the next year. Signs will be posted in the MBTA stations one day before each scheduled test.[237]" While it may not be realistically feasible to obtain informed consent from every single person

[235] Ibid.
[236] Ibid.
[237] US Department of Homeland Security. "Press Release: DHS, MBTA to Begin Series of Tests for Rapid Biological Response Sensors," (Washington, DC: 2012).

throughout the subway system who might be exposed to the test materials, it is possible to disseminate the relevant information so widely that everyone who might be exposed as a result of the testing is aware of a test's occurrence in advance. This appears to be the approach taken by DHS in the case of the MBTA tests. Presumably, their opinion is that riders who wish to avoid potential contact with the dispersed simulants can forego using the T on the specific days following the tests. While it would no doubt inconvenience these specific passengers to arrange for alternate transportation on the relevant days, this approach arms them with all the information necessary to make an informed decision about the situation and their potential exposure to it. Assuming that the information is adequately disseminated to all potential passengers sufficiently in advance, anyone choosing to ride the T on days following dispersal tests could be considered to be providing a "*de facto* informed consent." (The complications inherent to such large-scale tests that might expose such a large number of people as to make collecting traditional informed consent difficult or impossible is discussed further in Chapter 4.2.)

The environmental assessment provides valuable insight into the various options considered for the tests and the decision-making leading to the selection of Alternative 3. It is quite clear from the discussion of the options that the safety of the riders was DHS S&T's top priority. The assessment lists a multitude of facts supporting the claim that *B. subtilis* is a safe simulant and that exposure to the small amounts to be dispersed is unlikely to cause any harm to the public.

However, they then proceed to address the concern that a very small minority of riders might be particularly susceptible to infection by the bacterium. DHS concludes that even the extremely unlikely probability that even a small number of passengers might become infected as a result of the testing is too great a risk. As a result, the assessment proposes combining the two safest alternatives – killed spores and after-hours dispersal – that can still accomplish a realistic challenge test for the new biosensors.

Because the particular biosensors tested in these experiments were capable of detecting killed spores, it was not necessary to use viable organisms for these tests. Although the operational details of these biosensors have not been publicly released, it can be assumed based on their viability-independent nature that they likely function using a PCR- or immunoassay-based technology. All the tests described in the previous sections of Chapter 3 occurred prior to the invention of technologies such as PCR, which was first described in 1986.[238] Because the only detection method available for the earlier dispersal tests was culturing, viable simulants were required. This raises a very salient question: if UV-irradiated spores are still detectable, why bother trying to improve the safety of biologically viable simulants? In some cases where the simple detection of the physical presence of an organism is desired, such as in the MBTA tests, killed spores would function equally well. However, as was discussed in Chapter 2.4, many types of tests might require viable organisms. For example, any viability

[238] K Mullis *et al.*, "Specific Enzymatic Amplification of DNA *In Vitro*: The Polymerase Chain Reaction," *Cold Spring Harb Symp Quant Biol*, 51 Pt 1, 1986.

reduction countermeasures such as UV or gamma irradiation, would require live organisms. Because such techniques work by reducing the number of viable cells, killed spores such as those used in the MBTA tests could not be used: the number of bioaerosol particles would remain the same in both the pre- and post-countermeasure samples. Using viability (i.e., bacterial growth) as the parameter of detection allows the effects of the countermeasures in reducing the numbers of viable bioaerosol particles to be determined.

Various aspects regarding the way the MBTA open-air dispersal testing was conducted differentiate it from the tests described in previous sections of this chapter. First, various options were considered, and the least likely to put the public at risk was selected. Most importantly, the threshold for acceptable risk tolerance was set extremely low. Despite the proposed dispersal of a very safe simulant, spores of viable *B. subtilis* were judged to not be safe enough. The ultimate selection of experimental design was geared to protect the public in two ways: by using biologically inactivated spores and by dispersing them during the overnight hours after the closure of the T to passengers. Secondly, extensive information was provided to the potentially exposed public well in advance of the performance of the tests. This included an official assessment that described the proposed tests in sufficient detail as to be scientifically accurate and informative but still understandable to the layperson, public forums, press releases, and an extensive use of the media to inform the public about the tests. Thirdly, although there is no evidence of a traditional informed consent process, the detailed

information provided months prior to the tests and notification of riders on the days immediately preceding the tests enabled the public to make a decision as to their exposure to the simulant material – essentially a "*de facto* informed consent" process. The way in which the dispersal of *B. subtilis* spores to test biosensors within the MBTA system was conducted should be considered a model for the proper way in which such open-air dispersals should be conducted in the future.

3.12. Conclusions

The research priorities proposed in the 1948 "Report on Special BW Operations" (the Baldwin Report) set the course for both offensive and defensive biological weapons research in the United States from 1949 to 1973. Many of the experiments involved open-air testing of BW agents or their simulants, often with the intentional exposure of humans. For the myriad tests that exposed American civilian populations, there is no evidence that any type of informed consent or even dissemination of information occurred. Quite the contrary: many of the tests involved deliberate deceptions and cover stories to deceive the public. Those populations who are known to have been exposed, such as San Francisco, St. Louis, New York, and Washington, DC, have generally not learned of these experiments until several decades after they were conducted.

Although the United States' offensive BW program was terminated in 1969, defensive research into the threats posed by biological weapons has flourished under the banner of biodefense in the years since September 11,

2001, and the subsequent anthrax mailings. While most of this research is occurring in laboratories, we have once again become concerned about our vulnerability to bioterrorist attacks causing mass casualties, leading to a resumption of some open-air testing. Understanding the aerosolization and dispersal of biological agents, ensuring that aerosolized material can be properly detected and identified, and how to prevent exposure or decontaminate pathogenic environments are all critical components of biodefense. Fully characterizing the threat may in some cases require open-air testing.

It is clear that biodefense research by the US government involving open-air releases of biological agents and/or simulants has occurred in the past and is once again continuing today. It is important to understand whether the government has learned from its previous tests and is taking all reasonable precautions to ensure that the safety of the public and the environment are not jeopardized by these tests. Although the MBTA tests described in the previous section provide only a single data point with which to contrast the decades of clandestine intentional exposures, the deliberate precaution, informational dissemination, and public transparency with which the Boston tests were conducted suggest that perhaps attitudes toward the intentional exposure of the American public to potentially harmful substances without their knowledge has shifted dramatically for the better.

As this chapter has shown, dispersal of biologically viable simulants exposes populations (both intentionally targeted and collaterally exposed) to

potentially fatal opportunistic pathogens. Chapter 4 addresses specific flaws of the simulant systems used in the open-air tests described in this chapter and presents improvements that could be incorporated into these systems for the purpose of eliminating these flaws, resulting in a simulant system with increased utility, fidelity, and safety.

4. US TESTING CRITICAL ANALYSIS AND PROPOSED IMPROVEMENTS

With a historical chronology of previous simulant systems completed, the problems with those systems and the way they were used can be addressed. This chapter will focus on three aspects of the open-air tests described in Chapter 3. First, the biological pathogenicity of the agents or simulants dispersed will be discussed. Second, the varying attention given to arguably the most the critical component of human biomedical experimentation – informed consent – will be examined for each of these tests. Third, for those tests in which members of the military were exposed in the course of the experiments, the specific concern of military bioethics will be discussed. Table 4-1 summarizes how the tests described in Chapter 3 were performed with respect to each of these three issues. Each test/issue intersection contains two pieces of information. The first is the answer to the main question for the issue, respectively:

- Pathogenicity: Did this test series disperse an organism with known or potential pathogenicity?
- Informed Consent: Did this test series obtain informed consent (in some manner) from those human subjects exposed?

- Military Coercion: If military personnel were intentionally exposed during the test series, were efforts made to ensure they were not ordered or coerced into participating as human research subjects?

The second is a judgment as to whether the test was conducted properly (i.e., consistent with a current understanding of how the issue should be handled) or improperly (i.e., not consistent with a current understanding of how the issue should be handled) with respect to the issue.

Table 4-1: Ethical summary of open-air tests

Test	Pathogenicity[239]		Informed Consent		Military Coercion	
Pentagon	Yes	Improper	No	Improper	No	Proper[240]
Virginia	Yes	Improper	No	Improper	No	Improper
Operation Sea Spray	Yes	Improper	No	Improper	n/a	
Project St. Jo	No	Proper	No	Improper	n/a	
Project Whitecoat	Yes	Proper[241]	Yes	Proper	Yes	Proper
Operation LAC	No	Proper	No	Improper	n/a	
Project SHAD	Yes	Improper	No	Improper	No	Improper
Washington, DC	Yes	Improper	No	Improper	n/a	
NYC Subway	Yes	Improper	No	Improper	n/a	
MBTA	No	Proper	Yes	Proper	n/a	

n/a: not applicable.

[239] The pathogenicity listed refers to the major component of a test or the majority of tests in a test series.

[240] These tests occurred without the knowledge or consent of those exposed; therefore, military personnel served as research subjects but were not ordered or actively coerced into doing so. (See Chapter 4.1.1.1.)

[241] The pathogenicity of the organisms used was balanced by the informed consent process. (See Chapter 4.3.2.)

It should be noted that critical analyses such as this are typically conducted in such a manner as to avoid the judgment of historical events through the lens of modern-day ideals and information. This is typically known as the historian's fallacy. However, this chapter is less about a historical retrospective than it is about asking how biodefense research should be conducted in the future. The nature of the biological warfare and bioterrorism threat demands that research into detection, vulnerabilities, countermeasures, and therapeutics continue. It is incumbent upon science and policy, however, to examine their past methodologies and ask whether they are acceptable today. Is there anything ethically questionable, for example, about secretly releasing potentially pathogenic bacteria into the Pentagon or the New York subway system unbeknownst to all those inside? Whatever the justification at the time of the historical experiment, our modern understanding of science and ethics ***should*** be the basis of our judgment. To do otherwise risks the opposite fallacy, that is, the acceptance of methods that are unacceptable by the standards at the time of analysis (i.e., now). Thus, the tests are examined through the lens of the following question: if a dispersal experiment was conducted today, could it ethically be performed in this manner?

This chapter ends by providing an analysis of improvements that could be incorporated into the simulant systems discussed in Chapter 3 that would increase their utility, fidelity, and safety: in other words, what would be required of

a biological simulant in order to avoid all the problems described? This provides a direct segue into the proposed biological solution presented in Chapter 5.

4.1. Pathogenicity

The most critical component to the safe conduct of an open-air test with a biological simulant is its pathogenicity. In nearly all of the tests that dispersed a biological simulant, the presence of the organism was assayed by the ability to culture the organism. As such, an important characteristic of these agents and simulants is their biological viability. Without using viable organisms, the ability to culture (and therefore detect) whether the organism is present in a form that has retained its biological survivability after a dispersal would be eliminated. However, the fact that the organism is viable also means that it can survive and establish a culture in any environment it finds suitable – including any in which growth was unintended. Such locations could include environments within the human body that might be conducive to establishment of biological infections, such as the lungs or open wounds. This is of particular concern with individuals who are immunocompromised for any variety of reasons and would have a diminished ability to respond to and fight off any potential infections.

4.1.1. Tests with Viable Biological Organisms

The tests that dispersed viable biological agents can be divided into two categories. The first category includes those tests which intentionally dispersed organisms with known significant pathogenicity, while the second includes those tests which dispersed ostensibly safe biologically viable simulants. In general,

intentionally exposing individuals or groups of people to pathogenic organisms or biologically-derived toxins is considered highly unethical. If the people of one country were subjected to such an event by the government of a second country, the latter nation would be charged with waging biological warfare. Such methods are considered so abhorrent that the Biological Weapons Convention (BWC) was ratified by nearly every nation in the world to ensure that such weapons would not be used. The net effect of the BWC is to say this: wars will occur and people will kill each other, but the use of disease as a weapon is a barbarism that simply is unallowable. On a subnational scale, the cases of the 1984 Rajneeshees infections with *Salmonella enterica* and the 2001 anthrax letters illustrate that such intentional exposures are considered bioterrorism. On an individual level, thirty-three states in the US have a total of sixty-seven laws which criminalize knowingly infecting another person with HIV, known as criminal transmission of HIV.[242] Although the laws are controversial (based on their alleged discrimination against HIV-positive individuals[243]) and vary greatly between the states (ranging in severity from a misdemeanor to a first-degree felony[244]), the existence of such laws is clearly consistent with the idea that disease agents should not be used as a means of assault. Therefore, it is obvious that a clear

[242] JS Lehman *et al.*, "Prevalence and Public Health Implications of State Laws that Criminalize Potential HIV Exposure in the United States," *AIDS and Behavior*, 18(6), 2014.
[243] R Bennett-Carlson, D Faria, and C Hanssens, "Ending & Defending Against HIV Criminalization: A Manual for Advocates: State and Federal Laws and Prosecutions." (New York: CHLP's Positive Justice Project, The Center for HIV Law and Policy, 2014), http://www.hivlawandpolicy.org/sites/www.hivlawandpolicy.org/files/Criminalization%20Manual%20%28Revised%205.12.14%29_0.pdf.
[244] "HIV Criminalization: State Laws Criminalizing Conduct Based on HIV Status," (New York: Lambda Legal, 2010).

ethical norm exists against intentionally exposing people to infectious agents against their will.

4.1.1.1. Tests with Known Pathogens

As described in Chapter 3.8, five of the test series that made up Project SHAD – Shady Grove, Red Cloud, Watch Dog, Speckled Start, and DTC 69-75 – dispersed biological pathogens or biologically-derived toxins. These included *Coxiella burnetii*, *Francisella tularensis*, *Staphylococcus* enterotoxin, type B (SEB), and the agricultural pathogen *Puccinia graminis* var. *tritici*. The first two agents cause the generally nonlethal and treatable diseases Q fever and tularemia, respectively. SEB also causes severe but temporary incapacitation, and *Puccinia graminis* var. *tritici* is only known to infect cereal species such as wheat, barley, and rye.[245] The fact that these agents at worst cause nonlethal diseases in healthy humans is likely a factor in the US military's determination that exposing many of its own service members to these agents was acceptable in the name of BW research. Although there are no known cases of human infection or toxicity with these agents during these tests, the open-air dispersal of these agents and the microbiological techniques involved in their detection (e.g., culturing) inherently introduced a considerable level of risk to any of the personnel participating in Project SHAD. Based on the information released by the US government about these tests, there is no indication that the participants in these five Project SHAD experiments were a) informed of the pathogens and

[245] KJ Leonard and LJ Szabo, "Stem rust of small grains and grasses caused by *Puccinia graminis*," Molecular Plant Pathology, 6(2), 2005.

toxins to which they would be exposed and their potential effects, b) asked for consent to being exposed, or c) given any choice in the matter. (These latter two topics will be explored further in Chapters 4.2 and 4.3, respectively.)

Operation CD-22 and Project Whitecoat (Chapter 3.6) also exposed members of the military to a variety of pathogenic agents, including the etiological agents of tularemia, Q fever, typhoid fever, Rocky Mountain spotted fever, Rift Valley fever, sandfly fever, Eastern equine encephalitis, Western equine encephalitis, and Venezuelan equine encephalitis (EEE, WEE, and VEE, respectively).[246] However, this test program should be considered to be a special case, quite different from the exposures that occurred as a part of Project SHAD. The single biggest difference between Operation Whitecoat is the fact that it was designed from the beginning to be a completely voluntary program, as detailed in Chapter 3.6. This allowed experimentation on humans with otherwise dangerous organisms because the potential effects of the tests were fully disclosed in advance and the test subjects' participation was strictly voluntary. Thus, the pathogenicity of the organisms used was in a way balanced by the very clear informed consent process: the subjects ***chose*** to be exposed to pathogenic agents.

The major issue with the use of pathogenic agents in Project SHAD is that the nature of the exposures may have been unknown to those who participated in the tests. If this is the case, then these experiments were necessarily

[246] RL Mole and DM Mole, For God and Country: Operation Whitecoat: 1954 – 1973.

conducted without the consent of those exposed. Such a situation would be considered extremely unethical by any modern bioethical standard. In contrast, during Project Whitecoat those who were exposed to sublethal doses of pathogenic agents knew exactly what agents they were being exposed to, what the risks were, and the medical treatment that would be administered post-infection. Most importantly, the people exposed provided their voluntary and uncoerced consent. Therefore, while Project Whitecoat unquestionably exposed military service members to pathogenic agents, it did so in a highly responsible and proper manner.

4.1.1.2. Tests with Viable Simulants

The second category of tests that dispersed biological agents includes those tests which dispersed ostensibly safe biologically viable simulants, incapable of acting as pathogens. However, the existence of a truly nonpathogenic biological organism is debatable. According to Dr. Richard Goldstein "...any normally 'nonpathogenic' microbe... under certain conditions... can be pathogenic. Any simulant is potentially a pathogen.[247]" These simulants, then, are better considered to be biologically viable potential pathogens. Therefore, all the viable biological organisms discussed in this section are considered to be potentially pathogenic.

Serratia marcescens was one of the commonly used biological simulants in the early years of the US BW research program. The dispersals of *S.*

[247] LA Cole, Clouds of Secrecy: The Army's Germ Warfare Tests over Populated Areas.

marcesens into the ventilation system of the Pentagon that occurred in 1949 and 1950 (Chapter 3.2) released the bacterium into the air breathed by thousands of high-ranking officials of the US military. The 1950 dispersals off the coast of Virginia (Chapter 3.3) exposed the crews manning the aircraft carrier USS *Coral Sea* and the destroyer USS *Kenneth D. Bailey*, as well as the cities of Hampton, Newport News, and Norfolk, to aerosolized *S. marcesens*. The most publicized dispersals of *S. marcesens* (as a result of the death of Edward Nevin and the resulting lawsuit) were conducted as a part of Operation Sea Spray off the coast of San Francisco (Chapter 3.4). Despite medical evidence dating back to 1913 documenting the pathogenicity (albeit at a low level) of *S. marcesens*, those who selected this organism and conducted the tests acknowledged that it harbored pathogenic potential at "extremely large doses" and yet still considered it to be nonpathogenic.[248] Although it appears that the strain dispersed during Operation Sea Spray and the strain which led to the death of Edward Nevin possessed very different biological characteristics and therefore could not have been the same, the mere possibility of infection from an intentionally released pathogen should have given those planning the dispersal tests pause. Similarly, seven of the tests during Project SHAD dispersed *S. marcescens*: Half Note, Red Cloud, Watch Dog, Blue Tango, DTC 69-32, DTC 70-74, and DTC 73-30. The fact that those planning these experiments were aware of some level of pathogenicity and still chose to disperse the bacterium into the US military headquarters, into groups of

[248] US Army, Special Report 142: Biological Warfare Trials at San Francisco, California, 20-27 September 1950.

unsuspecting American service members, and over American cities suggests that they exercised poor judgment in the planning and execution of these tests.

Another organism that has been widely used as a biological simulant for dispersal studies is *Bacillus atrophaeus*. *B. atrophaeus* is a Gram-positive, spore-forming, rod-shaped bacterium that is in the same genus as *Bacillus anthracis*. As such, it shares many microbiological characteristics with the etiological agent of anthrax. As a Gram-positive bacterium, its vegetative cells are more resistant to environmental stresses than are Gram-negative bacteria such as *S. marcesens*. More importantly, it can form the same type of hardy spores as *B. anthracis*. These spores have similar aerobiological properties as anthrax spores, making them an excellent and far safer (albeit not completely safe) substitute. In addition to *S. marcesens*, both the Virginia naval tests and Operation Sea Spray also dispersed *B. atrophaeus*. All of the Project SHAD tests except for one, DTC 69-75, dispersed *B. atrophaeus*. Both of the tests in Washington, DC, (Chapter 3.9) and in the New York subway system (Chapter 3.10) dispersed *B. atrophaeus*, exposing passengers of the respective transportation systems to potential infection.

While these two organisms account for the majority of the ostensibly safe simulants used, two other simulants were utilized during various Project SHAD tests. Five different Project SHAD tests dispersed *Escherichia coli*: Half Note, Red Cloud, Watch Dog, Blue Tango, and DTC 69-32. Although *E. coli* is a natural commensal component of the human intestinal flora, a significant number

of pathogenic *E. coli* strains have been identified and have been responsible for a large number of illnesses and fatalities. A single Project SHAD test, DTC 73-30, dispersed T3 bacteriophage, a virus that infects *E. coli*. Although it is technically a pathogen, it is only pathogenic to *E. coli*. By nature of its very specific host range, T3 would not be expected to cause any detrimental health effects in humans. Therefore, because this dissertation is concerned on ***human*** pathogenicity, the use of T3 bacteriophage is considered to be a simulant rather than a known pathogen.

4.1.2. Tests without Viable Biological Organisms

Project St. Jo (Chapter 3.5) and Operation LAC (Chapter 3.7) both dispersed fluorescent particles of zinc cadmium sulfide (ZnCdS) as the sole experimental simulant. Ten of the Project SHAD tests dispersed ZnCdS: Big Jack (Phase A), Night Train, Shady Grove, West Side (both Phases I and II), Copper Head, Big Tom, Half Note, Blue Tango, and DTC 70-73. Although the potential health effects of ZnCdS have been questioned (and assessed by experts[249]), Chapter 4.1 is focused on the biological pathogenicity of simulants. Additionally, four Project SHAD tests dispersed other chemical tracers: Yellow Leaf dispersed a chemical called Tiara, Half Note and DTC 69-32 dispersed calcofluor, and Speckled Start dispersed uranine dye. Because these simulants are chemical compounds rather than microorganisms, for the purposes of this discussion, they are nonpathogenic simulants.

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[249] National Research Council: Subcommittee on Zinc Cadmium Sulfide, "Toxicologic Assessment of the Army's Zinc Cadmium Sulfide Dispersion Tests." (Washington, DC: National Academy Press, 1997).

The recent experiments in the MBTA subway system in Boston (Chapter 3.11) dispersed killed spores of *Bacillus subtilis*. Although this bacterium is clearly a biological agent, the spores were gamma-irradiated such that they lost all viability. Therefore, they are better considered as a nonbiological particulate simulant rather than a biological organism.

4.1.3. What is the Ideal Biological Simulant?

In a 1977 report to Congress entitled "US Army Activity in the US Biological Warfare Programs," the Army defined biological simulants as "living microorganisms, not normally capable of causing infection, representing the physical and biological characteristics of potential microbiological agents and considered medically safe to operating personnel and surrounding communities.[250]" This excellent definition captures the major requirements that an ideal simulant of a biological agent should meet. Parsing the definition allows its distillation into three criteria.

The first criterion is encompassed by the phrase "representing the physical and biological characteristics of potential microbiological agents..." Quite simply, the simulant should be a reasonably accurate representation of the pathogen to be modeled. Ultimately, the properties of the simulant should be as close as possible to the organism being simulated. Although some relevant data may be gathered from research using dissimilar simulants, the question must be asked: how accurately does the simulant model the organism being simulated?

[250] US Army, US Army Activity in the US Biological Warfare Programs.

After all, a simulant that varies too significantly in its characteristic properties from the agent of interest is of little value as a simulant, as it cannot be considered a reasonable approximation. The perfect simulant for *B. anthracis*, for example, would have closely matching physical and biological properties, while being medically safe. The similarity of *B. anthracis* spores to *B. atrophaeus* spores, including viability, was likely a large motivator for the selection of the latter as a simulant in many biological weapons dispersal tests, including those discussed in Chapter 3. The second criterion stems from the desire for maximizing similarity between the agent and the simulant: the simulant should be biological in nature, as opposed to the non-biological simulants described in Chapter 2.3. By using a viable biological agent, the biological effects of the environment, the dispersal, and any countermeasures can be analyzed. However, the use of a live agent introduces the possibility that the simulant could colonize undesired environments. This poses a serious potential danger if immunocompromised individuals may be exposed to the simulant, particularly in high doses. This was allegedly the case with the *Serratia marcescens* that infected Edward Nevin in San Francisco (Chapter 3.4), although the experiments and his death appear to not be causally linked. The third critical characteristic – and the most important for safety – is addressed twice within the definition: “not normally capable of causing infection” and “considered medically safe to

operating personnel and surrounding communities.[251]" Thus, a biological simulant should have an extremely low pathogenicity, ideally none at all.

The biological simulant systems previously used in military experiments included ostensibly nonpathogenic microbes. However, it is unlikely that a truly nonpathogenic biological organism exists. Echoing the statement of Dr. Richard Goldstein (Chapter 4.1.1.2), Dr. George H. Connell of the US Centers for Disease Control and Prevention (CDC) testified to Congress, "There is no such thing as a microorganism that cannot cause trouble... If you get the right concentration at the right place, at the right time, and in the right person, something is going to happen.[252]" The risks from otherwise innocuous organisms are greatest in populations with severe immunodeficiencies.

From the standpoint of pathogenicity, the ideal simulant is one that is completely nonpathogenic. However, the use of nonviable simulants limits their research usefulness, necessarily eliminating the study of any defensive equipment or techniques that function on the basis of eliminating or reducing viability. For example, if there is concern that a pathogen could be released into the ventilation system of an office or government building, one countermeasure might be to install some type of irradiation equipment upstream of the vents exhausting into the occupied areas. The intention would be to kill any introduced pathogens so that, even if they were inhaled, the organisms would be dead and incapable of causing infection. However, testing the installed system to validate

[251] Ibid.
[252] LA Cole, Clouds of Secrecy: The Army's Germ Warfare Tests over Populated Areas.

that it performs as intended is not possible without a biologically viable organism to kill. But again, the use of viable microorganisms carries the risk of unintended infections.

Thus, it would seem that the ideal simulant should be both biologically viable and nonviable, two requirements which are clearly mutually exclusive. No *naturally-occurring* viable system exists that can safely be considered completely nonpathogenic. However, if a biologically viable system could be created that could adequately address each of the aforementioned concerns, most importantly the requirement for a reasonably complete lack of pathogenicity, it could be considered to be a safe simulant.

4.2. Informed Consent

A great deal of literature concerning informed consent has been written. This section reviews opinions on informed consent, focusing on its origins in autonomy, and its role in biodefense research.

4.2.1. Autonomy as the Origin of Informed Consent

Before specifically addressing the principle of informed consent, it is necessary to review one of the fundamental principles of medical ethics and bioethics. Of the four primary principles forming the basis of modern bioethics – respect for autonomy, beneficence, nonmaleficence, and justice[253] – respect for autonomy is often considered a *primus inter pares* (that is, "first among

[253] TL Beauchamp and JF Childress, Principles of Biomedical Ethics (New York; Oxford: Oxford University Press, 2012).

equals").[254] Autonomy is the capacity of individuals to independently make their own choices and determine their own actions: "autonomy or self-determination reflects the cognitive capacity of human beings to legislate or determine their own ends and vision of the good life. As individuals contemplate these ends and the means necessary to realize them, they exercise the freedom to shape their own lives.[255]" Respect for autonomy, then, is understanding that individuals have the right of self-determination and allowing them the free exercise of this right. Respect for autonomy – akin to a *laissez-faire* philosophy regarding the actions of individuals – naturally has its limits. Nearly all legal systems, at a bare minimum, impose restrictions upon the ability for an individual's actions to infringe upon the respect for another individual's autonomy.

The principle of respect for autonomy is closely related to the philosophical position of libertarianism.[256] During the Enlightenment, John Locke, one of the original modern libertarian philosophers, published his classic work Two Treatises of Government. In the Second Treatise, Locke bases his discussion of the personal property rights of man on the natural right of self-ownership: "Though the earth, and all inferior creatures, be common to all men,

[254] B Jennings, "Autonomy," in The Oxford Handbook of Bioethics, Ed. B Steinbock (Oxford; New York: Oxford University Press, 2009).
[255] ML Gross, Bioethics and Armed Conflict: Moral Dilemmas of Medicine and War (Cambridge, MA: MIT Press, 2006).
[256] Despite the similarity in name, the basic tenets of libertarian philosophy greatly predate the modern political ideology of the same name, which espouses essentially analogous views: "Libertarianism is the view that each person has the right to live his life in any way he chooses so long as he respects the equal rights of others." (D Boaz, Libertarianism: A Primer (New York; London; Toronto: The Free Press, 1997).) The political Libertarian ideal of a limited, minimally obtrusive government stems from a respect for the autonomy of individuals.

yet every man has a *property* in his own *person*: this [nobody] has any right to but himself.[257]" [italics in original]

Understanding the nature of personal autonomy as the essential cornerstone underpinning informed consent in biomedical research warrants quoting at length from the most recent edition of the seminal work of Beauchamp and Childress, Principles of Biomedical Ethics:

> "The word *autonomy* [is] derived from the Greek *autos* ('self') and *nomos* ('rule,' 'governance,' or 'law')... At a minimum, personal autonomy encompasses self-rule that is free from both controlling interference by others and limitations that prevent meaningful choice, such as inadequate understanding. The autonomous individual acts freely in accordance with a self-chosen plan, analogous to the way an independent government manages its territories and sets it policies. In contrast, a person of diminished autonomy is in some material respect controlled by others or incapable of deliberating or acting on the basis of his or her desires and plans... We analyze autonomous action in terms of normal choosers who act (1) intentionally, (2) with understanding, and (3) without controlling influences that determine their action... To respect autonomous agents is to acknowledge their right to hold views, to make choices, and to take actions based on their values and beliefs. Such respect involves respectful *action*, not merely a respectful *attitude*... [italics in original] Respect, so understood, involves acknowledging the value and decision-making rights of autonomous persons and enabling them to act autonomously, whereas disrespect for autonomy involves attitudes and actions that ignore, insult, demean, or are inattentive to others' rights of autonomous action... As a positive obligation, the principle requires both respectful treatment in disclosing information and actions that foster autonomous decision making... **Respect for autonomy obligates professionals in health care and research involving human subjects to disclose information, to probe for and**

[257] J Locke, Second Treatise of Government (1690).

ensure understanding and voluntariness, and to foster adequate decision making.[258]" [emphasis added]

The requirement for informed consent derives from respect for autonomy. If one agrees that autonomous individuals have the rights of self-ownership and self-determination regarding their physical person, then it follows that acting upon an individual's body without their permission violates that autonomy. Because performing medical tests or research experiments upon otherwise autonomous individuals against their will is a violation of their personal autonomy, such actions are generally considered an egregious violation of bioethics as well as the physician-patient and/or researcher-subject relationships. With respect to ethics in biomedical research, the quoted passage from Beauchamp and Childress shows that autonomous decision-making requires three components: intentionality regarding their decisions, an understanding of their decisions, and a lack of undue influence or coercion leading to the decision.[259]

Although we may consider informed consent to be one of the fundamental tenets of medical research and clinical practice today, the modern interpretation of the principle is fairly recent.

4.2.2. The Evolution of Modern Informed Consent: A Timeline

The development of our modern concept of informed consent begins with the World War II Nazi medical atrocities prosecuted during the post-war Nuremberg Doctors Trial, known formally as *United States v. Karl Brandt et al*. In

[258] TL Beauchamp and JF Childress, Principles of Biomedical Ethics.
[259] The latter ethical issue with respect to military service members as human experimental subjects is addressed in Chapter 4.3.

developing a framework against which ethical and legal judgments of the Nazi doctors' actions could be made, a code outlining the conditions under which human experimentation could be ethically performed was developed.[260] This framework is now referred to as the Nuremberg Code. The very first directive of the Code states in no uncertain terms:

> "**The voluntary consent of the human subject is absolutely essential.** This means that the person involved should have legal capacity to give consent; should be so situated as to be able to exercise free power of choice, without the intervention of any element of force, fraud, deceit, duress, over-reaching, or other ulterior form of constraint or coercion; **and should have sufficient knowledge and comprehension of the elements of the subject matter involved as to enable him to make an understanding and enlightened decision.** This latter element requires that before the acceptance of an affirmative decision by the experimental subject there should be made known to him the nature, duration, and purpose of the experiment; the method and means by which it is to be conducted; all inconveniences and hazards reasonable to be expected; and the effects upon his health or person which may possibly come from his participation in the experiment.[261]" [emphasis added]

After its initial use in judging whether the Nazi doctors' experimentation on prisoners of war was outside the bounds of medical ethics, the Nuremberg Code became a *de facto* guide to ensuring that medical experiments involving human subjects were performed a) in an ethical manner, and b) in a manner that

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[260] SE Lederer, "The Cold War and Beyond: Covert and Deceptive American Medical Experimentation," in Military Medical Ethics, Ed. DE Lounsbury and RF Bellamy (Falls Church, Virginia: Office of The Surgeon General, Department of the Army, United States of America, 2003).

[261] *United States v. Karl Brandt et al*., The Medical Case, Trials of War Criminals Before the Nuremberg Military Tribunals Under Control Council Law No. 10.

protects the subject from harm. However, this concern for protecting the subject's physical well-being (despite its obviously great importance) as the basis for requiring informed consent has come to be replaced by respect for a subject's personal autonomy as an end unto itself.[262] With respect for autonomy as the underlying foundation, informed consent becomes less about ensuring experimental subjects do not get hurt than about ensuring the sanctity of their personal choices with respect to their person. Does the individual wish to have medical experiments (or more generally, medical procedures in a clinical setting) performed upon them? A negative answer should ethically be the end of any further consideration of that individual's participation. Assuming that the potential subject is capable of making an autonomous choice, the benefits or risks that the proposed experiments or procedures might entail are irrelevant. This includes, for example, treatments or experiments that could potentially save the life of a terminally-ill patient. If he or she has made a properly autonomous choice, that choice should be respected.[263]

On the other hand, a subject may make a choice to submit to medical experimentation. This leads back to the tripartite requirement of autonomous actions as described by Beauchamp and Childress. The individual's positive

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[262] RR Faden, TL Beauchamp, and NMP King, A History and Theory of Informed Consent (New York: Oxford University Press, 1986); NC Manson and O O'Neill, Rethinking Informed Consent in Bioethics (New York: Cambridge University Press, 2007).

[263] This, of course, has end-of-life implications that may result in ethical conflicts between a physician's respect for patient autonomy and the principles of beneficence and nonmaleficence. Because respecting such a patient's autonomy would necessarily cause physical harm to the patient, these principles are mutually exclusive. Although most of the medical community (as well as this author) tend to view respect for autonomy as the overriding principle, discussion of such conflicts between bioethical principles is outside the scope of this work.

decision to be the subject of experiments clearly demonstrates the intentionality component of autonomous action; thus, consent has been given. However, the second component is that the decision and its potential implications (positive and negative) be properly understood by the subject. If the individual has been a) informed of the experiment, its methods, and the possible benefits and risks, b) understood the nature of the provided information, and c) freely consented to have the procedures performed upon himself or herself, then the subject is considered to have given informed consent. A great deal of bioethics literature has been written regarding the depth of understanding which a subject (often a layperson untrained in science) can achieve regarding highly technical medical procedures. Often, a truly complete understanding of the underlying science is not known even to the experts, hence the need for experimentation. Thus, a layperson research subject cannot be expected to achieve a *complete* understanding of the experiments. However, the individual must be provided with the requisite relevant information presented at a level which is simultaneously a) detailed and accurate enough to convey the nature of the proposed procedures and b) in simple enough terms that the subject can achieve enough understanding upon which to make an informed decision.[264] Put quite succinctly, "The act of informing alone has no bearing on the principle of

[264] However, it should not be presumed that all research subjects will require a simplified version of the information. Individuals with backgrounds in the medical or biological sciences may be capable of comprehending the information at a level of technical detail generally reserved for the researchers.

informed consent.[265]" Thus, the onus for providing the individual with the properly presented information falls squarely upon the researchers.

The third element of autonomous action is the lack of undue controlling influences over the individual's decision. Hypothetically, the subjects of Nazi research could have been presented with comprehensible information regarding the atrocities which were about to occur; the subjects could have even signed forms consenting to the experiments. However, this would not be considered an autonomous decision. The fact that the subjects were prisoners of war placed them in an inherently coercive situation. The authority which captors have over their prisoners means that prisoners may not be (or feel) able to make a truly autonomous decision without fear of punishment or reprisal. The presence of any influence which affects the individual's ability to make an independent decision eliminates the autonomy of any resulting decisions. Such influence with respect to members of the military is examined in greater detail in Chapter 4.3.

On February 26, 1953, President Dwight D. Eisenhower's secretary of defense, Charles E. Wilson, issued a top secret memorandum to the heads of each service branch. This document, which came to be known as the Wilson Memorandum, gave approval for the military to conduct experiments on human subjects in pursuit of defenses against nuclear, chemical, and biological warfare. However, the memorandum also outlined specific conditions under which such

[265] ML Gross, Bioethics and Armed Conflict: Moral Dilemmas of Medicine and War.

experimentation could occur. The stipulations essentially replicate the principles resulting from the Nuremberg Doctors Trial, including a verbatim first condition:

> "**The voluntary consent of the human subject is absolutely essential**... This means that the person involved should have legal capacity to give consent; should be so situated as to be able to exercise free power of choice, without the intervention of any element of force, fraud, deceit, duress, overreaching, or other ulterior form of constraint or coercion; and should have sufficient knowledge and comprehension of the elements of the subject matter involved as to enable him to make an understanding and enlightened decision... The concept [*sic*: consent] of the human subject shall be in writing... The Secretaries of the Army, Navy and Air Force are authorized to conduct experiments in connection with the development of defenses of all types against atomic, biological and/or chemical warfare agents involving the use of human subjects **within the limits prescribed above**.[266]" [emphasis added]

The Wilson Memorandum upheld and expanded upon the principles that came out of Nuremberg. This document made these principles, foremost that of informed consent, the policy of the US military. Following the Wilson Memorandum, the individual military branches enacted policies at various times throughout the remainder of the 1950s and 1960s. Just four months after the memorandum from the secretary of defense, the chief of staff for the US Army issued CS:385, a memorandum that not only implemented the principles described in the Wilson Memorandum, it added the stipulation that relevant research had to be reviewed and approved by both the Army surgeon general

[266] CE Wilson. "Use of Human Volunteers in Experimental Research," (Washington, DC: 1953). Reprinted in: SE Lederer, "The Cold War and Beyond: Covert and Deceptive American Medical Experimentation."

and the secretary of the Army.[267] (Though originally issued as Top Secret, CS:385 was declassified in 1954.[268]) In March of 1954, the Surgeon General of the US Army issued "Use of Human Volunteers in Medical Research: Principles, Policies, and Rules," which expanded the research covered beyond nuclear, chemical, and biological warfare to include all medical research using volunteer human subjects.[269] In 1962, the US Army published Army Regulation (AR) 70-25, "Use of Volunteers as Subjects of Research," which further codified the principles outlined in the Wilson Memorandum and CS:385 as US Army policy:

> "These regulations prescribe policies and procedures governing the use of volunteers as subjects in Department of the Army research, including research in nuclear, biological, and chemical warfare, wherein human beings are deliberately exposed to unusual or potentially hazardous conditions. These regulations are applicable worldwide, wherever volunteers are used as subjects in Department of the Army research... Voluntary consent in [*sic*: is] absolutely essential... The consent of the volunteer will be in writing... No research with nuclear, biological, or chemical agents using volunteers will be undertaken without the consent of the Secretary of the Army.[270]"

The US Navy publication "Manual of the Medical Department" which requires that "experimental studies of a medical nature... [involving] personnel of the Naval Establishment (military and civilian)" be approved by the secretary of the Navy actually predates the Wilson Memorandum, as it was published in

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[267] JC Oakes. "Use of Volunteers in Research," Secretary of the General Staff; Department of the Army, (Washington, DC: 1953).
[268] Advisory Committee on Human Radiation Experiments (ACHRE), Final Report (Washington, DC: US Government Printing Office, 1995).
[269] Office of the Surgeon General. "Use of Volunteers in Medical Research, Principles, Policies, and Rules of the Office of the Surgeon General," US Army, (Washington, DC: 1954).
[270] US Army. "Use of Volunteers as Subjects of Research," (Washington, DC: 1962).

1951.[271] (However, it was not until 1967 when the manual was updated to include a requirement for written consent from volunteers.[272]) In 1965, the US Air Force published "Medical Education and Research – Use of Volunteers in Aerospace Research," which further strengthened the first principle of the Nuremberg Code with the addition of a single word: the "voluntary **informed** consent of the human subject is absolutely essential." [emphasis added] These regulations required human subjects participating in research which might result in "distress, pain, damage to health, physical injury, or death" to be volunteers and to provide written informed consent.[273]

In 1964, the most significant international bioethical effort since the Nuremberg Doctors Trial occurred as the World Medical Association (WMA) adopted guidelines designed to differentiate between ethical and unethical biomedical research, known as the Declaration of Helsinki.[274] The following decade saw the strengthening of American bioethical protections for both military and civilian research through the enactment of numerous major pieces of legislation. In 1972, the United States Code was amended to add 10 USC 980, which directed that "Funds appropriated to the Department of Defense may not be used for research involving a human being as an experimental subject

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[271] US Navy. "Manual of the Medical Department, sec. IV, research article 1-17," Bureau of Medicine and Surgery, (Washington, DC: 1951).
[272] US Navy. "Manual of the Medical Department, 20-8, Change 36," (Washington, DC: 1967).
[273] US Air Force, "Medical Education and Research – Use of Volunteers in Aerospace Research," in AFR 169-8 (October 8, 19651965).
[274] "Declaration of Helsinki: Recommendations Guiding Medical Doctors in Biomedical Research Involving Human Subjects [adopted by the 18th World Medical Assembly, Helsinki, Finland, 1964]," *New England Journal of Medicine*, 271(9), 1964; RR Faden, TL Beauchamp, and NMP King, A History and Theory of Informed Consent.

unless... the informed consent of the subject is obtained in advance.[275]" In 1974, Congress passed the National Research Act (Public Law 93-348), establishing the institutional review board (IRB) process and the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research.[276] The commission, which was tasked with "conduct[ing] a comprehensive investigation and study to identify the basic ethical principles that should underlie the conduct of biomedical and behavioral research involving human subjects," published the "Belmont Report" in 1978. The commission's recommendations, including voluntary informed consent, were justified according to the ethical principles of beneficence, respect for autonomy, and justice. The Belmont Report has been updated five times since its initial publication and is now considered to be the standard for the ethical conduct of experiments involving human subjects.[277] The most recent major protective measure is Title 45, Part 46 of the US Code of Federal Regulations (45 CFR 46) entitled "Protection of Human Subjects" but usually referred to as the Common Rule.[278] 45 CFR 46 was originally approved in 1974, took effect in 1991, and most recently revised in 2009. The Common Rule "applies to all research involving human subjects conducted, supported or otherwise subject to regulation by any federal department or agency which takes appropriate administrative action to make the policy applicable to such research.

[275] 10 USC 980, *Limitation on the Use of Humans as Experimental Subjects*.
[276] Public Law 93-348, *National Research Act*, (July 12, 1974).
[277] National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research. "The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research," (Washington, DC: 1978).
[278] The DoD-specific implementation of this legislation is 32 CFR 219.

This includes research conducted by federal civilian employees or military personnel.[279]" According to the Common Rule,

> "[N]o investigator may involve a human being as a subject in research covered by this policy unless the investigator has obtained the legally effective informed consent of the subject or the subject's legally authorized representative. An investigator shall seek such consent only under circumstances that provide the prospective subject or the representative sufficient opportunity to consider whether or not to participate and that minimize the possibility of coercion or undue influence. The information that is given to the subject or the representative shall be in language understandable to the subject or the representative. No informed consent, whether oral or written, may include any exculpatory language through which the subject or the representative is made to waive or appear to waive any of the subject's legal rights, or releases or appears to release the investigator, the sponsor, the institution or its agents from liability for negligence.[280]"

For the purposes a convenient comparison, it is useful to construct a joint timeline of the tests detailed in Chapter 3 coupled with relevant legal and policy highlights regarding bioethics and the adoption of these principles into the operations of the US military (Figure 4-1). The open-air dispersal experiments are labeled above the timeline and the bioethical events are labeled below the timeline. This timeline will be referenced in the following sections as the tests are examined with respect to informed consent and respect for autonomy.

[279] 45 CFR §46.101, *Protection of Human Subjects*, "To what does this policy apply?" (July 14, 2009).
[280] 45 CFR §46.116, *Protection of Human Subjects*, "General requirements for informed consent." (July 14, 2009).

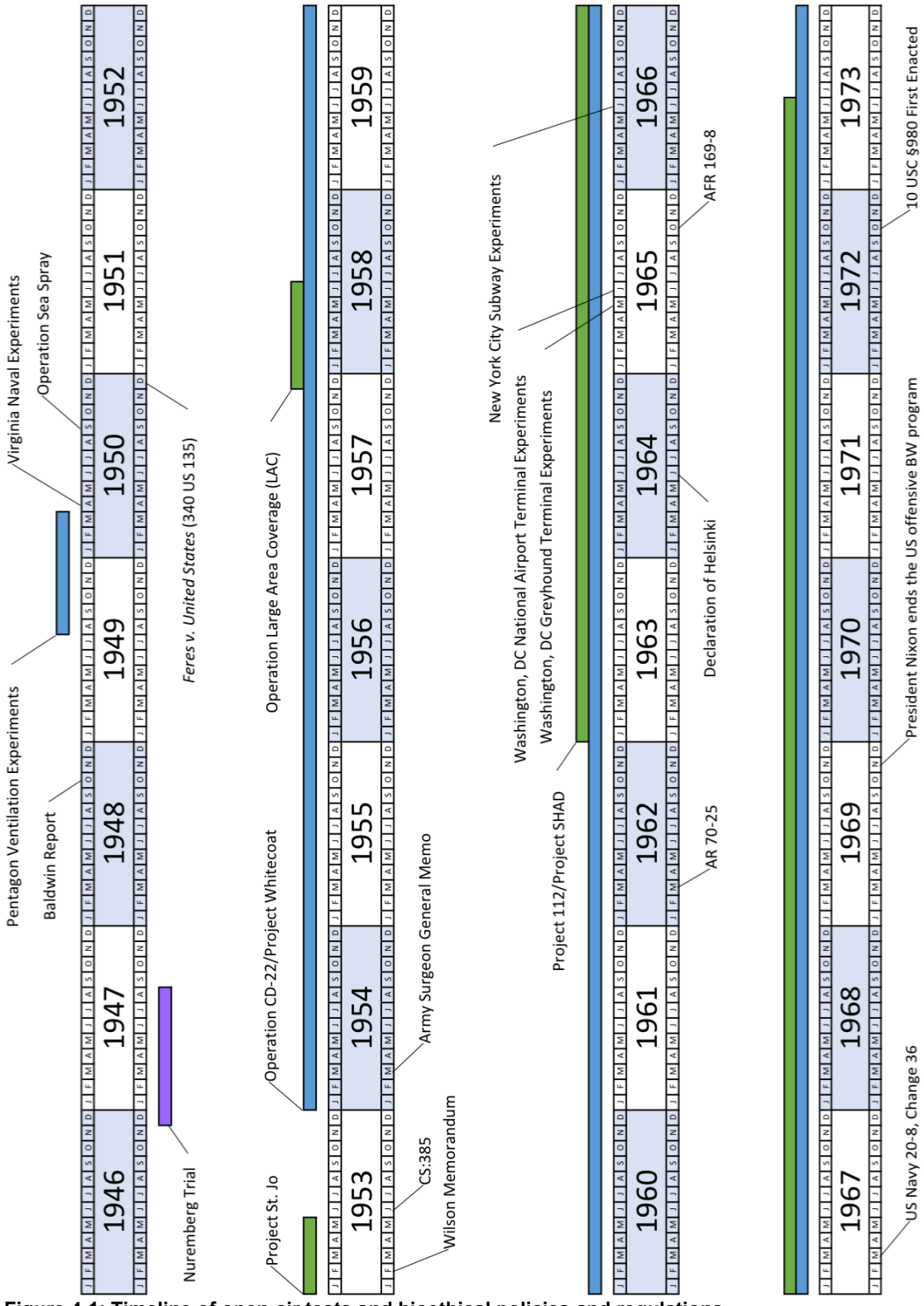

Figure 4-1: Timeline of open-air tests and bioethical policies and regulations

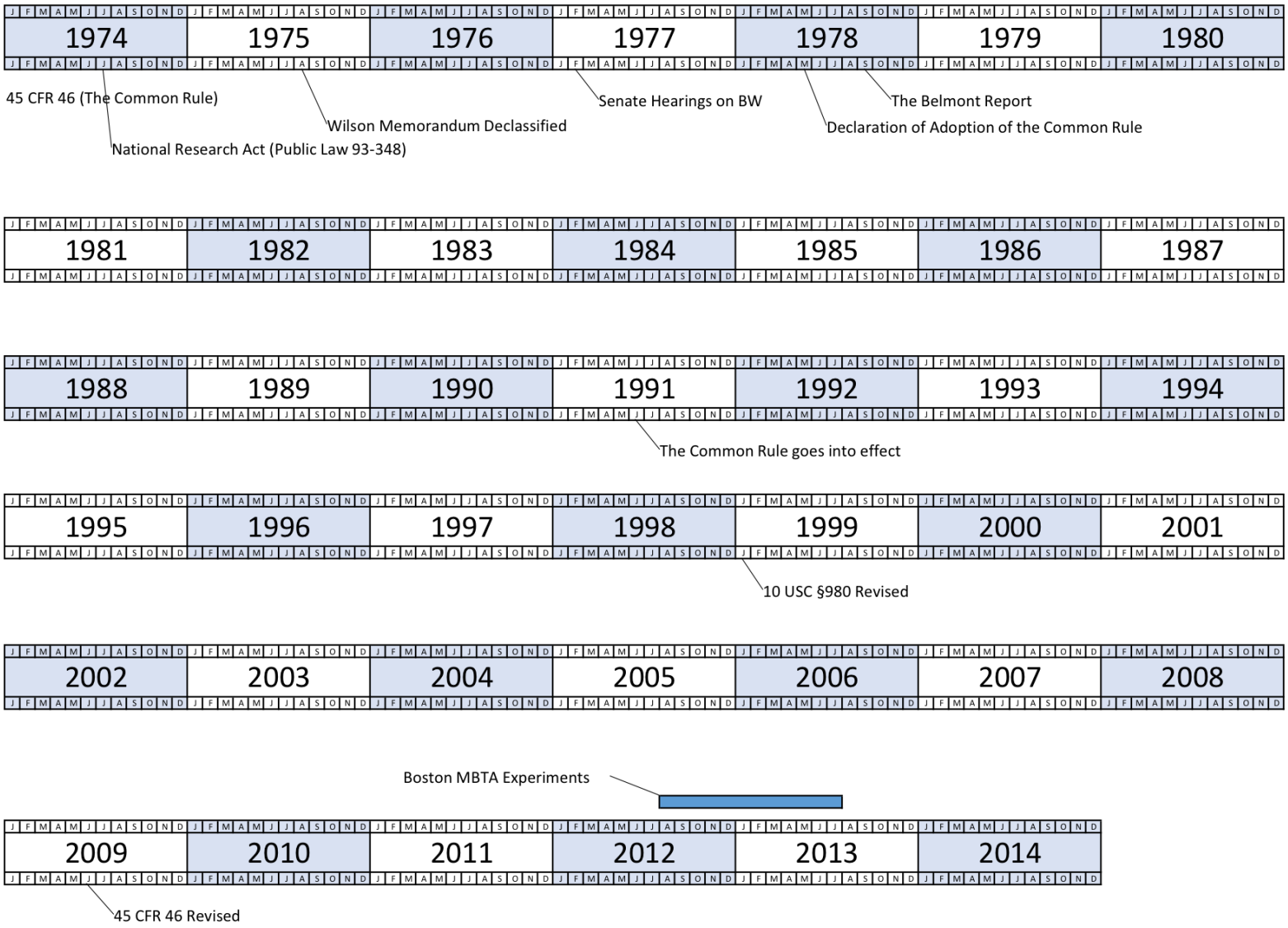

Figure 4-1 (continued): Timeline of open-air tests and bioethical policies and regulations

4.2.3. Informed Consent was Not Obtained

In nearly all of the tests described in Chapter 3, it is either known that informed consent was not obtained or no evidence is known that informed consent was obtained.

The dispersals of *Serratia marcescens* into the ventilation system of the Pentagon (Chapter 3.2) intentionally exposed thousands of military and civilian employees, engulfing the nation's top brass in clouds of a pathogenic bacterial species. There is some suggestion that the most senior leadership at the Pentagon authorized the experiment.[281] However, even if this were true, the test almost certainly took place without the knowledge of most of the individuals in the building. Ethically, such an experiment cannot be performed without obtaining the informed consent of all the military and civilian personnel exposed. Because the unwitting subjects of these experiments were neither informed nor asked for consent, there is clearly no possibility of informed consent. Two major ethical criteria were violated in the course of the Pentagon dispersal. First, in spraying the people inside the Pentagon with bacteria without their explicit consent, the personal autonomy of those exposed was not respected. Second, because this bacterium was at the time known to have some level of pathogenicity, those exposed were put at risk of harm, violating the principle of nonmaleficence.

Similar to the dispersals at the Pentagon, the dispersals of *S. marcescens* and *Bacillus atrophaeus* connected to the Virginia naval tests (Chapter 3.3) appear to have been unconnected with any type of informed consent. As

[281] E Regis, The Biology of Doom: The History of America's Secret Germ Warfare Project.

mentioned previously, information regarding this series of tests is particularly scant. However, it can be presumed that three groups were exposed or potentially exposed: the crew of the USS *Coral Sea*, the crew of the USS *Kenneth D. Bailey*, and the civilians living, working, or traveling through the exposed surrounding areas. Although the aircraft carrier USS *Coral Sea* was docked, its participation as the target of the dispersals suggests that it was probably at least partially manned by its crew. This is supported by the 1977 US Army report to Congress indicating the secondary goal of testing the performance of prototype electronic systems for detection of biological warfare agents: personnel likely would have been present on the USS *Coral Sea* to operate the equipment.[282] Even so, the fact that *B. atrophaeus* spores were dispersed means that the entire ship likely became contaminated with these hardy spores. Without very stringent shipwide decontamination protocols, any off-ship personnel simply would have been exposed upon their return. Secondly, the personnel aboard the USS *Kenneth D. Bailey* conducting the dispersals, particularly any who may have been topside, may possibly have been exposed to the aerosolized agents. Lastly, the people within the cities of Hampton, Newport News, and Norfolk, as well as the surrounding areas would also have been exposed to the biological agents directed at the USS *Coral Sea*. It is not known whether the crews of both ships were told of the details of their missions; they may not have known that air was being filled with living (and therefore potentially

[282] US Army, US Army Activity in the US Biological Warfare Programs.

infectious) bacteria. Conducting such an experiment today would likely require the informed consent of all the military personnel on both ships. There is no evidence that this occurred during these tests in 1950.

The spraying of the San Francisco Bay Area during Operation Sea Spray in 1950 (Chapter 3.4) shows a similar disregard. The use of the city as an experimental testbed came to light in 1976 and was posited as a potential cause of a rare outbreak of *Serratia marcescens* infections at a hospital in Stanford, California. Again, spraying viable bacteria with a known level of pathogenicity over a major American metropolitan area should require informed consent from the entire population of the target cities and likely the crews of the dispersing naval vessels. However, despite the declassification of the complete Army document "Special Report 142: Biological Warfare Trials at San Francisco, California, 20-27 September 1950," no mention is made of any such procedures. Quite the contrary, the experiments remained hidden from the public for over 25 years.

Obtaining informed consent from those exposed would be required in order to ethically perform such experiments today. Although the tests described above appear to have made no attempts to even provide information let alone obtain consent, the timeline in Figure 4-1 shows that these requirements were not part of military policy prior to the publication of the Wilson Memorandum and the later policies which would have regulated such experiments. Thus, an argument could be made that these military experiments predate the policy

requirement (if not the ethical requirement) for the military to obtain informed consent from its experimental human subjects. However, these tests all occurred only a few years after the United States prosecuted German doctors and scientists for their conduct of wartime experiments. As discussed in the previous section, the Nuremberg Code which arose from these trials used respect for the autonomy of the experimental subjects as the lynchpin principle upon which human experiments could be ethically conducted. The Nazi experiments themselves may have been barbaric and torturous, but according to the principles that emerged from the Nuremberg trials, the primary ethical offense was the violation of the subjects' sovereign personal autonomy. Thus, the sanctimonious ethical judgment of the charges[283] and the voracity with which they were prosecuted only a short time prior makes the fact that these first three test programs occurred before the Wilson Memorandum codified informed consent as military policy at best an extremely flimsy argument. However, no matter how weak that argument may be, the remaining six experiments discussed in this section have no such defense because they occurred after the publication of the Wilson Memorandum.

The dispersals of zinc cadmium sulfide (ZnCdS) during Project St. Jo (Chapter 3.5) occurred between 1953 and 1954. During these experiments, the citizens of Minneapolis and St. Louis were exposed to aerosolized chemical

[283] This description should not be construed as a repudiation of the Nuremberg Code or its underlying ethical principles, but merely an emphasis of the hypocrisy displayed by the application of two completely opposite standards.

simulants without their knowledge. The cover stories and elaborate attempts to disguise the true nature of the experiments represent an attitude dichotomous with the principles underlying informed consent.

Operation Large Area Coverage (Chapter 3.7) dispersed ZnCdS across large swaths of the United States in 1957 and 1958. While these tests did not involve the deception that was used in Project St. Jo, the entire test series was conducted in secret, a methodology clearly mutually exclusive with informed consent.[284] Based on the large areas of dispersal, it can be assumed that millions of Americans were potentially exposed to the chemical simulant.

Project 112 and Project SHAD (Chapter 3.8) were major chemical and biological test programs conducted between 1963 and 1973 in various places throughout the world but focused primarily in the Pacific Ocean. Military personnel were exposed to a variety of pathogenic agents, toxic chemicals, and simulants that were not fully characterized.

The 1965 experiments with *Bacillus atrophaeus* spores inside the Washington, DC, bus and airport terminals (Chapter 3.9) and the 1966 releases of *B. atrophaeus* spores inside the New York City subway system (Chapter 3.10) were designed to clandestinely disperse biological agents amidst an unknowing target population. In both experiments, knowledge of the tests was deliberately withheld from the public and local officials. Additionally, the SOD operatives involved in dispersing the agents carried forged credentials to aid in the

[284] Obtaining traditional informed consent from the many millions of people potentially exposed, however, would be realistically infeasible.

deception of anyone who became suspicious of their activities. Clearly, these activities are not consistent with informed consent.[285]

As Figure 4-1 shows, the Wilson Memorandum was published approximately halfway through Project St. Jo. Proper communication of the requirements issued by Secretary Wilson should have resulted in, at the very least, the reevaluation of the experimental protocols (namely the clandestine dispersal of chemicals over unsuspecting American cities). There is no evidence that this occurred, however. Figure 4-1 also clearly shows that the remaining test series in this section all occurred after the publication of the Wilson Memorandum, CS:385, and the memorandum from the Army Surgeon General. Additionally, AR 70-25 and the Declaration of Helsinki predate the last four test series (although the latter only predates approximately 80% of Project 112/Project SHAD). Further, US Navy 20-8, Change 36 was enacted prior to the chronological midpoint of Project SHAD, which once again should have spurred a reexamination of the experimental protocols in place for the remaining program tests.

Thus, the obvious question arises: why did the US military disregard its own policy requirements, particularly as outlined in the earliest documents, the Wilson Memorandum and CS:385? Four scenarios appear possible.[286] In the

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[285] JD Moreno, Undue Risk: Secret State Experiments on Humans (New York: W.H. Freeman, 2000).

[286] This disregards a possible fifth scenario: that informed consent was obtained and all these experiments were fully compliant with the Wilson Memorandum. Three facts suggest this could not have been the case: a) the documented deceptive measures described in Project St. Jo, the Washington, DC tests, and the New York subway tests, b) the difficulties in constructing lists of

first (and perhaps most innocuous) scenario, the individuals conducting the experiments could have been unaware of the requirements stipulated by the various governing policies. This, to some degree, appears to have been the case. In attempting to uncover "the magnitude of covert and deceptive medical research that had been ongoing in this country throughout most of the Cold War," the Advisory Committee on Human Radiation Experiments (ACHRE) examined the publication and implementation of the Wilson Memorandum, which also would have covered such nuclear tests.[287] The findings published by ACHRE show that the Wilson Memorandum was distributed no further down the chain of command than the Secretaries of the Army, Navy, and Air Force.[288] If this is the case, one would expect that no individuals directly involved in the conduct of such experiments would be aware of the governing policies. This seems to be consistent with the facts. For example, in 1994 testimony before ACHRE, the then-director of the Human Resources Research Organization (HumRRO), an Army contractor which conducted some of the atomic bomb experiments, stated that he was unaware of the Wilson Memorandum.[289] Similarly, an Air Force

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service members potentially exposed during Project 112/SHAD, and c) the sheer number of people exposed during Project St. Jo, the Washington, DC tests, the New York subway tests, and Project 112/SHAD. If informed consent was obtained and written documentation preserved as per the Wilson Memorandum, it is reasonable to assume that a) deceptive measures would have been unnecessary, b) lists of those exposed (with their permission) would exist, and c) at least one of the many millions of individuals exposed would have objected and/or come forward to made public these experiments. Because the possibility that these test series were conducted within the stipulations outlined by the Wilson Memorandum seems inconceivable and inconsistent with the known facts, it is given no further consideration.

[287] SE Lederer, "The Cold War and Beyond: Covert and Deceptive American Medical Experimentation."

[288] Advisory Committee on Human Radiation Experiments (ACHRE), Final Report.

[289] Ibid.

general who flew through the radiation cloud resulting from a nuclear explosion stated that he was unaware of the Wilson Memorandum.[290] Although these instances might exculpate the experimenters from *intentionally* disobeying the orders of the Secretary of Defense, it is indicative of a serious breakdown in the communication of important orders. In the second scenario, although the individuals conducting the experiments could have been fully aware of the Wilson Memorandum and its requirements, they may have chosen to willfully ignore them and proceed with their experiments in violation of the orders issued by the Secretary of Defense. However, if any unit-level documentation exists which shows commanding officers intentionally misinterpreting the Wilson Memorandum requirements as inapplicable or otherwise justifying proceeding with the experiments in spite of them, such documents are not known to the public. In the third scenario, the experiments could have been granted an approval by the chain of command waiving the requirements of the Wilson Memorandum. However, the approval process stipulated in the memorandum is quite rigorous:

> "In each instance in which an experiment is proposed pursuant to this memorandum, the nature and purpose of the proposed experiment and the name of the person who will be in charge of such experiment shall be submitted for approval to the Secretary of the military department in which the proposed experiment is to be conducted. No such experiment shall be undertaken until such Secretary has approved in writing the experiment proposed, the

[290] Ibid.

person who will be in charge of conducting it, as well as informing the Secretary of Defense.[291]"

If such a waiver of the informed consent requirement was indeed provided, it too likely would have needed approval from the Secretaries of the Army, Navy, and/or Air Force as well as the Secretary of Defense. Presumably such a waiver would need to lay out the specific factors at play that warrant overriding the stipulations of the Wilson Memorandum. Because such a waiver would effectively negate standing military policy on a case-by-case basis, it would likely not be granted lightly. If such documents exist, they remain classified and unknown to the public. In the fourth scenario, later orders effectively repealing the Wilson Memorandum may have been issued. Again, the existence of such documents is unknown to the public. However, given that AR 70-25 effectively reiterated the same policy in 1962 (and was further strengthened upon its revision in 1990), it seems that the Army was committed to the principles of the Wilson Memorandum, at least officially. Thus, documents negating the Wilson Memorandum are not expected to exist. Of these four possible scenarios, the only one with any support from publicly known documentation or testimony appears to be the first scenario.

[291] CE Wilson, Use of Human Volunteers in Experimental Research. Reprinted in: SE Lederer, "The Cold War and Beyond: Covert and Deceptive American Medical Experimentation."

4.2.4. Informed Consent was Obtained

Only two of the test series described in Chapter 3 can be considered as having obtained informed consent: the long-running Operation CD-22/Project Whitecoat and the dispersal testing within the 2012 Boston MBTA system.

In the Operation CD-22/Project Whitecoat test programs (Chapter 3.6), US Army volunteers who were members of the Seventh-day Adventist Church were recruited to be exposed to infectious microorganisms to further the defensive research of the biological weapons program. The manner in which Operation CD-22 and Project Whitecoat were conducted stand in stark contrast to the tests described in the previous section. Great care was taken to ensure that the soldiers' decision to participate was entirely voluntary. By examining the procedures used through the prism of the threefold requirement for autonomous decision-making as outlined by Beauchamp and Childress, it is abundantly clear that a deliberate attempt was made to obtain proper informed consent. The first requirement, intentionality regarding a decision, can be seen occurring repeatedly throughout the recruitment process. First, potential recruits were interviewed and provided information about Project Whitecoat; the medically qualified volunteers became part of a human subject pool. When a specific research study was recruiting volunteers, the soldiers in the subject pool were given a detailed briefing about the study, including the risks involved. The Project Whitecoat pool volunteers were then asked whether they wished to participate in the specific study. Those who volunteered for the recruiting study signed written consent forms which detailed the experiments, the subject's role,

and the risks to the subject.[292] The amount and detail of information provided coupled with the layers of opportunities to volunteer or decline participation leaves little doubt that anyone who served as a subject did so in a manner that was not voluntary. The second requirement, an understanding of the decision, was assured by providing the volunteers with detailed information and having the volunteer sign the consent form indicating his understanding of the information. Although one can present an individual with voluminous information, it is difficult if not impossible to ***absolutely*** ascertain or ensure the understanding of the information by the individual. While the burden of providing sufficient information upon which to make a decision falls upon the researchers, at some point the onus must necessarily shift to the volunteer. The volunteers were given numerous opportunities to ask questions; presumably, this includes requesting clarification of information which was presented but not fully understood. Providing a signature itself does not ensure understanding, but an individual voluntarily – and honestly – assenting to understanding information should be considered sufficient. The third requirement, a lack of undue influence or coercion leading to the decision, was met at various points throughout the process. From the very outset, the initial briefings of recruits at Fort Sam Houston, Texas were intentionally delivered by individuals outside their chain of command. This was done to ensure the potential recruits did not feel they were being given orders. Thus, it is clear that the methods employed by Project

[292] RL Mole and DM Mole, For God and Country: Operation Whitecoat: 1954 – 1973.

Whitecoat respected the autonomy throughout the entire process and ensured informed consent by the volunteer human research subjects.

In the recent Boston MBTA experiments (Chapter 3.11), a careful assessment of various experimental protocol options was made before deciding upon the one which best balanced the risk to the public with the benefit of the most realistic testing of the detection sensors: the dispersal of killed *B. subtilis* spores during after-hours periods. Although the primary dispersals occurred while the MBTA system was closed and no passengers were present, the system was operational the following day. Thus, the possibility exists of rider exposure to spores still aerosolized and/or deposited as residue on surfaces. Even though the spores were biologically nonviable, ethically exposing the passengers to this simulant material requires treating them as human research subjects and therefore subject to informed consent requirements. However, it does not appear that a traditional informed consent process was followed. Presumably because obtaining signed informed consent documentation from anyone who might be exposed as a result of these tests would be virtually impossible, those planning the experiments appear to have opted for an alternative method. Significant dissemination of information occurred using a wide variety of methods, including press releases, online information, public forums, local and national media coverage, and notification within the T stations prior to the experiments. Such informational saturation prior to the experimental dispersals can be considered to serve as a "*de facto* informed consent" process: riders of the T, armed with

information about the imminent tests, could make a choice whether to expose themselves to the simulant materials. For those who chose to ride the T on the days following the experimental dispersals, their choice satisfies the three requirements for autonomous decision-making as stated by Beauchamp and Childress. As a result, the conduct of the MBTA experiments respected the autonomy of the citizens of Boston by obtaining *de facto* informed consent from those willing to be exposed to the killed simulant spores.

4.2.5. When is Informed Consent Necessary, and Why?

Chapter 4.2.3 addressed the lack of informed consent in eight different military research programs, while Chapter 4.2.4 described the informed consent procedures (both traditional and *de facto*) utilized during the conduct of two additional test series. The differences between the conduct of these two sets of experiments, as well as the attitudes displayed toward the human research subjects involved, are stark.

The simplest and most ethical answer to the title question is that respect for autonomy requires that informed consent always be obtained. This is a reasonable requirement which has become standard for biomedical research experiments on individual subjects as well as routine clinical procedures.[293] However, it has been argued that population-level research poses unique difficulties:

[293] In the clinical setting, properly executed informed consent not only serves to ensure the autonomy of the patient is respected but also to protect the physician from litigation.

> "[E]xperimentation on large populations precludes traditional, individual informed consent measures... Because of the effort of bioterrorists to disperse bioweapon agents over large populations, human subject experimentation... on populations is inevitable in biodefense research. It cannot include the informed consent of every individual in the population because the procedure of obtaining consent would likely destroy the experiment: the behavior of the participants would likely be altered, and many could be expected to flee to avoid exposure to the unknown consequences of an experiment.[294]"

Perhaps the foremost difficulty in obtaining traditional informed consent for population-level experiments would be the sheer logistics of disseminating information to and collecting signed consent documentation from every single person who would (or even possibly could) be exposed. For open-air dispersal experiments such as the ones discussed in Chapters 4.2.3 and 4.2.4, a sudden change in the direction of the wind could expose a whole new unintended population. In this type of research, adherence to the traditional informed consent process would be next to impossible. Thus, a different type of consent process – one which still embodies respect for the autonomy of the subjects – is required. The best alternative appears to be the *de facto* informed consent procedure used during the experiments in Boston's MBTA system in 2012.

However, one could argue that informed consent is unnecessary if the subjects incur no additional risk. Chapter 4.1 discussed the pathogenicity of the simulants dispersed by the various research programs. If a simulant to be

[294] V Sutton, "A Multidisciplinary Approach to an Ethic of Biodefense and Bioterrorism," *The Journal of Law, Medicine & Ethics*, 33(2), 2005.

dispersed as part of population-level biodefense research is not pathogenic or harmful, is informed consent required from those within a population who might be exposed? Once again, the most ethical answer is yes. Subjecting individuals to a substance against their will violates their autonomy. In the most widely accepted interpretation of the principle of respect for autonomy, it makes no difference whether the substance is harmless or even beneficial. Thus, if the government chooses to conduct open-air dispersal experiments in the future, it should take all possible measures to ensure that informed consent (even if only *de facto*) is obtained, even when the simulant is ostensibly harmless.

4.3. Military Bioethics

One of the most critical components of military operation is discipline. The nature of military operations requires soldiers to follow orders without necessarily having a complete understanding of the full situation. Heated battles cannot be paused for a commanding officer to stop and provide a full briefing to his troops on *why* they are being given their mission. Further, soldiers must be willing to follow their orders unquestioningly, even (and especially) when they know that doing so could result in harm to themselves. A breakdown in such discipline erodes the effectiveness of military units. Soldiers cannot simply decline to participate in a mission because they feel it is too dangerous or think they might get killed.

But soldiers are human individuals as well, and individuals have both autonomy and the right to life. The individual's desire to live is perhaps the

closest that humanity has to a universal constant throughout each of us. The right to life is something that normally is neither surrendered easily nor taken from individuals lightly.[295] However, when soldiers are ordered into battle, they must follow that order regardless of the threat to their lives. This obligation (both legal and moral) is fairly unique within our society. While heroic and perilous actions are performed by many (e.g., police officers and firefighters), none have such an inflexible requirement to follow legal orders knowing that they will not survive. Similarly, in no other segment of our society do group leaders have the right to a) issue their subordinates commands knowing that their deaths will result, and b) expect these commands to be unquestioningly followed. As soldiers, individuals have both diminished autonomy and right to life relative to that enjoyed by civilians. What rights, then, are necessarily relinquished in the line of duty, and what autonomy is retained? A particularly salient summary of the diminished rights of soldiers is provided in the book Bioethics and Armed Conflict:

> "Soldiers enjoy but a conditional right to life that they will lose once they don a uniform and take up arms against one another... Military service, regardless of a nation's state of war, limits a person's autonomy, right to self-determination, and derivative civil liberties. Military personnel do not enjoy full autonomy. The state usurps this right out of consideration for the task military personnel must perform in the service of the common good... Because military service, particularly during war, limits autonomy, it necessarily

[295] Perhaps the most notable instance of the deprivation of an individual's right to life is the death penalty. The sentencing of a criminal to be executed is reserved for the most heinous of crimes. Even still, the high thresholds that must be met for such a sentence to be delivered and the many years of legal wrangling that occur before someone is actually put to death serve to illustrate the solemnity with which our society and legal system views the deprivation of this right.

> curtails its derivative rights. These include both medical rights (informed consent, privacy, and confidentiality) and civil liberties (freedom of movement, assembly, and representation).[296]"

The rights of soldiers generally do not include refusal of medical treatment. A soldier ordered to take medicine or accept vaccinations has essentially no choice in the matter. This basic right, paramount to the biomedical ethics of properly autonomous civilians, is denied to those who risk their lives in service of our country. However, this should not be considered an injustice. Instead, it is precisely ***because*** they are in service of our country that it must be so:

> "As collective interests overwhelm individual welfare, the information at one's fingertips recedes and decision making passes to others... But individual welfare is not the focus of military medical care. While medical personnel work to provide good medical care, they are obligated to provide the care necessary to maintain soldiers as a fighting *force* — that is, a corporate personality... [italics in original] Soldiers do not receive medical care to guarantee their health as individuals but to preserve the health of a larger organism, a common good quite distinct from the interests of the soldier as patient.[297]"

Chapter 4.2 discussed patients and/or research subjects and their personal autonomy as ends unto themselves. However, the diminished autonomy of soldiers is indicative of their role as a means rather than an end (the end, in this case, being the national defense). If soldiers are required to follow orders without being fully informed of the risks and must also accept medical

[296] ML Gross, Bioethics and Armed Conflict: Moral Dilemmas of Medicine and War.
[297] Ibid.

treatment, this would suggest that they can be ordered to participate in military medical research and must comply or, worse, can be experimented upon without explicit informed consent. After all, such a role would be in the greater service of the nation.

This, however, would not be considered to be ethical. The soldier may at times be faced with considerable risk; this is necessarily inherent in the role. But when soldiers face risks that are both supererogatory (that is, "above and beyond the call of duty") and distributed disproportionately (that is, one or more individuals carry a greater portion of the risks than others), it becomes necessary to obtain consent from the soldier.[298] An example is the situation faced by a commander who is planning a tactical maneuver which is clearly a suicide mission for the soldier who executes it. Choosing a soldier and ordering him to complete the mission is akin to murder. This differs from ordering an entire squad or platoon to engage an enemy force that greatly outnumbers them. In the latter case, the risk is shared equally among the individuals and is in the performance of their normal military duties. In the former case, a) the extreme risk must be borne by a single individual alone, and b) the risk is supererogatory. Thus, the commander briefs his troops on the mission, including its strategic importance and the negligible likelihood of survival, and he asks for volunteers for the mission. Whether spurred by patriotism or a sense of duty, any solider

[298] Ibid.

who volunteers for the suicide mission has clearly gone above and beyond the call of duty.

Like suicide missions, medical experimentation does not fall within the expected role of a soldier. To be clear, there is nothing wrong with such military medical experimentation. Quite the contrary, such research is critical to the protection of and development of effective medical treatment for our fighting forces. However, participation in such experiments is supererogatory and thus cannot be ordered. The risks involved in the research may be great and, more importantly, are disproportionately distributed to those participating in the experiment. The benefits that stem from the research, however, might be enjoyed by the entire military. This is counter to the basic principle of justice: the proportional distribution of both the risks and rewards of research.[299] Thus, the ethical conduct of such experiments requires the voluntary participation of soldiers. However, in order for their participation to be fully voluntary, it is required that the volunteers be provided with all the necessary information to willingly make an autonomous choice that they feel is best for them: full and properly executed informed consent.

From the very first major attempt at crafting a framework delineating ethical biomedical research, the Nuremberg Code, it was understood that the concept of informed consent was useless if the subject was not in a position to

[299] TL Beauchamp and JF Childress, Principles of Biomedical Ethics.

make a freely voluntary decision. The first principle of the Nuremberg Code states:

> "The voluntary consent of the human subject is absolutely essential. This means that the person... should be so situated as to be able to exercise free power of choice, without the intervention of any element of force, fraud, deceit, duress, over-reaching, or other ulterior form of constraint or coercion.[300]"

As discussed in Chapter 4.2.2, those incarcerated and tortured by the Nazis were not in a position to exercise their personal autonomy by virtue of their status as prisoners. Soldiers are hardly in a similar circumstance, but are they truly free to make a choice that is free from any coercion?

In research involving civilians as experimental subjects, the avoidance of coercion or undue influence is more easily achieved than in military medical research. However, the rank dynamics of the military structure inject an additional complexity into military medical research that is not present in identical research performed on civilian subjects. It may be difficult for military personnel to truly grant consent when asked to participate in experiments.[301] From a soldier's very first day in boot camp, they are instilled with an ethos of subservience to the legal orders of their commanding officers. This has been described as the "generalized deference to authority inherent in military culture.[302]" Thus, even if an officer ***asks*** an enlisted soldier whether he or she

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[300] *United States v. Karl Brandt et al*., The Medical Case, Trials of War Criminals Before the Nuremberg Military Tribunals Under Control Council Law No. 10.
[301] V Sutton, "A Multidisciplinary Approach to an Ethic of Biodefense and Bioterrorism."
[302] Advisory Committee on Human Radiation Experiments (ACHRE), Final Report.

wishes to participate in a medical experiment, it is quite possible that the soldier would interpret this request as a "soft order," even if no such hidden meaning was intended. It is specifically for this reason that the final ACHRE report includes a recommendation against placing soldiers in such a potentially conflictive situation:

> Recommendation 12, Part 3: "Maximizing voluntariness: The service secretaries should consider the situations under which it would be appropriate ... [for] unit officers and senior noncommissioned officers (NCOs) who are not essential as volunteers in the research [to] be excluded from recruitment sessions in which members of units are informed of the opportunity and asked to participate in research by investigators.[303]"

In 2002, this recommendation was officially implemented as policy in DoD Directive 3216.2, §4.4.4.[304]

4.3.1. Military Members May Have Been Coerced

Of the four experimental series described in Chapter 3 which had military members as one of the primary (i.e., intended) exposed populations, only two are considered likely candidates for coercion into participation as an experimental subject occurring: the Virginia naval experiments and Project 112/Project SHAD. (The Pentagon ventilation experiments and Operation CD-22/Project Whitecoat are discussed in the next section.)

During the 1950 experiments off the coast of Virginia (Chapter 3.3), the destroyer USS *Kenneth D. Bailey* dispersed both *S. marcescens* and *B.*

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[303] Ibid.

[304] US Department of Defense, "Protection of Human Subjects and Adherence to Ethical Standards in DoD-Supported Research," in DoD Directive 3216.2 (Washington, DC2002).

atrophaeus. The target of the bacterial dispersals was the aircraft carrier USS *Coral Sea*, which was docked in Hampton Roads, Virginia. In addition to at least a partial crew complement aboard the USS *Coral Sea*, personnel would have likely been necessary for operation of the prototype electronic systems being evaluated for the detection of biological warfare agents.[305] It is unclear whether the service members who participated in these experiments were aware that they were being targeted with clouds of aerosolized bacteria. Most likely they were simply ordered to complete their tasks without any mention that their environment would be filled with invisible, potentially infectious organisms. However, it is possible that they were given some indication that this would be occurring.[306] If so, it may have been coupled with coercion from commanding officers to submit to these exposures. If individuals had reservations about being exposed, being given orders to participate in the tests despite their concerns would likely have compelled them to do so. Because essentially no details aside from the fact that these experiments occurred have been released, it is currently unknown whether any information was provided to the targeted military members or whether coercive methods such as those described in the previous section were used to ensure participation in the experiments.

As detailed in Chapter 3.8, many of the test series that comprised the decade-long research program known as Project 112/Project SHAD are either a) known to have exposed US military service members to a multitude of

[305] US Army, US Army Activity in the US Biological Warfare Programs.
[306] This is discussed strictly as a hypothetical scenario, as there is no evidence that this occurred.

pathogenic agents, toxic chemicals, and not thoroughly characterized simulants, or b) potentially resulted in such exposures. The pathogens and toxins included *Coxiella burnetii*, *Francisella tularensis*, *Staphylococcus* enterotoxin, type B (SEB), and *Puccinia graminis* var. *tritici*. The ostensibly safe biological simulants included *B. atrophaeus*, *E. coli*, *S. marcescens*, and T-3 coliphage. In most of the cases involving US Navy ships, the vessels involved in the tests were manned by their standard crew complement. It is not known whether the military personnel were informed of the agents to which they would be exposed or of the potential risks to their health. As with the experiments off the coast of Virginia, if the service members who participated in Project SHAD were given any advance notice of the nature of their role, it was most likely intended to be information rather and an attempt at informed consent. It is unlikely that they were given an opportunity to decline participation in the missions.

4.3.2. Military Members Were Not Coerced

Six of the experimental series described in Chapter 3 did not have military members as one of the primary (i.e., intended) exposed populations.[307] Operation Sea Spray (Chapter 3.4) targeted the San Francisco Bay Area, Project St. Jo (Chapter 3.5) targeted Minneapolis and St. Louis, and Operation LAC (Chapter 3.7) targeted large swaths of the country. In these tests, the primary

[307] It should be noted that some military personnel, simply as a virtue of their numbers within the general population, were likely in each of the target groups discussed in this section, e.g., living in San Francisco, traveling through Washington, DC's National Airport, or riding the New York City subway. However, because they were not the specifically recruited targets, the exposures of individual military personnel while amongst the generally targeted population is not considered within the scope of this section's discussion.

targets were civilian populations. The military personnel conducting the dispersals may have been exposed to the simulants, but for the purposes of this discussion, they exposed incidentally and were not coerced into participating as targeted human subjects. The tests in Washington, DC, and New York City (Chapters 3.9 and 3.10, respectively) targeted transportation systems in use by civilian populations and were performed by personnel from Fort Detrick's Special Operations Division; again, the incidental exposures of the SOD operatives that may have occurred are not considered as evidence of coerced participation. The recent dispersals in Boston's MBTA system (Chapter 3.11) were performed by the Department of Homeland Security, and military personnel were neither involved in nor a primary target of the dispersals. In all six of these tests, no military personnel were targeted, which implies that no coercion of military members to participate as human subjects is likely to have occurred. Thus, Table 4-1 lists the "Military Bioethics" description for these experiments as "not applicable".

The experimental dispersals of bacteria into in the Pentagon ventilation system (Chapter 3.2) exposed many members of the military as well as affiliated civilians. While military personnel were clearly the primary exposed population for these tests, this occurred without their knowledge or consent. Because they were not even aware that an experiment was being conducted, participation under coercion from superior officers is clearly not a concern. Examining *only* the coercion aspect of this experiment (and temporarily ignoring the flagrant

disregard for personal autonomy and informed consent), this experiment was conducted in a proper manner: military personnel served as research subjects but were not coerced into doing so.

Operation CD-22 and its successor Project Whitecoat (Chapter 3.5) were previously described in Chapter 4.2.4 as being constructed from the outset in such a manner as to ensure proper informed consent. The same appears to be true of its measures to ensure that its volunteers were uncoerced. Because soldiers are trained and required to follow the orders of superior officers, it appears that those who designed the recruitment sessions felt that being briefed for a volunteer project by their commanding officers could present the soldiers with a perceived conflict between being provided an opportunity to volunteer and being asked (in all but words) to volunteer. Removing the potential volunteers' commanding officers from the process was intended to eliminate any passive or indirect coercion the recruits might feel. These efforts were an integral part of the recruitment procedures decades before the recommendations of the ACHRE report or the implementation of DoD Directive 3216.2, §4.4.4. Once in the subject pool, those who declined participation in specific Project Whitecoat studies suffered no consequences, resumed their normal duties, and were provided the opportunity to enroll in future research studies. This ensures that subjects did not feel pressured to participate in a specific experiment lest they jeopardize their enrollment in or status as part of Project Whitecoat. Once again, the manner in which Operation CD-22/Project Whitecoat was conducted can be

viewed as a model for any military medical research involving human subjects: military personnel served as research subjects, but great lengths were taken to ensure that they were not coerced into doing so.

4.4. Improvements

For each of the open-air tests described in Chapter 3, this chapter examined three aspects of the experiments: the biological pathogenicity of the agents or simulants dispersed, informed consent, and military bioethics. With a critical analysis of the problems inherent to the previous simulant systems and/or their use completed, an analysis can be made of specific improvements that could be incorporated into those simulant systems and/or their use in order to avoid the problems described.

4.4.1. Pathogenicity

As was discussed in Chapter 4.1, an ideal simulant should exhibit essentially no pathogenicity. In discussing the desired lack of pathogenicity, it must be remembered that everything is dangerous. The 16th Century alchemist Paracelsus, regarded as the father of toxicology, famously stated: "What is there that is not poison? All things are poison and nothing [is] without poison. Solely the dose determines that a thing is not a poison.[308]" In that vein, the most poisonous toxin known – botulinum toxin – has therapeutic value if delivered at low enough a dose. On the contrary, while water is probably the most vital compound to human life, its overconsumption can result in a lethal electrolyte

[308] Quoted in: CD Klaassen, Casarett and Doull's Toxicology: The Basic Science of Poisons (New York: McGraw-Hill Medical Pub. Division, 2001).

imbalance known as water intoxication.[309] Thus, it is unrealistic to discuss a simulant having ***absolutely no*** pathogenicity or toxicity. After all, if water can be lethal, surely nothing can be entirely safe.

Therefore, we must adopt a more realistic view of pathogenicity. The two components that comprise the concept of risk are hazard and probability. Hazard encompasses the severity of the threat and its potential consequences. Probability simply refers to the statistical likelihood of that hazardous event occurring. A common method for risk analysis and prediction is through the use of a multiplicative risk equation, e.g.,

$$\text{Risk} = \text{Hazard} \times \text{Probability}$$
$$R = H \times P_H$$

Equation 4-1: Risk

Examining the risk components individually, a hazard with $H = 0$ would represent an event (in this case, exposure to a simulant) which has no effects whatsoever, whereas a hazard with $H = 1$ would represent an infection lethal to the person exposed. In assigning a value to H, it is useful to define an additional term, the maximal hazard, H_{max}. Quite simply, H_{max} represents the worst-case

[309] N Radojevic *et al.*, "Forensic aspects of water intoxication: Four case reports and review of relevant literature," *Forensic Science International*, 220(1), 2012.

scenario that could occur.[310] (For the sake of maximal safety, it is prudent to calculate the risk posed by a simulant such that $H = H_{max}$.) The probability, P, of an event (in this case, the specific hazard defined by H) occurring can be defined such that $P_H = 0$ would represent an event with no possibility whatsoever of occurring, whereas a $P_H = 1$ would represent a statistical certainty of the specific hazard occurring.[311] If $H = H_{max}$ in Equation 4-1, then the equation allows the calculation of the maximal risk, R_{max}:

$$\text{Maximal Risk} = \text{Maximal Hazard} \times \text{Probability}$$

$$R_{max} = H_{max} \times P_H$$

Equation 4-2: Maximal Risk

Because of the multiplicative nature of the equation, such an approach enables the general quantification of risk while factoring in values at either end of the spectrum for both hazard and probability. Based on these two comprising factors, risks can be generally classified into four categories. These are, in

[310] However, in doing so, we must be cautious to not take our considerations to unrealistic extremes. As mentioned at the beginning of this section, the fact that even water can be lethal in cases of overconsumption would suggest that H_{max} for absolutely everything should be equal to one. This, however, would defeat the purpose of incorporating hazard into the risk equation. While drinking water may ***technically*** have $H_{max} = 1$, drinking enough water such that a lethal dose is achieved is essentially not a realistic possibility. H_{max}, then, should be defined as the worst-case scenario that could ***realistically*** happen, even if it is statistically unlikely. (This, however, should not be confused with the probability, P.)

[311] It should be noted that P, as described here, is a relative but arbitrary factor. Thus, while $P = 0$ and $P = 1$ represent absolute uncertainty and certainty, respectively, the values in between are not necessarily linearly scaled; nor are they necessarily representative of actual statistical percentages. (E.g., $P = 0.5$ does not necessarily equate to a 50% likelihood of an event, nor is it exactly twice as likely as an effect with $P = 0.25$.) Because accurate hazard probabilities (P_H) for most situations are most likely unknowable, the use of P_H will typically be relegated to arbitrary relative values based upon estimation.

generally increasing order of risk: a) low consequence/low probability events, b) low consequence/high probability events, c) high consequence/low probability events, and d) high consequence/high probability events. In applying the risk equation to the pathogenicity of a simulant, the hazard becomes the severity of the disease or infection potentially caused, while the probability is the statistical chance of such an infection occurring.

Because both the hazard and probability contribute equally to the risk in Equation 4-2, one could imagine the calculated risks for both a low consequence/high probability event and a high consequence/low probability event being quantitatively equal. In one hypothetical scenario, a simulant causes a slight nasal irritation resulting in sneezing in everyone exposed. Because the effects are tangible but so minuscule, we can define H_{max} = 0.01. However, the likelihood that the simulant will have such an effect is P_H = 1, a complete certainty. Thus, we can assume that everyone exposed to the simulant will sneeze and suffer no other effects. The risk calculation for this scenario results in an R_{max} = 0.01. In a second hypothetical scenario, a simulant is capable of causing a systemic infection that is ultimately lethal. Because the effects are so severe, we can define H_{max} = 1. However, the likelihood that the simulant will have such an effect is P_H = 0.01, making this effect a fairly rare occurrence. Thus, we can assume that one in every one hundred people exposed will die as a result. The risk calculation for this scenario results in an identical maximal risk as in the first scenario, R_{max} = 0.01. Clearly, the implications of the former

scenario (making 100% of people exposed sneeze) and the latter scenario (killing 1% of people exposed) are quite different. Thus, it is useful to apply a factor that increases the contribution of the hazard to the calculated risk:

$$\text{Weighted Risk} = \left(\text{Maximal Hazard}\right)^2 \times \text{Probability}$$

$$R_W = (H_{max})^2 \times P_H$$

Equation 4-3: Weighted Risk

Recalculating the risks for the first scenario (H_{max} = 0.01) according to Equation 4-3 results in an R_W = 0.0001. Meanwhile, after recalculating the risks for the second scenario (H_{max} = 1) according to Equation 4-3, the weighted risk for the potentially lethal simulant remains R_W = 0.01. Using the weighted risk equation results in a first scenario risk that is 100 times less than that of the second scenario. The decision to modify the risk equation specifically by squaring the hazard is admittedly fairly subjective. A different specific modification utilized can be tailored to the tolerance for hazard in any given situation. However, Equation 4-3 provides a sufficient starting point for comparative analyses of the risk posed by different simulants.

It follows logically that the safest (i.e., with the lowest level of risk) simulant is one with both a low probability of an infection (P_H) taking hold and a negligible severity (H_{max}) if it does. Thus, the simulants least likely to cause infection would

be non-biological in nature.[312] However, as stated previously, use of such simulants would eliminate any experimental approaches contingent upon the viability of the simulant. The most accurate simulant for something is, of course, itself. Thus, as was mentioned in Chapter 2.1, the most accurate simulant for spores of pathogenic *B. anthracis* is spores of pathogenic *B. anthracis*. However, because both the probability of infection and its severity are high, using pathogenic spores in this manner would result a high consequence/high probability event that would be, to say the least, undesirable. On the contrary, a simulant which behaves entirely differently than the threat being simulated is of little value as a highly accurate simulant, no matter how safe it might be. Thus, the risk equation can be further adapted to incorporate the similarity of the simulant. An index of similarity, S, can be defined such that a range of similarity is represented numerically, where $S = 0$ is a simulant which is entirely dissimilar from the threat being simulated, and $S = 1$ is a simulant which is exactly similar (i.e., itself). Incorporating the index of similarity as an additional factor allows the calculation of the overall value of the simulant. Thus, for any simulant, its specific similarity (S_{sim}), the maximal hazard it poses (H_{max}), and probability of that hazard occurring (P_H) can be used to calculate the overall value of the specific simulant, V_{sim}:

[312] Chemical simulants could pose a risk of causing other toxic hazards, but this section is limited to biological pathogenicity.

$$\text{Simulant Value} = \frac{\text{Index of Similarity}}{\text{Weighted Risk}}$$

$$V_{sim} = \frac{S_{sim}}{(H_{max})^2 \times P_H}$$

Equation 4-4: Simulant Value

Returning to the earlier first scenario of the fairly safe, sneeze-inducing simulant with an H_{max} = 0.01 and a P_H = 1, the overall value of the simulant can be calculated according to Equation 4-4. Assuming the simulant has a high index of similarity (S_{sim} = 0.9), the value of the simulant is V_{sim} = 9,000. Intermediate (S_{sim} = 0.5) and low (S_{sim} = 0.1) indices of similarity result in V_{sim} values of 5,000 and 1,000, respectively. Returning to the earlier second scenario of the potentially lethal simulant with an H_{max} = 1 and a P_H = 0.01, the overall value of the simulant can be similarly calculated. Assuming the simulant has a high index of similarity (S_{sim} = 0.9), the value of the simulant is V_{sim} = 90. Intermediate (S_{sim} = 0.5) and low (S_{sim} = 0.1) indices of similarity result in V_{sim} values of 50 and 10, respectively. The approach presented in Equation 4-4 allows the estimation of a simulant's usefulness based on its similarities to the original threat being simulated while taking into account the probability of the simulant causing a hazard and (more heavily) the severity of the potential hazard.

While a simulant with a pathogenicity of zero likely does not exist, the risk of a simulant can be decreased to essentially zero by reducing either H_{max} or P_H

(or even better, both) to as close to zero as possible. The ideal simulant, then, would have a very low P_H, a very low H_{max}, and a high S_{sim}. The biological simulants most commonly used in the experiments described in Chapter 3 were *B. atrophaeus* and *S. marcescens*. Based on their known history of pathogenesis (even at the time), both the hazard and the probability factors are nonnegligible. (This should, in retrospect, be particularly true for *S. marcescens*. Although the strain of *S. marcescens* used during Operation Sea Spray appears to be different than the one which infected Edward Nevin, the bacterial species is clearly capable of causing potentially fatal opportunistic infections. Further, the historical literature and the outbreak in Stanford show clearly that the probability is by no means zero.)

For simulating the dispersal of pathogenic *B. anthracis* spores, the most accurate simulant (pathogenic *B. anthracis* spores, $S_{sim} = 1$) is clearly unusable due to its high H_{max} and P_H values. Table 4-2 shows the result of Equation 4-4 for this scenario as $V_{sim} = 1.0$. The simulant with the next highest similarity would be nonpathogenic *B. anthracis* spores (e.g., Sterne strain). Because they should be virtually identical in every way, with the exception of the expression of pathogenic traits (i.e., capsule genes and/or toxin genes), nonpathogenic *B. anthracis* spores are assigned an $S_{sim} = 0.99$. However, it must be kept in mind that the genes for these traits are naturally present in the environment, in both *B. anthracis* and other *Bacillus* species. If the nonpathogenic spores used as a simulant were to acquire the missing genes, the result would be fully virulent *B.*

anthracis. Thus, the maximal hazard value of 1.0 is retained. However, the probability of that occurring is low and is assigned a P_H = 0.2. Spores of *B. cereus* or *B. thuringiensis* could be used instead. The genotypic and phenotypic similarity of these species to *B. anthracis* results in a high, though slightly lower, S_{sim} = 0.95. Again, because the lethal genes could be acquired, H_{max} = 1, though this is probably even less likely and is assigned a P_H = 0.1. Spores of *B. atrophaeus* or *B. subtilis*, simulants which were used in many of the experiments described in Chapter 3, are more distantly related to *B. anthracis* than *B. cereus* or *B. thuringiensis* but are still very similar and are assigned a S_{sim} = 0.9. However, the historical literature shows that these species could still have some potential for infection (P_H = 0.1), though such opportunistic infections tend to be relatively minor (H_{max} = 0.25).

Table 4-2: V_{sim} and associated parameters for various simulants

Simulant	S_{sim}	H_{max}	P_H	V_{sim}
Pathogenic *B. anthracis* spores	1.0	1.0	1.0	1.0
Nonpathogenic *B. anthracis* spores	0.99	1.0	0.2	4.95
B. cereus or *B. thuringiensis* spores	0.95	1.0	0.1	9.5
B. atrophaeus or *B. subtilis* spores	0.9	0.25	0.1	144
Synthetic *B. subtilis* spores	0.9	0.001	0.001	9×10^8

S_{sim}: Index of similarity; H_{max}: Maximal hazard; P_H: Probability of hazard occurring; V_{sim}: overall value of the simulant.

The results shown in Table 4-2 rank the V_{sim} for the various simulant options considered thus far in an order consistent with their respective risk based on *Bacillus* biology. However, as safe as spores of *B. atrophaeus* or *B. subtilis* might be, the risk should still be considered unacceptably high for general use as an open-air simulant. In order to overcome this risk, a synthetic strain of *B. subtilis* can be designed such that it would retain its similarity to *B. anthracis* but have virtually no chance of causing an undesired infection. Extremely low values of both H_{max} and P_H increase the V_{sim} for such a simulant to levels that place it as the clearly preferable choice over any of the others (Table 4-2).

Such a simulant would meet the three-part criteria for an ideal biological simulant as described in Chapter 4.1.3: similarity, biological viability, and essentially no pathogenicity. Although no *naturally-occurring* viable system exists that can be regarded as completely nonpathogenic, a synthetic biologically viable system with parameters similar to those shown in Table 4-2 could be considered to be a safe and accurate simulant. Such a system would be a significant improvement over the simulant systems used in the tests described in Chapter 3: it would be a biological simulant, allowing biological viability to be assayed, while simultaneously avoiding the pathogenic risk inherent to those previously used biological simulant systems. The design of such a novel simulant is described in Chapter 5.

4.4.2. Informed Consent

The words printed in the Nuremberg Code, were later reprinted (often verbatim) in the Wilson Memorandum, CS:385, AR 70-25, and numerous ethical policy documents that followed: "The voluntary consent of the human subject is absolutely essential.[313]" This alone should highlight the fundamental nature of informed consent to ethical biomedical research. However, Chapter 4.2 outlined the complete disregard for informed consent that was displayed during most of the experiments in Chapter 3. Such tests would be considered appallingly unethical, if not downright illegal, today. To be fair, however, our understanding of informed consent during most of these experiments was neither as fully solidified nor as ubiquitously understood as it is now. The purpose of this section is less to render judgment on the past than it is to ensure that the mistakes of the past are not repeated. The informed consent methods used by two of the test series stood out from the remainder (which essentially had none): the traditional informed consent procedures utilized during Operation CD-22/Project Whitecoat and the alternative "*de facto* informed consent" utilized during the Boston MBTA experiments.

Traditional informed consent methods are more easily applicable to biomedical experiments conducted at the level of individual subjects/patients. The type of biodefense research that would be conducted at the individual level (e.g., therapeutic treatments, diagnostics, vaccines) would likely be governed by

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[313] *United States v. Karl Brandt et al*., The Medical Case, Trials of War Criminals Before the Nuremberg Military Tribunals Under Control Council Law No. 10.

existing research and/or medical care regulations regarding informed consent. For the most part, these informed consent policies are easily interpreted, well understood, and already commonly practiced.

However, traditional informed consent measures are not so easily applied to population-level research, as most open-air dispersal tests would be. The sheer logistics of obtaining traditional informed consent forms from anyone who will or might be exposed is likely prohibitively difficult. Thus, it becomes necessary to adapt procedures to large populations that are likely spread geographically and/or temporally. Such an alternative approach has not been employed often, and its requisite methodologies and potential legal ramifications are not well understood. However, there is essentially no valid argument that can support the idea that populations deserve fewer protections than do specific individuals.

One recent set of experiments where such an alternative method was used is the series of dispersals in the Boston MBTA system in 2012. In the months, weeks, and days prior to the dispersals, such a significant level of information was distributed as to essentially saturate the community with notification and description of the impending experiments. Because the provided information also included the dates of the tests, the riders of the subway system had the ability to avoid using the T on the specific days following the dispersals (i.e., decline participation in the experiments). Thus, respect for the autonomy of the community members was maintained, since they were not denied the

opportunity to exert their autonomy regarding the conditions to which they would be exposed.

The procedures used during these experiments are doubtlessly not ideal. Individuals who could have been exposed might have been unwilling or unable to attend one of the various public forums. They may have been similarly unwilling or unable to visit one of the various websites which provided information (both official information from the Department of Homeland Security and media information covering news of the impending tests). One might argue that an unwillingness to encounter the information about upcoming experiments is in and of itself an autonomous choice made by individuals and is tantamount to consent through apathy: if these individuals didn't care enough to seek out the information, then they have little right to complain.[314] However, such an argument would not be applicable to individual-level experiments: instead, a potential subject who was unwilling to be informed cannot provide valid informed consent and would be quickly removed from the potential study. To conduct experiments on such individuals despite their lack of informed consent would be a violation of their autonomy and would effectively return biomedical research to the pre-Nuremberg days. Why, then, would such an approach be acceptable in population-level research?

However, issues such as a lack of access to the information or an inability to fully understand the information provided are more likely to arise. Such issues

[314] An analogous argument is often applied to those who choose not to vote in elections.

might prove to have a higher prevalence in socioeconomically disadvantaged areas, placing residents in these areas in danger of their autonomy being violated. This could have implications regarding the bioethical principle of justice, which emphasizes that the risks from research should be distributed evenly.[315] It would be difficult to argue that the autonomy of an individual was respected if that person was unable to obtain or incapable of comprehending information about the experiment. Because a prerequisite of informed consent is being informed, there is essentially no realistic chance that such a situation could be considered as providing informed consent. Can an experiment on a population be ethically conducted if it is uncertain that the information necessary to make a decision has been disseminated and understood by all members of the population? These issues are interesting areas of bioethics which, though outside the scope of this already voluminous dissertation, likely warrant further exploration.

In order to ethically conduct open-air dispersal experiments that will or might expose populations to simulants, the most critical and urgently required improvement is the establishment of defined ethical and methodological protocols for informed consent at the population level. For such experiments, the methods used during the Boston MBTA tests appear to provide at least minimal protection for autonomy and informed consent and should be considered an

[315] On its own, the socioeconomic status of such individuals could have such implications, but this is further compounded by the fact that residents of socioeconomically disadvantaged neighborhoods are also more likely to be from racial and ethnic minority groups.

excellent model upon which to begin the formation of a population-level biodefense research ethic.

4.4.3. Military Bioethics

The ethics of military medicine is complex, and a comprehensive review of the various topics involved goes well beyond the scope of this dissertation. Instead, the current focus is on the coerced participation of military members as human research subjects in biomedical experiments.

Experiments which expose military personnel are likely essential to the protection of our fighting forces from agents of biological warfare. To suggest that completely avoiding exposure of soldiers is the most prudent approach is to put our entire military at risk. The situations that soldiers may encounter are unique, and the information necessary to protect them might not be able to be gleaned from civilian, population-level biodefense research. Thus, it may be necessary that some risks be taken in order to protect the overall health of our greater defense force. However, it is critical that those soldiers who participate as research subjects do so voluntarily of their own volition. As discussed in Chapter 4.3, the personal autonomy that would normally be enjoyed by soldiers is necessarily curtailed as a condition of their service in the military. Soldiers can be ordered into extremely dangerous situations in the course of their duties, and they have a moral and legal obligation to obey lawful orders. However, participation in biomedical experiments is supererogatory and thus cannot be

ordered.[316] For such experiments to be ethically conducted on soldiers, their participation must be fully voluntary, which also requires that they be provided with all the information necessary to make a properly autonomous choice.

An additional reason military service members and their personal autonomy deserve extra protection from coercive participation in biomedical experiments is because the "Feres Doctrine" prevents the federal government from being sued by military personnel.[317] Any physical or psychological injuries suffered by a civilian research subject may be addressed through litigation, particularly in cases of negligence. Soldiers likely have no such recourse. Without adequate protection of their rights, it is unethical to put them in such a situation without their completely uncoerced and voluntary participation.

The necessary improvement with regard to coercion of military personnel is fairly obvious: ensure that it does not happen. The real question becomes: ***how*** do we ensure that military personnel are not forced or coerced into participating as human subjects in biomedical research? An understanding of two critical factors can accomplish this. First, military leaders at all levels need to understand exactly what types of activities qualify as research and when soldiers

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[316] It should be noted that many of the individuals who were exposed or potentially exposed in the military tests described in Chapter 3 may have been drafted into military service. As such, they would have been conscripts who may not have joined the military of their own volition. The justice of restricting someone's personal autonomy as a condition of their enlistment when they were forced to join the military in the first place, then using that diminished autonomy as the basis for exposure to potentially dangerous medical experiments is highly debatable. This, however, is not currently an issue due to the elimination of conscription in the United States and the shift to an all-volunteer military.

[317] *Feres v. United States*,(1950); PJ Amoroso and LL Wegner, "The Human Volunteer in Military Biomedical Research," in Military Medical Ethics, Ed. TE Beam and LR Sparacino, ***Textbook of Military Medicine*** (Washington, DC: Office of The Surgeon General, Department of the Army, United States of America, 2003).

cross the line into research and should be considered to be human subjects. Second, those planning, recruiting for, and conducting the experiments must understand the variety of potential issues and the rank dynamics inherent to the military culture and the implications that these could have on the ability for soldiers to provide true voluntary informed consent.

Fortunately, much of that work has already been accomplished. The recommendations and requirements outlined by the Belmont Report, the ACHRE report, and the Common Rule (32 CFR 219), and DoD Directive 3216.2, §4.4.4 address most of these issues. This, however, is not to say that there is no work left to be done. One only need to remember that the unquestionably clear requirements of the Wilson Memorandum – "The voluntary consent of the human subject is absolutely essential[318]" – were effectively meaningless without the proper communication of those orders to the relevant people conducting and participating in military research.

An excellent example of methods to ensure that military research subjects are recruited in a completely voluntary and uncoerced manner is Operation CD-22/Project Whitecoat. Another example is the US Army Natick Soldier Systems Center (NSSC), located in Natick, Massachusetts and often referred to simply as "Natick Labs". The mission of this organization is to perform research geared toward developing systems, techniques, and equipment to enhance the effectiveness and survivability of American soldiers. A description of the

[318] CE Wilson, Use of Human Volunteers in Experimental Research. Reprinted in: SE Lederer, "The Cold War and Beyond: Covert and Deceptive American Medical Experimentation."

procedures used by Natick Labs to ensure fully voluntary participation in research projects reads very much like a description of Project Whitecoat.[319]

One way to avoid issues with non-dissemination of orders as happened with the requirements of the Wilson Memorandum is to centralize the conduct of military research. Having people all across the military conducting experiments raises the possibility that the experimenters have not received such important orders or are using procedures which may not be proper. Instead, having a specialized unit devoted to conducting military research reduces the probability of this occurring (assuming everyone within the unit is properly trained and managed). Units such as the Natick Labs help protect our military service members from experiments which could violate their personal autonomy and potentially place them at an unethical risk of harm.

4.5. Conclusions

Beginning in 1949 and continuing through 1973, the United States government (primarily the military) conducted numerous open-air dispersal experiments that intentionally exposed human subjects to a variety of simulants. In most of these cases, the simulants used were known to have some nonnegligible level of pathogenicity, putting those exposed at risk. Additionally, most of these tests exposed people – either the American public or US military service members – without their knowledge or consent. Although informed consent is now considered to be the cornerstone of ethical biomedical research,

[319] PJ Amoroso and LL Wegner, "The Human Volunteer in Military Biomedical Research."

the lack of informed consent in these tests means that the personal autonomy of those exposed was violated. Also concerning is the possibility that American soldiers may have been ordered to or coerced into participating in potentially hazardous medical research involving pathogenic microorganisms. The intentions of US Cold War-era defensive BW research may be laudable, but the manner in which most of these experiments were conducted would be considered egregious today.

Both biological warfare waged upon our military and bioterrorism upon our American citizens have been and continue to be significant concerns with high consequences. Thus, it is critical to the national security of the United States that the types of biomedical experiments described in Chapter 3 continue to be conducted today. However, the manner in which most of these experiments were performed in the past is now considered unethical and would be wholly unacceptable today.

In order to safely perform open-air testing with biologically viable simulants, the experiments should be conducted in a manner that favorably addresses each of the three ethical issues described in this chapter. First, the simulants used should be incapable of colonizing undesired environments, as this carries the risk of unintended infections. Second, anyone who may be exposed should provide some manner of informed consent. Where possible, traditional informed consent is highly preferable; however, in cases where it is not realistically possible, an alternative process such as *de facto* informed consent

should be applied. Such a process should be developed, refined, and approved prior to its application to ensure that it meets all ethical guidelines for human experimentation as understood today. Third, if any military personnel serve as human subjects, great care should be taken to ensure that their participation in such experiments is completely and unquestionably voluntary. The latter two issues are critical and must be addressed, but their further exploration is left to others in the bioethics community.

Instead, the remainder of this dissertation focuses on the first issue. As has been made clear, the most useful and accurate simulants for modeling pathogenic microorganisms are biologically viable, but the safest simulants in terms of pathogenicity are nonviable. It would appear that these are mutually exclusive criteria which cannot be achieved simultaneously. A proposed biological solution to this apparent conundrum is presented in Chapter 5.

5. *IN SILICO* DESIGN OF NOVEL *BACILLUS SUBTILIS* STRAIN

Chapter 2 of this dissertation discussed why simulation of bioaerosol dispersal is necessary and various types of models created to meet that need. While the use of biologically viable simulants is ideal for simulation of bioaerosol dispersal, their use poses a risk of pathogenicity. Chapter 3 presented a historical chronology of bioaerosol testing (using both infectious organisms and simulants) conducted by the US government, while Chapter 4 addressed the various bioethical flaws in the historical tests, including the wonton disregard shown by the government in the dispersal of potentially pathogenic agents amongst unsuspecting US citizens.

The potential for undesired viability is inherent to the use of a living bacterial simulant. However, if the simulant could be "conditionally viable" (that is, viable only when and where desired, but otherwise non-viable), it could be considered a safe simulant. Chapter 5 describes such a simulant system designed to essentially eliminate the possibility of colonization in undesired environments. First, creation of a novel simulant system through the genetic modification of *Bacillus subtilis* is described generally in Chapter 5.1. The logical culmination of this dissertation – the *in silico* design of the genetic constructs for creating such a strain – is presented in the remainder of the chapter.

5.1. *Bacillus subtilis*

Bacillus subtilis is a species in the same genus as *Bacillus anthracis*. Like *B. anthracis*, *B. subtilis* is a Gram-positive, spore-forming, aerobic bacterium that occurs ubiquitously in soil. Although the concentration of *B. subtilis* in the environment has not been established, bacilli in general are found in soil at an approximate concentration of 10^6 – 10^7 bacteria/gram.[320] Between 60% – 100% of soil bacilli are in the form of spores. The spores formed by *B. subtilis* and *B. anthracis* exhibit slight differences in size and shape. *B. subtilis* spores are smaller in diameter (mean: 1.07 μm) and longer in aspect ratio (2.23), which corresponds to a rod shape, while *B. anthracis* spores are larger (mean: 1.42 μm) with a more ellipsoid aspect ratio (mean: 1.74).[321]

B. subtilis is considered to be non-pathogenic in humans, animals, and plants and is Generally Regarded As Safe (GRAS) by the US Food and Drug Administration (FDA). However, as with most bacteria, *B. subtilis* should more accurately be considered an opportunistic pathogen. *B. subtilis* infections, though rare, have been documented. Most cases have occurred in people with suppressed or compromised immune systems. Reported cases include endocarditis after drug abuse, three fatal cases of pneumonia and bacteremia in patients with leukemia, septicemia in a patient with metastatic breast carcinoma, and infection of a necrotic axillary tumor in another breast cancer patient, as well

[320] M Alexander, Introduction to Soil Microbiology (New York: John Wiley and Sons, Inc., 1977).
[321] M Carrera *et al.*, "Difference between the spore sizes of *Bacillus anthracis* and other *Bacillus* species."

as a fatal septicemia in a patient with chronic lymphocytic leukemia.[322] Over a period of eight years, 18 febrile cancer patients at a hospital in Baltimore were diagnosed with bacteriemias caused by a variety of *Bacillus* species, including *B. cereus*, *B. circulans*, *B. subtilis*, and *B. pumilus*.[323] In 1990, a pseudoepidemic at a hospital in Taiwan resulted in 15 patients being infected with *B. cereus* from contaminated ethanol.[324] A hospital in Turkey reported infections with *B. licheniformis*, *B. cereus*, and *B. pumilus* in 12 patients with acute leukemia or non-Hodgkin's lymphoma, two of which patients died. Though the *Bacillus* infections "were not attributable factors for the death, [they] were thought to be major factors in the course of disease.[325]" Additionally, inoculation of dermal abrasions with *Bacillus* species has been documented; the likelihood of infection is exacerbated further by the presence of an immunocompromised state.[326]

Furthermore, the numbers of immunocompromised or immunosuppressed individuals in the United States has increased dramatically as the use of immunosuppressive drugs has increased, as well as through the spread of

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[322] NA Logan, "*Bacillus* species of medical and veterinary importance."; MR Oggioni *et al.*, "Recurrent septicemia in an immunocompromised patient due to probiotic strains of *Bacillus subtilis*," *J Clin Microbiol*, 36(1), 1998.
[323] C Banerjee *et al.*, "*Bacillus* infections in patients with cancer," *Arch Intern Med*, 148(8), 1988.
[324] PR Hsueh *et al.*, "Nosocomial pseudoepidemic caused by *Bacillus cereus* traced to contaminated ethyl alcohol from a liquor factory," *J Clin Microbiol*, 37(7), 1999.
[325] V Ozkocaman *et al.*, "*Bacillus* spp. among hospitalized patients with haematological malignancies: clinical features, epidemics and outcomes," *J Hosp Infect*, 64(2), 2006.
[326] S Boulinguez and R Viraben, "Cutaneous *Bacillus cereus* infection in an immunocompetent patient," *J Am Acad Dermatol*, 47(2), 2002; Centers for Disease Control and Prevention (CDC), "Outbreak of cutaneous *Bacillus cereus* infections among cadets in a university military program--Georgia, August 2004," *MMWR Morb Mortal Wkly Rep*, 54(48), 2005; KJ Henrickson *et al.*, "Primary cutaneous *Bacillus cereus* infection in neutropenic children," *Lancet*, 1(8638), 1989; D Tena *et al.*, "Cutaneous infection due to *Bacillus pumilus*: report of 3 cases," *Clin Infect Dis*, 44(4), 2007.

immunocompromising diseases such as AIDS. For example, the Centers for Disease Control and Prevention (CDC) estimates nearly 1.2 million people in the US were living with either diagnosed or undiagnosed HIV/AIDS at the end of 2011. Of these, an estimated 50,199 new infections occurred in 2011.[327] Thus, while *B. subtilis* is generally regarded as safe, there is a non-negligible (and increasing) population who face potential risk from exposure to *B. subtilis*. Because of this, using wild-type *B. subtilis* as a simulant to model release of *B. anthracis* in an indoor or outdoor environment is potentially hazardous to a vulnerable population subset, and is therefore an unacceptable risk. Instead, a safer alternative must be used.

5.1.1. Genetically Modified *B. subtilis* Strain as Simulant

A genetically-engineered novel strain of *B. subtilis* could be created that would inhibit growth of bacteria unless a predefined very specific set of conditions is met. (The reason for using a strain of *Bacillus subtilis* rather than *Bacillus atrophaeus* is because the former has been a model organism for many decades and is well-characterized. Additionally, the genome of the most commonly studied and well-characterized laboratory strain – *B. subtilis* subsp. *subtilis* strain 168 – has been fully sequenced[328] and better characterized, facilitating the design of genetic manipulations. These two species are extremely

[327] Centers for Disease Control and Prevention (CDC). "HIV Surveillance Report, 2011," (2013).
[328] F Kunst *et al.*, "The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*."

closely related, more so than either of them are to *B. anthracis*.[329]) Using inducible promoters, the expression of certain critical genes in the bacteria can be regulated so that the bacteria will survive only if supplied with specific chemicals and would die under other circumstances. Thus, the bacteria can be engineered to survive only where and when desired. Due to the growth regulation exhibited by this novel strain, it should hypothetically be even safer than wild-type *Bacillus subtilis*, which itself is generally regarded as safe. Spores of this novel strain could then be released into an environment to directly test the dissemination patterns and potential effects of countermeasures. This method of direct testing would allow for the accurate measurements of *Bacillus* spore dispersal, rather than relying on mathematical models or dissimilar physical simulants, while maintaining a very high margin of safety for vulnerable populations. There is an additional benefit to this system. Because the system is biological rather than purely physical, the detection endpoint of the simulation is growth of bacteria from the spores. This adds an important advantage over non-biological simulants: it enables the system to detect the effects of countermeasures aimed at the biological (e.g., killing or otherwise inactivating the bacteria) rather than being restricted to purely the physical (e.g., filtration of the simulant).

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[329] V Bhandari *et al.*, "Molecular signatures for *Bacillus species*: demarcation of the *Bacillus subtilis* and *Bacillus cereus* clades in molecular terms and proposal to limit the placement of new species into the genus *Bacillus*."; HS Gibbons *et al.*, "Genomic signatures of strain selection and enhancement in *Bacillus atrophaeus* var. *globigii*, a historical biowarfare simulant."; D Xu and JC Cote, "Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3' end 16S rDNA and 5' end 16S-23S ITS nucleotide sequences," *Int J Syst Evol Microbiol*, 53(Pt 3), 2003.

The proposed system uses inducible promoters to control the expression of two types of genes: essential genes and lethal genes. Essential genes are genes without which the bacterium is unable to survive or replicate. A systematic inactivation of the over 4,100 genes in the *Bacillus subtilis* genome showed that only 261 of these genes are essential for growth or division when inactivated singly.[330] If the bacteria are unable to express any one of these genes, they will either be non-viable and will quickly die or will be unable to reproduce. In contrast, lethal genes are those whose protein products are fatally toxic or damaging to the bacterial cells. Some of these genes must remain entirely unexpressed in order for the bacteria to survive. Others can be expressed at certain times, (e.g., cell wall degradation enzymes), but can be detrimental or lethal if expressed at inappropriate times. The genome of *B. subtilis* contains such lethal genes. Using inducible promoter systems, the expression of selected essential and lethal proteins can be controlled. While the use of inducible promoters to control gene expression at the transcriptional level is not new, the proposed approach combines the restoration of expression of proteins essential to the survival of *B. subtilis* (using positive regulation) with the silencing of gene expression of genes lethal to the bacteria (using negative regulation). In a positive regulatory system, the default state for a gene is to not be transcribed, i.e., "off"; regulation of the gene by a specific stimulus activates the gene's

[330] FM Commichau, N Pietack, and J Stulke, "Essential genes in *Bacillus subtilis*: a re-evaluation after ten years," *Mol Biosyst*, 9(6), 2013; K Kobayashi *et al.*, "Essential *Bacillus subtilis* genes," *Proc Natl Acad Sci U S A*, 100(8), 2003.

transcription, i.e., turns the system "on." In a negative regulatory system, the default state is the transcription of a gene, i.e., "on"; regulation of the gene by a specific stimulus halts the gene's transcription, i.e., turns the system "off."[331] Examples of such stimuli include temperature, stress, or the presence or absence of specific chemicals.

By placing several of the essential genes under positive regulation, their expression will be silenced by default. Thus, the bacterium will be unable to survive outside of its protective spore. Only when the environment is suitable (i.e., the stimulus is applied) will transcription of an essential gene occur. On the contrary, placing multiple lethal genes under negative regulation would result in a bacterium that naturally produces toxic products that result in its demise (i.e., bacterial "suicide"). When properly stimulated, expression of these lethal genes would cease, thus allowing the bacteria to live. The use of either (or both) of these systems allows the experimenter to define the conditions under which the bacteria can live.

The proposed system would use chemically-induced promoter systems to regulate gene expression. Ideally, such a system would use a variety of different promoter-inducing chemicals. Examples of various promoter inducing systems include xylose[332], tetracycline[333], vancomycin[334], and isopropyl β-D-1-

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[331] DL Hartl and EW Jones, "Molecular Mechanisms of Gene Regulation," in Genetics: Analysis of Genes and Genomes (Sudbury, Massachusetts: Jones and Bartlett, 2001).
[332] L Kim, A Mogk, and W Schumann, "A xylose-inducible *Bacillus subtilis* integration vector and its application," *Gene*, 181(1-2), 1996.
[333] M Gossen and H Bujard, "Tight control of gene expression in mammalian cells by tetracycline-responsive promoters," *Proc Natl Acad Sci U S A*, 89(12), 1992.

thiogalactopyranoside (IPTG). Because the chemical stimuli for some of the common inducible promoter systems are drugs (e.g., tetracycline), it is conceivable that a person taking one of these drugs may be able to support induction of such a promoter system, resulting in bacterial growth. For this reason, multiple promoter systems with different induction chemicals will be used. Additionally, several of the genes will be placed under the induction of chemicals that are not drugs or are of negligible therapeutic or human nutritional value, e.g., xylose or IPTG. This would reduce the probability of the proposed *B. subtilis* strain encountering the stimulus in a human environment because it is highly unlikely that a person will have the chemical in their system. The result is an organism whose growth could be tightly controlled, occurring only in a highly-specific "cocktail" of inducing chemical compounds. Hypothetically, an organism designed in this way could be released into an environment and would have virtually no chance of survival except in specifically-designed growth media (either liquid or solid).

Figure 5-1 shows a simplified diagram of an essential/lethal system as described above. In the figure, the system is composed of one essential gene (under the transcriptional control of Chemical A) and one lethal gene (under the transcriptional control of Chemical B). The first scenario (depicted in Figure 5-1A) is the only one in which the bacteria can survive, because both Chemicals A

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[334] AT Ulijasz, A Grenader, and B Weisblum, "A vancomycin-inducible *lacZ* reporter system in *Bacillus subtilis*: induction by antibiotics that inhibit cell wall synthesis and by lysozyme," J Bacteriol, 178(21), 1996.

and B are present. In the remaining three situations (Figure 5-1B – Figure 5-1C), one or both are missing; this results in a situation where an essential gene is not produced, a lethal gene is produced, or both. While this simplified scenario illustrates the principle behind the essential/lethal transcriptional gene control system, a realistic implementation of this system would use more genes, each under the control of a different promoter system. Each new inducible promoter system added to the genome decreases the probability of a chance encounter with the proper combination of chemical inducers required for bacterial survival. Ideally, the final version of the modified bacteria would be under the control of 5 or more different chemicals. Using multiple promoters is important because some level of loss of control is often seen. A review of suicidal genetic elements states that a fraction (10^{-3} – 10^{-6}) of bacteria always survive. This can be ameliorated, however, by either using two identical systems (e.g., the same suicidal gene) or using two different systems.[335] For example, if the failure rate of one inducible promoter system is 1 bacterium in 1,000 (a rate of 10^{-3}), adding a second inducible promoter system with a similar failure rate would make the total failure rate (i.e., the probability of a single bacterium having a mutation that bypasses both inducible promoter systems) 10^{-6}. Because any one inducible promoter system might fail (through mutation, etc.) or have inherent promoter leakiness, using multiple inducible promoters simultaneously builds redundancy into the system.

[335] S Molin *et al.*, "Suicidal genetic elements and their use in biological containment of bacteria," *Annu Rev Microbiol*, 47, 1993.

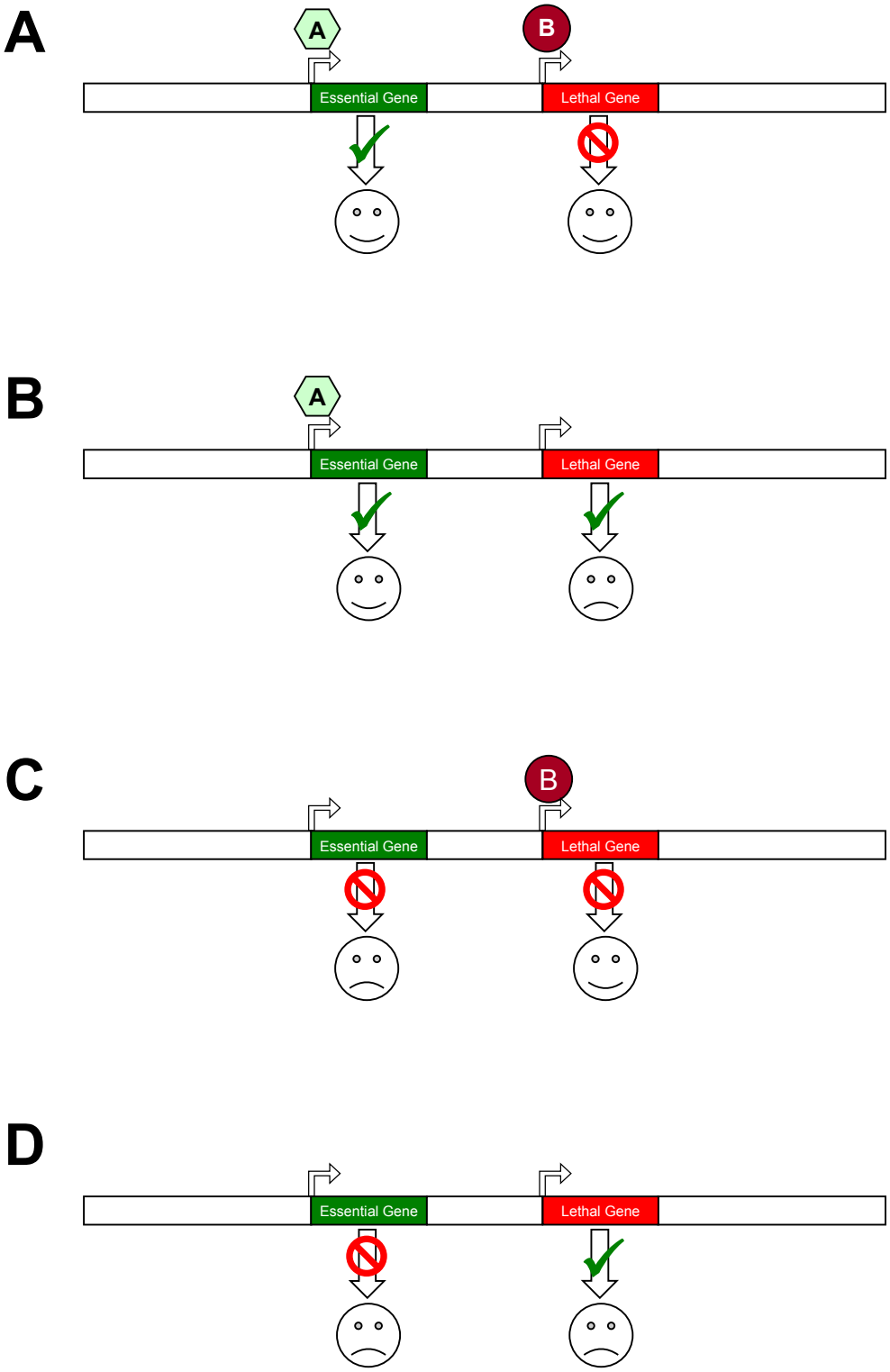

Figure 5-1: Simplified diagram of an essential/lethal regulatory system

A very important corollary to the growth restriction exhibited by the proposed bacterial system manifests itself in the detection of this novel strain. Because this strain would only be able to survive when supplied with the proper chemicals, the detection endpoint becomes the growth of the bacteria. Bacteria that find the specifically-designed media will germinate and multiply. Those that do not find the suitable growth medium will a) perish rapidly if they germinate and enter a vegetative state, or b) remain in spore form until a later date, when they will perish upon germination. Thus, the detection system that would be used for such bacteria is inherent in the bacteria itself.

(An important side note is that the bacterium must be controlled in this manner – germination cannot be directly prevented using inducible promoters. This is because germination is essentially a passive process, as neither protein synthesis nor production of adenosine triphosphate (ATP) is necessary to begin germination. All the components necessary to break down the spore coat and bring the bacterium back to life are produced during the sporulation process; these molecules reside inside the spore until needed for germination.[336] By knocking out four genes critical to germination (*gerD*, *cwlJ*, *sleB*, and *cwlD*, which all play roles in the process of spore coat breakdown), a strain of *B. subtilis* has been recently designed to be incapable of germination.[337] This arrangement means that cells that form spores without having expressed these genes prior to

[336] M Paidhungat and P Setlow, "Spore Germination and Outgrowth," in *Bacillus subtilis* and Its Closest Relatives: From Genes to Cells, Ed. AL Sonenshein, JA Hoch, and RM Losick (Washington, DC: ASM Press, 2002).
[337] LMU-Munich iGEM Team, "**Bead**zillus: How do Sporulation & Germination Work?," http://2012.igem.org/Team:LMU-Munich/Germination_Stop/Knockout.

sporulation will be unable to emerge from within the spore coat. However, deletion of these genes inhibits *all* germination regardless of circumstances, meaning that the process is irreversible. Thus, while such an arrangement might be ideal, germination of spores cannot be prevented using inducible promoters. The best alternative is to prevent the bacterium's survival after germination.)

Essential genes to be placed under inducible regulation would be selected such that multiple critical pathways are each redundantly controlled. For example, two or more essential genes from the DNA replication pathway would both be placed under different inducible promoters, as would two or more essential genes from various critical metabolic pathways. By assuring that critical pathways are redundantly controlled, this reduces the probability of bacterial escape from inducible promoter control.

Because of the multiple genetic constructs that would be placed into the *Bacillus subtilis* genome, using antibiotic selection markers in the standard manner would a) require a great number of different antibiotic resistance genes in order to select the serially transformed bacteria at each step, and b) would ultimately result in a *B. subtilis* strain with an extensive repertoire of antibiotic resistance. Although *B. subtilis* is generally regarded as safe, this is still a potentially precarious situation. Additionally, the end result could possibly be a strain with other altered biological properties relative to wild-type *B. subtilis*. Thus, a better method must be used.

The proposed method uses the transient insertion of an antibiotic resistance gene (Ab^R) via homologous recombination for the purposes of selection, followed by the removal of the Ab^R via excision using the *cre/lox* recombinase system. Colonies with successful removal of the Ab^R would be isolated via replica plating and verified via PCR and/or DNA sequencing. Removal of the Ab^R amounts to the insertion of the inducible promoter without the use of permanent antibiotic resistance markers. Employing this "markerless" approach would avoid creating such a multiply-resistant strain of *B. subtilis* and would avoid the unnecessary alteration of *B. subtilis* biology.

5.1.2. Synthetic Biology

Synthetic biology is an emerging field within the biological sciences. Although the first "genetic engineering" based on molecular biology techniques occurred in 1972 with the use of bacterially-derived restriction enzymes to mediate DNA recombination[338], the term "genetic engineering" is a misnomer, as the process is not based on true engineering principles. The field of synthetic biology seeks to bring a true engineering approach to the biological sciences. Through the use of concepts and techniques such as rational design, standardized biological parts, orthogonal systems, and refactoring, synthetic biology aims to legitimize biology as a true engineering discipline by allowing the predictable, from-the-ground-up design and construction of novel biological devices in a manner similar to architectural or electrical engineering.

[338] DA Jackson, RH Symons, and P Berg, "Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*," Proc Natl Acad Sci U S A, 69(10), 1972.

One of the primary goals of synthetic biology is to develop a “toolbox” of well-defined biological parts, including promoters, ribosome binding sites (RBSs), coding sequences (CDS), and terminators. The analogy that is most often used among synthetic biologists compares these standardized biological parts to LEGO® bricks. The desired ideal is that the individual parts can be attached end-to-end to build predictably working biological systems. The modular design and construction of these systems allows for interchangeability of parts as well. For example, a lactose-responsive promoter upstream of an RBS-CDS-terminator group could be easily swapped for a promoter that responds to arabinose. This replacement should have little to no impact on the function of the remaining parts, other than the expected switch in input signal.

One of the most popular formats for standardized biological parts is the BioBrick™ format. In the BioBrick™ format, parts each contain a defined set of restriction enzyme cut sites at the 5’ end and a different defined set of restriction enzyme cut sites at the 3’ end. By cutting the DNA parts with the appropriate restriction enzymes, individual parts can be strung together into composite parts that perform a biological function.

An alternative method to the piecemeal construction of such composite parts is *in silico* design, also known as biological computer-aided design (CAD) or BioCAD. Using the same general principles, long chains of parts can be organized in a biologically meaningful arrangement and assembled using software. Once a sequence is designed, it can be uploaded to the website of any

of a number of commercial vendors which can synthesize the desired DNA sequence with high fidelity and ship it to the customer. The cost of custom DNA synthesis has dropped so dramatically that designing and simply ordering such customized biological systems is reasonably within the reach of many labs. Although this dissertation does not entail the physical production and *in vivo* testing of the sequences designed, this would be the logical final step in bringing the idea to its full fruition.

5.2. Methods: General

5.2.1. Software

Graphics for figures were created using TouchDraw for iPad. Graphics were based on a modified version of the Synthetic Biology Open Language[339] Visual (SBOL Visual), available online at http://www.sbolstandard.org/. SBOL Visual is an open-source graphical notation that supports the description and specification of genetic parts, devices, modules, and systems. Codon optimization and determination of Codon Adaptation Index (CAI) values were performed using the Java Codon Adaptation Tool (JCat)[340], available online at http://www.jcat.de. The organism selected as the reference set was *Bacillus subtilis* (Strain 168). Alignment of DNA and protein sequences was performed using the multiple sequence alignment program Clustal Omega v.1.2.1[341], available online at http://www.ebi.ac.uk/Tools/msa/clustalo/. Three-dimensional

[339] M Galdzicki *et al.*, "Standard biological parts knowledgebase," *PLoS One*, 6(2), 2011.
[340] A Grote *et al.*, "JCat: a novel tool to adapt codon usage of a target gene to its potential expression host," *Nucleic Acids Res*, 33(Web Server issue), 2005.
[341] F Sievers *et al.*, "Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega," *Mol Syst Biol*, 7, 2011.

structures of unknown proteins were modeled using the automated homology modeler ESyPred3D Web Server v.1.0[342], available online at http://www.unamur.be/sciences/biologie/urbm/bioinfo/esypred/. Molecular graphics and analysis of three-dimensional protein structures were performed with the UCSF Chimera v.1.7 extensible molecular modeling package[343], available online at http://www.cgl.ucsf.edu/chimera/. Prediction of RNA secondary structure was performed using the RNAfold WebServer[344], available online at http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi. Assembly of individual genetic parts into constructs was performed using the j5 DNA Assembly Design Automation Software v.2.5.8[345], the DeviceEditor visual biological CAD canvas v.2.1.6[346], and Vector Editor display and annotation software v.1.7.4.[347] The j5 suite is available online at http://j5.jbei.org/.

5.2.2. General Strategy

The synthetic constructs intended primarily for protein production were designed according to the general pattern shown in Figure 5-2. The promoter-RBS-CDS arrangement is necessary for the proper transcription of the gene DNA into mRNA and the subsequent translation of the encoded message into protein.

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[342] C Lambert *et al.*, "ESyPred3D: Prediction of proteins 3D structures," *Bioinformatics*, 18(9), 2002.
[343] EF Pettersen *et al.*, "UCSF Chimera--a visualization system for exploratory research and analysis," *J Comput Chem*, 25(13), 2004.
[344] AR Gruber *et al.*, "The Vienna RNA websuite," *Nucleic Acids Res*, 36(Web Server issue), 2008.
[345] NJ Hillson, RD Rosengarten, and JD Keasling, "j5 DNA assembly design automation software," *ACS Synth Biol*, 1(1), 2012.
[346] J Chen *et al.*, "DeviceEditor visual biological CAD canvas," *J Biol Eng*, 6(1), 2012.
[347] TS Ham *et al.*, "Design, implementation and practice of JBEI-ICE: an open source biological part registry platform and tools," *Nucleic Acids Res*, 40(18), 2012.

The terminator downstream of the CDS terminates the transcription process, ensuring that the gene is monocistronic and is expressed independently of any neighboring genes. The UpStream Homologous Region (USHR) and DownStream Homologous Region (DSHR) at either end of the constructs define where the construct will insert into the genome via homologous recombination. (These regions are described in more detail in Chapter 5.3.7.) Immediately inside of both the USHR and DSHR, a terminator has been added to either end of each construct to ensure that the genes of the constructs are regulatorily isolated from the surrounding genes (i.e., the genes of the construct are not driven by the promoters of the genes in the region of insertion, or vice versa).

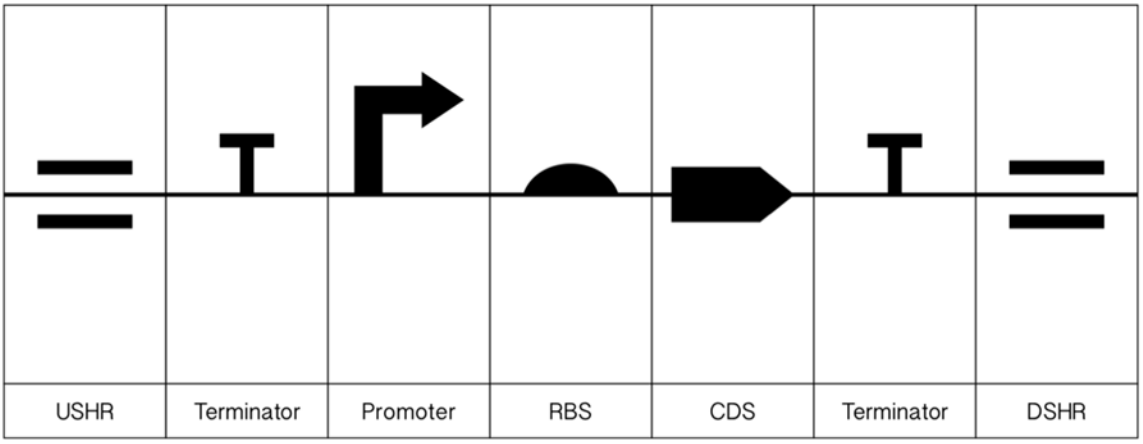

Figure 5-2: SBOL Visual design for protein-producing constructs

The promoter replacement constructs were designed according to the general pattern shown in Figure 5-3.

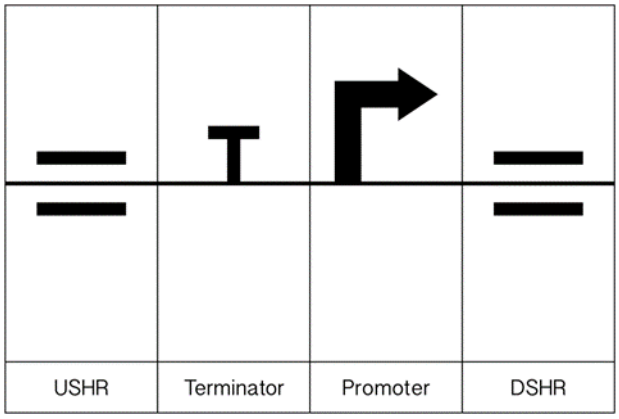

Figure 5-3: SBOL Visual design for promoter replacement constructs

Because the intent of the construct is to change the regulatory signals governing expression of the downstream gene, these constructs were not designed with a terminator at the 3' end. All the DSHRs were designed such that at their 5' end was the transcription start site (TSS) of the essential gene. Where the operon immediately upstream of the region of insertion was in the same orientation as the essential gene to be replaced, the USHR was designed such that at its 3' end was the terminator of the upstream operon (Figure 5-4A). This arrangement should ensure that any native promoters, operators, or other regulatory sequences are replaced by the insertion of the cassette. Additionally, the incorporation of the native sequence between the TSS and the 5' end of the gene into the DHSR increases the likelihood that the gene can be expressed at levels approximating those in the wild-type state (apart from the primary effects of the promoter replacement). Where the operon immediately upstream of the region of insertion was in the opposite orientation as the essential gene to be replaced, the USHR was designed such that at its 3' end was the upstream

promoter's identifiable regulatory element closest to the essential gene (Figure 5-4B). Again, this arrangement should eliminate any of the native regulatory sequences governing expression of the essential gene, while not interfering with the regulation of the upstream operon. All the USHR and DSHR sequences were designed to span a length of 150 bp each.

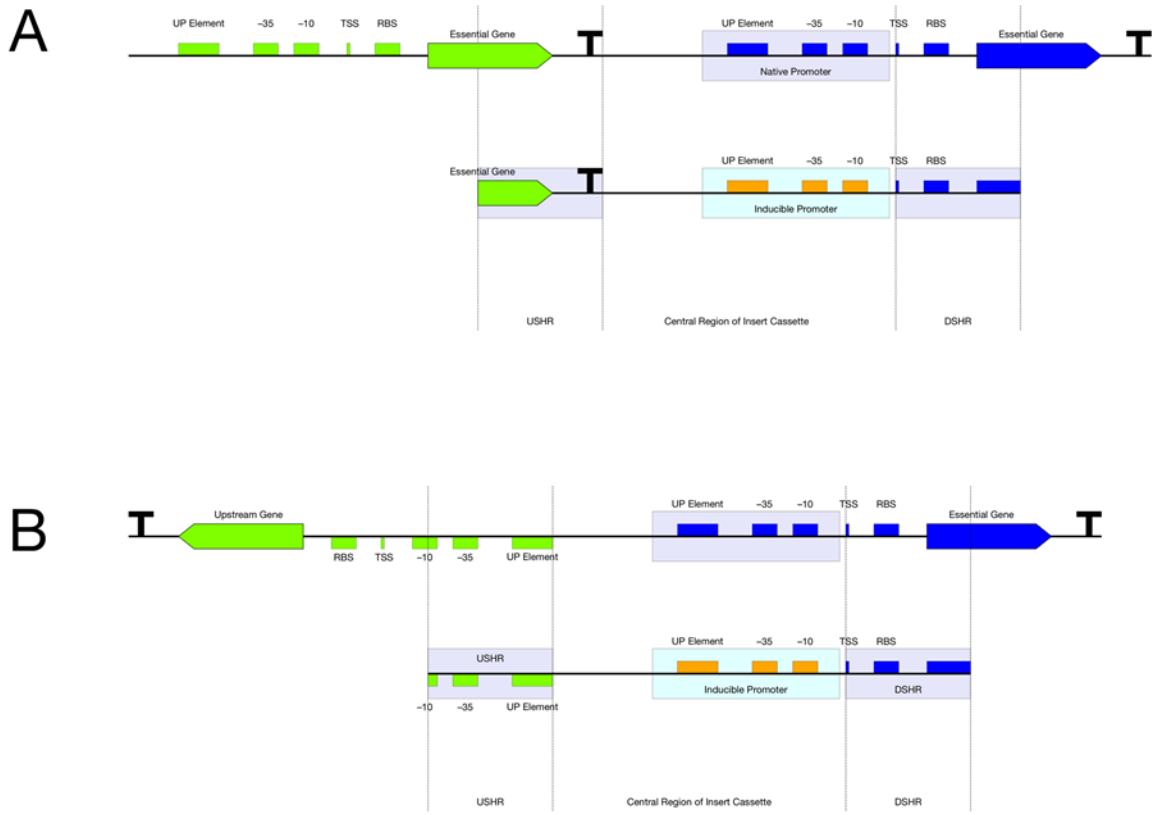

Figure 5-4: Overlap of construct USHRs and upstream native regulatory elements

5.2.3. Optimization of Genetic Part Sequences

Where appropriate, the genetic parts described in this chapter were optimized prior to their use in the construction of the synthetic constructs.

The primary optimization that was performed was correction for codon bias in the genes to be expressed. The anticodon on tRNA molecules recognizes a specific codon on the DNA strand. At the aminoacyl end of the molecule, the amino acid specific to the codon is added to the nascent peptide chain. In most cases, the same amino acid is transferred by multiple tRNAs; for example, `tgt` and `tgc` each encode cysteine. (This redundancy should be intuitively obvious: 61 amino acid-encoding tRNAs exist to encode only 20 unique amino acids.) These are known as synonymous codons. However, the frequency at which these synonymous codons occur is often skewed. For example, in the highly-expressed genes (HEG) in *Bacillus subtilis*, cysteine is encoded by `tgt` in 63% of codons and `tgc` in only 37% of codons. This favoring of certain codons over others is called codon bias. Codon bias allows the regulation of gene expression at the level of translational efficiency. Because the expression level of the individual tRNA molecules is strongly correlated with codon bias, a higher cellular concentration exists of the tRNA molecules for the more frequently occurring codons. Consequently, in genes with non-optimal codons, the continued translation of nascent proteins can be delayed while the ribosome waits to encounter the less frequent tRNA molecules.

Codon bias can also vary between organisms. For example, while `ctg` accounts for 85% of leucine codons in *E. coli*, it only accounts for 4% of leucine

codons in *B. subtilis*. In *B. subtilis*, the predominant codon encoding leucine is `ctt` (51%). Thus, the expression of a foreign gene in an organism with a different codon bias can be inefficient unless adequate compensations for the codon bias differences are made. One method for compensating for the relative scarcity of certain tRNAs is to provide the organism with a supplementary source of these rare molecule types. One such system for use in *E. coli* is the pRARE plasmid.[348] A second method of compensation is the optimization of the gene's codons to better reflect the codon bias of the target organism. In the case of the example above, an *E. coli* gene with codons for leucine could be optimized for expression in *B. subtilis* by switching the `ctg` codons to `ctt` codons.

The protein-encoding genes were optimized in the latter manner using JCat. For all optimized genes, verification that codon bias optimization did not affect the primary sequence of the encoded polypeptide is presented.

5.3. Methods: Genetic Parts

5.3.1. Essential Genes

Essential genes were selected based on three criteria: 1) a demonstrated lack of viability in *Bacillus subtilis* single-gene knockouts or mutants, 2) monocistronic transcription, and 3) distribution amongst different critical pathways. Recent publications have identified and confirmed 261 genes as essential.[349] However, not all these genes would make ideal candidates for the

[348] NV Kirienko *et al.*, "Significance of codon usage and irregularities of rare codon distribution in genes for expression of BspLU11III methyltransferases," *Biochemistry (Mosc)*, 69(5), 2004.
[349] FM Commichau, N Pietack, and J Stulke, "Essential genes in *Bacillus subtilis*: a re-evaluation after ten years."; K Kobayashi *et al.*, "Essential *Bacillus subtilis* genes."

proposed work. In particular, the polycistronic nature of bacterial transcription introduces potential complications. Replacement of a polycistronic gene's native promoter in order to achieve manipulation of expression would likely change the expression conditions of any downstream genes within the same co-transcribed operon. Thus, in addition to the primary criterion of essentiality, monocistronic genes were selected such that minimal effect on expression of other genes would be expected.[350] Finally, in order to ensure the maximal redundancy of essentiality, candidate genes were selected from a variety of different important biochemical pathways. By assuring that critical steps in various essential pathways are redundantly controlled, the probability of bacterial escape from inducible promoter control is reduced. Genes that meet these three criteria would be expected to be ideal choices for the proposed work. Two such genes were selected.

5.3.1.1. *hbs*

The *hbs* gene is 276 bp in length and encodes Hbsu, a 92 aa histone-like protein approximately 9 kDa in size that binds DNA nonspecifically. A homologue of the *E. coli* histone-like HU proteins, it has been shown to play a critical role in the packaging of bacterial DNA and is essential to the viability of *Bacillus subtilis*.[351] Hbsu is one of the most abundant proteins in *B. subtilis*. It is present in similarly high levels (approximately $3 – 5 \times 10^4$ monomers/cell) in

[350] V Vagner, E Dervyn, and SD Ehrlich, "A vector for systematic gene inactivation in *Bacillus subtilis*," *Microbiology*, 144 (Pt 11), 1998.
[351] FM Commichau, N Pietack, and J Stulke, "Essential genes in *Bacillus subtilis*: a re-evaluation after ten years."; K Kobayashi *et al.*, "Essential *Bacillus subtilis* genes."; SubtiWiki, "Hbs," http://subtiwiki.uni-goettingen.de/wiki/index.php/Hbs.

both spores and vegetative cells.[352] As would be expected from a DNA-binding protein, Hbsu localizes in the cell nucleoid, where dimers bind to the chromosome approximately every 140 – 170 bp.[353]

In vegetative cells, σ^A-mediated transcription of the *hbs* gene is regulated by two overlapping promoters, known as *P*1 and *P*3. Both of these promoters are fairly strong, as would be expected from promoters regulating an essential gene. Compared to the *B. subtilis* consensus σ^A -10 and -35 sequences, both the *P*1 and *P*3 promoters have identical nucleotides in 8/12 (66.6%) positions.[354] In sporulating cells, however, transcription is mediated by σ^H or σ^C and driven from the *P*2 promoter.[355] Because of this three-promoter scheme, creation of a strain which transcribes *hbs* only in the presence of an inducing chemical requires the ablation of all three (*P*1, *P*2, and *P*3) promoters. Conditional mutants with Hbsu expression regulated by the IPTG-induced P_{spac} promoter show a dramatically reduced rate of growth. However, no viable Δ*hbs* mutants

[352] S Fernandez and JC Alonso, "*Bacillus subtilis* sequence-independent DNA-binding and DNA-bending protein Hbsu negatively controls its own synthesis," *Gene*, 231(1-2), 1999; MA Ross and P Setlow, "The *Bacillus subtilis* HBsu protein modifies the effects of α/β-type, small acid-soluble spore proteins on DNA," *J Bacteriol*, 182(7), 2000.
[353] S Fernandez and JC Alonso, "*Bacillus subtilis* sequence-independent DNA-binding and DNA-bending protein Hbsu negatively controls its own synthesis."; MA Ross and P Setlow, "The *Bacillus subtilis* HBsu protein modifies the effects of α/β-type, small acid-soluble spore proteins on DNA."
[354] S Fernandez and JC Alonso, "*Bacillus subtilis* sequence-independent DNA-binding and DNA-bending protein Hbsu negatively controls its own synthesis."; H Jarmer *et al.*, "Sigma A recognition sites in the *Bacillus subtilis* genome," *Microbiology*, 147(Pt 9), 2001.
[355] B Micka and MA Marahiel, "The DNA-binding protein HBsu is essential for normal growth and development in *Bacillus subtilis*," *Biochimie*, 74(7-8), 1992.

could be created by gene interruption, consistent with the classification of *hbs* as an essential gene.[356]

The DNA sequence for the *hbs* gene was obtained from the annotated full-genome sequence of *B. subtilis* subsp. *subtilis*, strain 168.[357]

5.3.1.2. *trxA*

The *trxA* gene is 312 bp in length and encodes thioredoxin, a 104 aa protein approximately 11 kDa in size that acts as an efficient hydrogen donor for a variety of redox reactions critical to *Bacillus subtilis* metabolism. Thioredoxin is also involved in protecting other proteins against oxidative damage and is essential to the viability of *B. subtilis*.[358] As would be expected from an essential gene, insertional *trxA* mutants could not be created. Conditional mutants under the regulation of the IPTG-inducible P_{spac} promoter showed a drastic retardation of growth rate at absent or low concentrations of IPTG.[359]

The transcription of *trxA* is regulated by two promoters. The further upstream promoter, P_B, is recognized by the σ^B-containing RNAP molecules that is generally associated with transcription of genes in response to environmental

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[356] B Micka *et al.*, "Molecular cloning, nucleotide sequence, and characterization of the *Bacillus subtilis* gene encoding the DNA-binding protein HBsu," J Bacteriol, 173(10), 1991.
[357] F Kunst *et al.*, "The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*."; National Center for Biotechnology Information (NCBI), "*Bacillus subtilis* subsp. *subtilis* str. 168 chromosome, complete genome (NCBI Reference Sequence: NC_000964.3)," http://www.ncbi.nlm.nih.gov/nuccore/nc_000964.
[358] FM Commichau, N Pietack, and J Stulke, "Essential genes in *Bacillus subtilis*: a re-evaluation after ten years."; K Kobayashi *et al.*, "Essential *Bacillus subtilis* genes."; C Scharf *et al.*, "Thioredoxin is an essential protein induced by multiple stresses in *Bacillus subtilis*," J Bacteriol, 180(7), 1998; SubtiWiki, "TrxA," http://subtiwiki.uni-goettingen.de/wiki/index.php/TrxA.
[359] C Scharf *et al.*, "Thioredoxin is an essential protein induced by multiple stresses in *Bacillus subtilis*."

stresses.[360] P_B is likely a strong stress-response promoter, as its -10 and -35 regions share 10/12 (83.3%) of their combined nucleotide sequence with the σ^B consensus recognition sequence, including all 10 of the positions critical for recognition by the σ^B-containing RNAP.[361] P_A, on the other hand, is relatively weaker, as its -10 and -35 regions share only 7/12 (58.3%) of their combined nucleotide sequence with the σ^A consensus recognition sequence.[362] Replacement of the native transcriptional regulation should replace both promoters with a single inducible promoter.

The DNA sequence for the *trxA* gene was obtained from the annotated full-genome sequence of *B. subtilis* subsp. *subtilis*, strain 168.[363]

5.3.2. Lethal Genes

Lethal genes were selected based on a demonstrated ability to kill or prevent the replication of *Bacillus* species. Putative lethal genes where such a capability had not previously been demonstrated were analyzed to determine whether the mechanism of lethality was likely compatible with *Bacillus subtilis*. Three such genes were selected.

5.3.2.1. *spoIISA*

General Description The *spoIISA* gene is 744 bp in length and encodes a stable toxic 248 aa protein approximately 28 kDa in size involved in programmed

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[360] WG Haldenwang, "The sigma factors of *Bacillus subtilis*," *Microbiol Rev*, 59(1), 1995.
[361] C Scharf *et al.*, "Thioredoxin is an essential protein induced by multiple stresses in *Bacillus subtilis*."
[362] Ibid.
[363] F Kunst *et al.*, "The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*."; National Center for Biotechnology Information (NCBI), "*Bacillus subtilis* subsp. *subtilis* str. 168 chromosome, complete genome (NCBI Reference Sequence: NC_000964.3)."

cell death. Immediately downstream of *spoIISA* is the *spoIISB* gene, which is 168 bp in length and encodes a labile 56 aa protein approximately 6 kDa in size that is involved in sporulation. The ORFs for *spoIISA* and *spoIISB* overlap by a single base pair. SpoIISB binds to SpoIISA and neutralizes its lethal effects; thus, expression of SpoIISA without SpoIISB is lethal to the cell. Together, *spoIISA* and *spoIISB* form a type II toxin-antitoxin (TA) gene pair. Such a system is often also referred to as an "addiction module." This is because the cell cannot survive without (i.e., is "addicted" to) the antitoxin, in this case SpoIISB.

Because *spoIISA* overlaps and is bicistronic with its antitoxin *spoIISB*, the proposed work requires the functional separation of the two genes. Multiple obvious strategies for accomplishing this are apparent. In the first, *spoIISA* is placed under negative regulatory control, while *spoIISB* remains unmodified. In environments where the promoter's regulatory chemical is present, the expression of SpoIISA is turned off, resulting in cell viability. Without the chemical, SpoIISA is expressed. However, its lethality is potentially mitigated by the concomitant expression of SpoIISB. In a second strategy, *spoIISB* is placed under positive regulatory control, while *spoIISA* remains unmodified. In environments where the promoter's regulatory chemical is present, the expression of SpoIISB is turned on, resulting in cell viability. Without the chemical, SpoIISB is not expressed, leaving the cell vulnerable to the lethal effects of SpoIISA. A third strategy is the complete removal of the *spoIISB* antitoxin gene and the placement of *spoIISA* under negative regulation. This

strategy appears to have the highest stringency, as any chance of the lethality of SpoIISA being circumvented by SpoIISB is eliminated by its absence. Because the cell cannot produce the antitoxin, the expression of *spoIISA* should be invariably lethal. The only way to ensure viability of the cells is by repressing transcription of the gene, meaning that the survival of the strain is inseparably tied to the presence of the promoter system's corepressor molecule.

Optimization The DNA sequence for the *spoIISA* gene was obtained from the annotated full-genome sequence of *B. subtilis* subsp. *subtilis*, strain 168.[364] The CAI of the wt *spoIISA* gene is 0.3804. Optimization using JCat resulted in the alteration of 163 nucleotides (22%) relative to the original sequence (Appendix 2). The CAI of the optimized *spoIISA* gene is 1.0. The proteins encoded by both the original and the optimized *spoIISA* genes are identical (Appendix 2).

The use of the *spoIISA* gene in the inducible manner proposed first requires the complete deletion of the entire native *spoIISAB* locus. After creation of a Δ*spoIISAB* strain, the optimized *spoIISA* gene under the control of an inducible promoter can be introduced into the strain. The reasons for this are twofold. First, the translation of the SpoIISB antitoxin could allow the neutralization of SpoIISA. Because the intention is for *spoIISA* to kill the cell unless its transcription is repressed by the presence of a particular chemical

[364] F Kunst *et al.*, "The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*."; National Center for Biotechnology Information (NCBI), "*Bacillus subtilis* subsp. *subtilis* str. 168 chromosome, complete genome (NCBI Reference Sequence: NC_000964.3)."

substrate, the potential transcription of the *spoIISB* gene threatens to uncouple viability from the intended stringent regulation. Thus, the *spoIISB* gene should be deleted in order to ensure the desired regulation of viability via *spoIISA*. Second, the deletion of *spoIISB* presents an additional dilemma. If the native copy of *spoIISA* was left intact and an additional optimized copy of *spoIISA* under the control of an inducible promoter were introduced into the cell, transcription from the latter gene can be shut off in the presence of the proper chemical. However, the native copy of *spoIISA* might still be transcribed. Without a copy of *spoIISB* available to neutralize any errant SpoIISA proteins produced by the native gene, the cell could succumb to its lethal effects in spite of the presence of the proper inducer for the inserted gene. Thus, the proposed solution involves the deletion of the entire native *spoIISAB* operon in one of the initial steps, after which the optimized *spoIISA* gene can be introduced and operate as intended.

A dedicated deletion construct could be designed, such that the USHR and DSHR of the insertion cassette flank the *spoIISAB* operon. When the cassette is transformed into the cell, homologous recombination would occur. The cassette would take the place of *spoIISAB*, effectively deleting it from the genome. (Although the original DNA sequence is would briefly still be present within the cell as a linear fragment, the cellular degradation machinery would eliminate it fairly rapidly. Additionally, without an origin of replication, it could not continue to persist as an extrachromosomal element.) However, rather than design a cassette specifically to delete the *spoIISAB* operon, one of the

accessory insertion cassettes already planned will be designed to have a USHR and DSHR with homology to the regions flanking the *spoIISAB* operon. This will allow the cassette to perform two duties simultaneously: the insertion of required accessory genes and the deletion of the undesired *spoIISAB* operon.

5.3.2.2. *lysB4*

General Description Bacteriophages are viruses that infect bacteria; they replicate by hijacking the normal cellular machinery of the bacteria and using it to create more viral particles instead. One of the genes produced by such phages is termed an endolysin, which encodes an enzyme that degrades the bacterial cell wall. This allows the newly formed virions inside the host cell to escape and infect other cells, starting the process over. A recently described endolysin produced by the *Bacillus cereus*-infecting bacteriophage B4 has shown significant lytic activity against both *B. cereus* and *B. subtilis*.[365] The *lysB4* gene is 789 bp in length and encodes the LysB4 protein, an enzyme approximately 28 kDa in size and belonging to the L-alanoyl-D-glutamate family of endopeptidases. LysB4 contains a domain similar to the *B. subtilis* CwlK cell wall hydrolase.[366] An important component of the bacterial cell wall is peptidoglycan. In *Bacillus subtilis*, N-acetyl glucosamine and N-acetylmuramic acid repeats form long chains. The N-acetylmuramic acid rings are populated with tripeptide chains

[365] B Son *et al.*, "Characterization of LysB4, an endolysin from the *Bacillus cereus*-infecting bacteriophage B4," *BMC Microbiol*, 12, 2012.
[366] National Center for Biotechnology Information (NCBI), "*Bacillus* phage B4 LysB4 (*lysB4*) gene, complete cds (GenBank: JN616385.1)," http://www.ncbi.nlm.nih.gov/nuccore/jn616385; B Son *et al.*, "Characterization of LysB4, an endolysin from the *Bacillus cereus*-infecting bacteriophage B4."

composed of L-alanine, D-glutamic acid, and meso-diaminopimelic acid; approximately 33% of these tripeptides are cross-linked by D-alanine to the tripeptide on the N-acetylmuramic acid ring of an adjacent chain, forming a tetrapeptide chain joining them.[367] LysB4 cleaves the bond between the L-Ala and D-Glu groups of the cell wall peptidoglycan cross-links. Because peptidoglycan is such a major component of the cell wall (accounting for as much as 40% of the weight of a Gram-positive bacterial cell wall[368]), cleaving the cross-links between peptidoglycan chains would be expected to have a severely detrimental effect on the cell wall structure and bacterial viability, causing the cell's peptidoglycan layer to unravel.[369]

Application of 5 µg of purified recombinant LysB4 to a culture of vegetative *B. cereus* cells reduced the number of viable cells by 99.99% within only 15 minutes; a similar level of lytic activity was seen when applied to *B. subtilis*.[370] Thus, *lysB4* is an excellent choice for introduction into this strain of *B. subtilis*. It is hypothesized that placing *lysB4* under the control of a repressible promoter will result in the production of an efficient, lethal, cell wall-degrading enzyme that will destroy the bacterial cell from the inside-out, while allowing repression of lethal LysB4 when the proper inducing chemical is present in the growth media.

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[367] SJ Foster and DL Popham, "Structure and Synthesis of Cell Wall, Spore Cortex, Teichoic Acids, S-Layers, and Capsules," in *Bacillus subtilis* and Its Closest Relatives: From Genes to Cells, Ed. AL Sonenshein, JA Hoch, and RM Losick (Washington, DC: ASM Press, 2002).
[368] GD Shockman and JF Barrett, "Structure, function, and assembly of cell walls of gram-positive bacteria," *Annu Rev Microbiol*, 37, 1983.
[369] B Son *et al.*, "Characterization of LysB4, an endolysin from the *Bacillus cereus*-infecting bacteriophage B4."
[370] Ibid.

Optimization The original DNA sequence for the *lysB4* gene was obtained from NCBI.[371] The CAI of the wt *lysB4* gene is 0.6926. Optimization using JCat resulted in the alteration of 141 nucleotides (18%) relative to the original sequence (Appendix 2). The CAI of the optimized *lysB4* gene is 1.0. The proteins encoded by both the original and the optimized *lysB4* genes are identical (Appendix 2).

5.3.2.3. *ccdB$_{Ec}$*

General Description The *ccdB* gene from the *E. coli* F plasmid produces a toxic poison that binds to the key dimerization domain of subunit A of gyrase, an essential bacterial topoisomerase. This prevents the enzyme from introducing negative supercoils into bacterial closed circular dsDNA, an assistive step critical to the process of bacterial DNA replication. In particular, the arginine residue located at position 462 (R462) in the *E. coli* GyrA protein has been shown to be critical for the interaction with CcdB; mutation of this residue has been shown to eliminate the cytotoxic effect of CcdB.[372] Inhibition of DNA gyrase (by a different mechanism) is also the mechanism of action of various classes of antibiotics, most notably the quinolones.[373] In *E. coli*, the CcdB toxin has been shown to be lethal when expressed in the absence of its antidote, CcdA. (The *ccdB* gene on the *E. coli* F plasmid should not be confused with the identically named, but

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[371] National Center for Biotechnology Information (NCBI), "*Bacillus* phage B4 LysB4 (*lysB4*) gene, complete cds (GenBank: JN616385.1)."; B Son *et al.*, "Characterization of LysB4, an endolysin from the *Bacillus cereus*-infecting bacteriophage B4."

[372] P Bernard and M Couturier, "Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes," *J Mol Biol*, 226(3), 1992.

[373] CD Klaassen, Casarett and Doull's Toxicology: The Basic Science of Poisons.

unrelated *B. subtilis ccdB* gene, which is also known by the synonyms *yneI* and *yoxH*.[374] Similarly, the *ccdA* gene also on the *E. coli* F plasmid should not be confused with the identically named, but unrelated *B. subtilis ccdA* gene, which encodes a membrane protein involved in the synthesis of cytochrome C.[375] To avoid ambiguity, the *E. coli* genes and proteins will be annotated with a subscript "*Ec*", e.g. $ccdB_{Ec}$. Similarly, the *B. subtilis* genes and proteins will be annotated with a subscript "*Bs*", e.g. $ccdB_{Bs}$.)

Optimization The original DNA sequence for the $ccdB_{Ec}$ gene was obtained from was obtained from the annotated sequence of the *E. coli* F plasmid.[376] The CAI of the wt $ccdB_{Ec}$ gene is 0.3187. Optimization using JCat resulted in the alteration of 71 nucleotides (33%) relative to the original sequence (Appendix 2). The CAI of the optimized $ccdB_{Ec}$ gene is 1.0. The proteins encoded by both the original and the optimized $ccdB_{Ec}$ genes are identical (Appendix 2).

Prediction of Lethal Activity Although the $CcdB_{Ec}$ protein has been shown to bind to the *E. coli* GyrA ($GyrA_{Ec}$) subunit, this activity has not yet been shown in *B. subtilis*. Although it is unclear whether expression of $CcdB_{Ec}$ in *B. subtilis* will result in the same lethal inhibition of gyrase, three-dimensional molecular modeling provides some insight suggesting that it may behave similarly. While

[374] UniProtKB, "P45709: Protein CcdB - *Bacillus subtilis* (strain 168)," http://www.uniprot.org/uniprot/P45709.
[375] C von Wachenfeldt and L Hederstedt, "Respiratory Cytochromes, Other Heme Proteins, and Heme Biosynthesis," in *Bacillus subtilis* and Its Closest Relatives: From Genes to Cells, Ed. AL Sonenshein, JA Hoch, and RM Losick (Washington, DC: ASM Press, 2002).
[376] National Center for Biotechnology Information (NCBI), "*Escherichia coli* K-12 plasmid F DNA, complete sequence (NCBI Reference Sequence: NC_002483.1)," 2013.

the structure of the *B. subtilis* GyrA ($GyrA_{Bs}$) subunit has not been definitively determined by X-ray crystallography, the structure of the $GyrA_{Ec}$ has been determined in this manner.

Alignment of $GyrA_{Bs}$ to $GyrA_{Ec}$ shows that the two protein sequences are 58.5% identical (Figure 5-5). More importantly, the 75.4% degree of overall amino acid conservation is very high, suggestive of conserved three-dimensional structure and function between the proteins from both species. Of specific note is that each protein contains the arginine residue critical for the interaction with CcdB at the same location (highlighted in red in Figure 5-5), further suggesting a conservation of susceptibility to CcdB.

The three-dimensional structure of the interaction between $GyrA_{Ec}$ and the $CcdB_{Ec}$ toxin has also been determined by X-ray crystallography (PDB: 1X75[377]). Figure 5-6A shows a three-dimensional model of the interaction between a 14 kDa fragment of the dimerization domain of $GyrA_{Ec}$ ($GyrA14_{Ec}$) in blue and cyan (with each R462 highlighted in green) and the $CcdB_{Ec}$ dimer chains in yellow and orange (with each W99 highlighted in magenta). Figure 5-6B shows a close-up of the region where the $GyrA14_{Ec}$ arginine residues interact with the $CcdB_{Ec}$ tryptophan side chains.[378] (Both rotamers of each R462 side chain are shown.) Figure 5-6C shows the same molecular interaction as Figure 5-6A, rotated approximately 45° to better show the individual R462 side chains. Figure 5-6D

[377] MH Dao-Thi *et al.*, "Molecular basis of gyrase poisoning by the addiction toxin CcdB," *J Mol Biol*, 348(5), 2005.
[378] Ibid.

and Figure 5-6E show different close-up views of the area of interaction between GyrA14$_{Ec}$ and CcdB$_{Ec}$. The molecular interaction between these two proteins was elucidated by Doa-Thi, *et al*.[379]

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CLUSTAL O(1.2.1) multiple sequence alignment

GyrA_Bs           MSEQNTPQVREINISQEMRTSFLDYAMSVIVSRALPDVRDGLKPVHRRILYAMNDLGMTS     60
GyrA_Ec_1AB4      ------------------------------VGRALPDVRDGLKPVHRRVLYAMNVLGNDW     30
                                               *.****************:***** **

GyrA_Bs           DKPYKKSARIVGEVIGKYHPHGDSAVYESMVRMAQDFNYRYMLVDGHGNFGSVDGDSAAA     120
GyrA_Ec_1AB4      NKAYKKSARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLRYMLVDGQGNFGSIDGDSAAA     90
                  :* ******:**:***************:::***** *. *******:*****:*******

GyrA_Bs           MRYTEARMSKISMEILRDITKDTIDYQDNYDGSEREPVVMPSRFPNLLVNGAAGIAVGMA     180
GyrA_Ec_1AB4      MRYTEIRLAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSSGIAVGMA     150
                  ***** *::**: *:: *: *:*:*: *****:*: * ***:::*******::*******

GyrA_Bs           TNIPPHQLGEIIDGVLAVSENPDITIPELMEVIPGPDFPTAGQILGRSGIRKAYESGRGS     240
GyrA_Ec_1AB4      TNIPPHNLTEVINGCLAYIDDEDISIEGLMEHIPGPDFPTAAIINGRRGIEEAYRTGRGK     210
                  ******:* *:*:* **  :: **:*  *** *********. * ** **.:**.:***.

GyrA_Bs           ITIRAKAEIEQT-SSGKERIIVTELPYQVNKAKLIEKIADLVRDKKIEGITDLRDESDRT     299
GyrA_Ec_1AB4      VYIRARAEVEVDAKTGRETIIVHEIPYQVNKARLIEKIAELVKEKRVEGISALRDESDKD     270
                  : ***:**:*   .:*:* *** *:*******:******:**::*::***: ******:

GyrA_Bs           GMRIVIEIRRDANANVILNNLYKQTALQTSFGINLLALVDGQPKVLTLKQCLEHYLDHQK     359
GyrA_Ec_1AB4      GMRIVIEVKRDAVGEVVLNNLYSQTQLQVSFGINMVALHHGQPKIMNLKDIIAAFVRHRR     330
                  *******::*** .:*:*****.** **.*****::** .****::.**: :  :: *::

GyrA_Bs           VVIRRRTAYELRKAEARAHILEGLRVALDHLDAVISLIRNSQTAEIARTGLI--------     411
GyrA_Ec_1AB4      EVVTRRTIFELRKARDRAHILEALAVALANIDPIIELIRHAPTPAEAKTALVANPWQLGN     390
                   *: *** :*****. ******.* *** .:* :*.***.: *   *:*.*:

GyrA_Bs           -------------------------EQFSLTEKQAQAILDMRLQRLTGLEREKIEEEYQ     445
GyrA_Ec_1AB4      VAAMLERAGDDAARPEWLEPEFGVRDGLYYLTEQQAQAILDLRLQKLTGLEHEKLLDEYK     450
                                           : ***:*******:***:*****:**: :**:

GyrA_Bs           SLVKLIAELKDILANEYKVLEIIREELTEIKERFNDERRTEIVTSGLETIEDEDLIEREN     505
GyrA_Ec_1AB4      ELLDQIAELLRILGSADRLMEVIREELELVREQFGDKRRTEIT-----------------     493
                  .*:. ****  **..  :::*:*****  ::*:* *:*****.

An * (asterisk) indicates positions which have a single, fully conserved residue.
A : (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250
        matrix.
A . (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250
        matrix.
```

Figure 5-5: Alignment of GyrA$_{Bs}$ and GyrA$_{Ec}$

[379] Ibid.

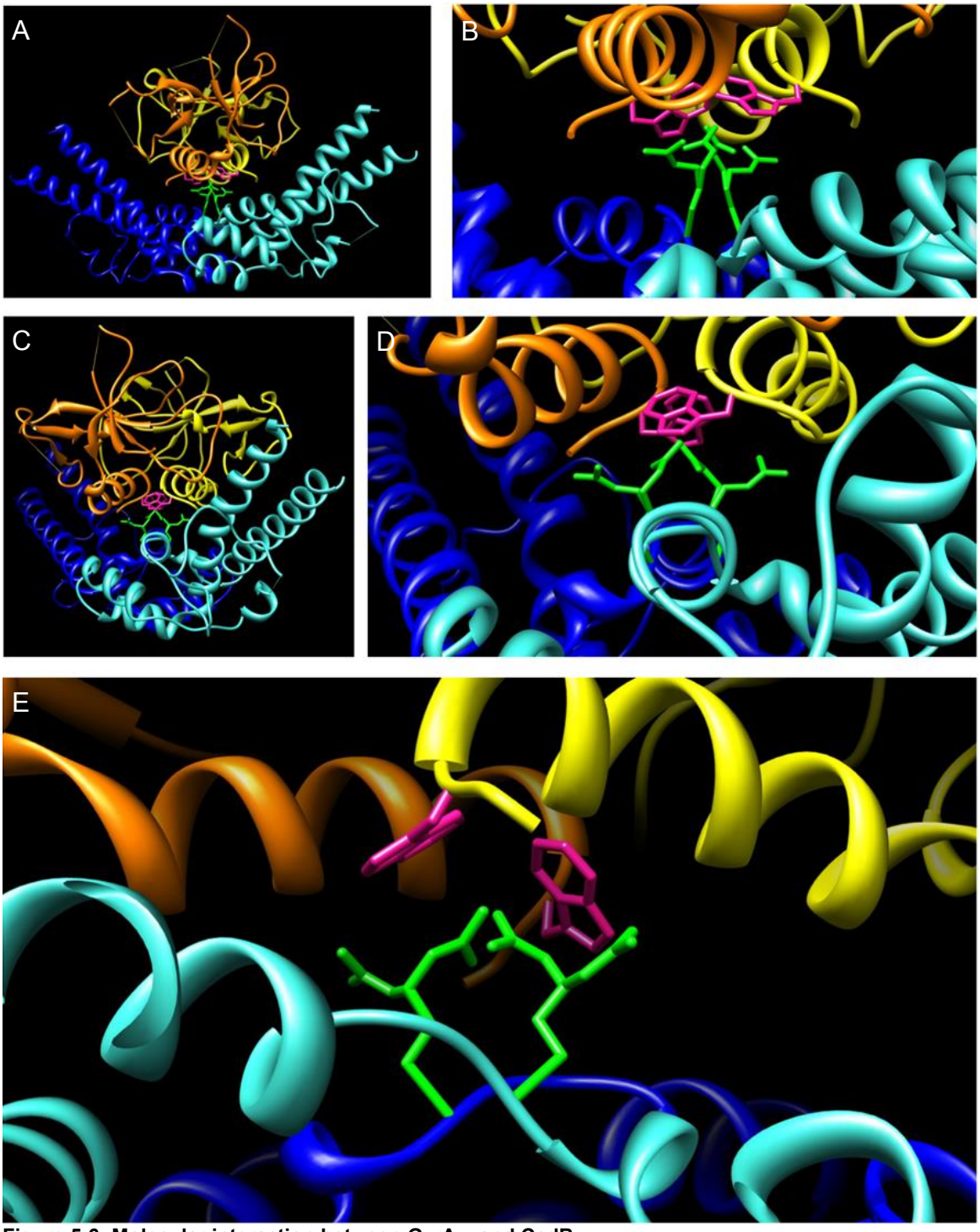

Figure 5-6: Molecular interaction between $GyrA_{Ec}$ and $CcdB_{Ec}$

When ESyPred3D was used to model the three-dimensional structure of $GyrA_{Bs}$ using the X-ray diffraction-determined three-dimensional structure of a monomer of $GyrA_{Ec}$ (PDB: 1AB4[380]) as a template, the predicted overall structure shows remarkable similarity. Figure 5-7A shows the determined $GyrA_{Ec}$ structure with the R462 side chain highlighted in green, while Figure 5-7B shows the predicted $GyrA_{Bs}$ structure with the R428 side chain similarly highlighted in green. In particular, the side chain of the arginine residue (R462) previously shown to be critical for the interaction with $CcdB_{Ec}$ is in nearly the exact same location in $GyrA_{Bs}$ relative to the overall peptide.

A three-dimensional alignment of the predicted structure of $GyrA_{Bs}$ with the determined structure of $GyrA14_{Ec}$ interacting with $CcdB_{Ec}$ shows a highly similar structure in the relevant dimerization domain. Figure 5-8A shows $GyrA_{Bs}$ in red overlaid on $GyrA14_{Ec}$ in blue; the R462 of $GyrA14_{Ec}$ is highlighted in green, while the R428 of $GyrA_{Bs}$ is shown in white. Figure 5-8B shows a close-up view of the two arginine residues. Figure 5-8C shows the same overlay as in a rotated 90°, while Figure 5-8D shows a close-up view. Figure 5-8E shows an additional close-up view rotated to more clearly show the two arginine residues.

[380] JH Morais Cabral *et al.*, "Crystal structure of the breakage-reunion domain of DNA gyrase," *Nature*, 388(6645), 1997.

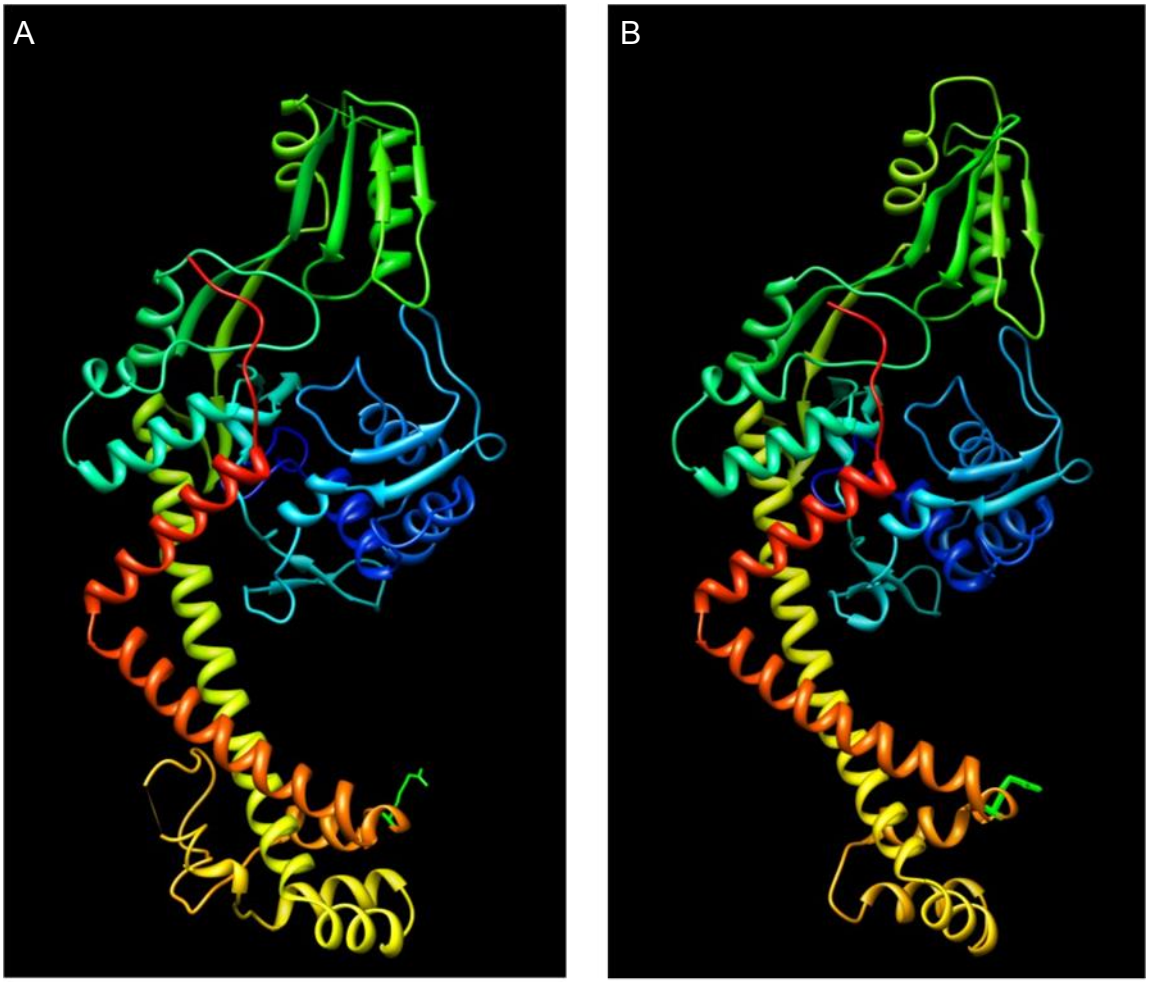

Figure 5-7: Molecular structure of GyrA$_{Ec}$ and predicted structure of GyrA$_{Bs}$

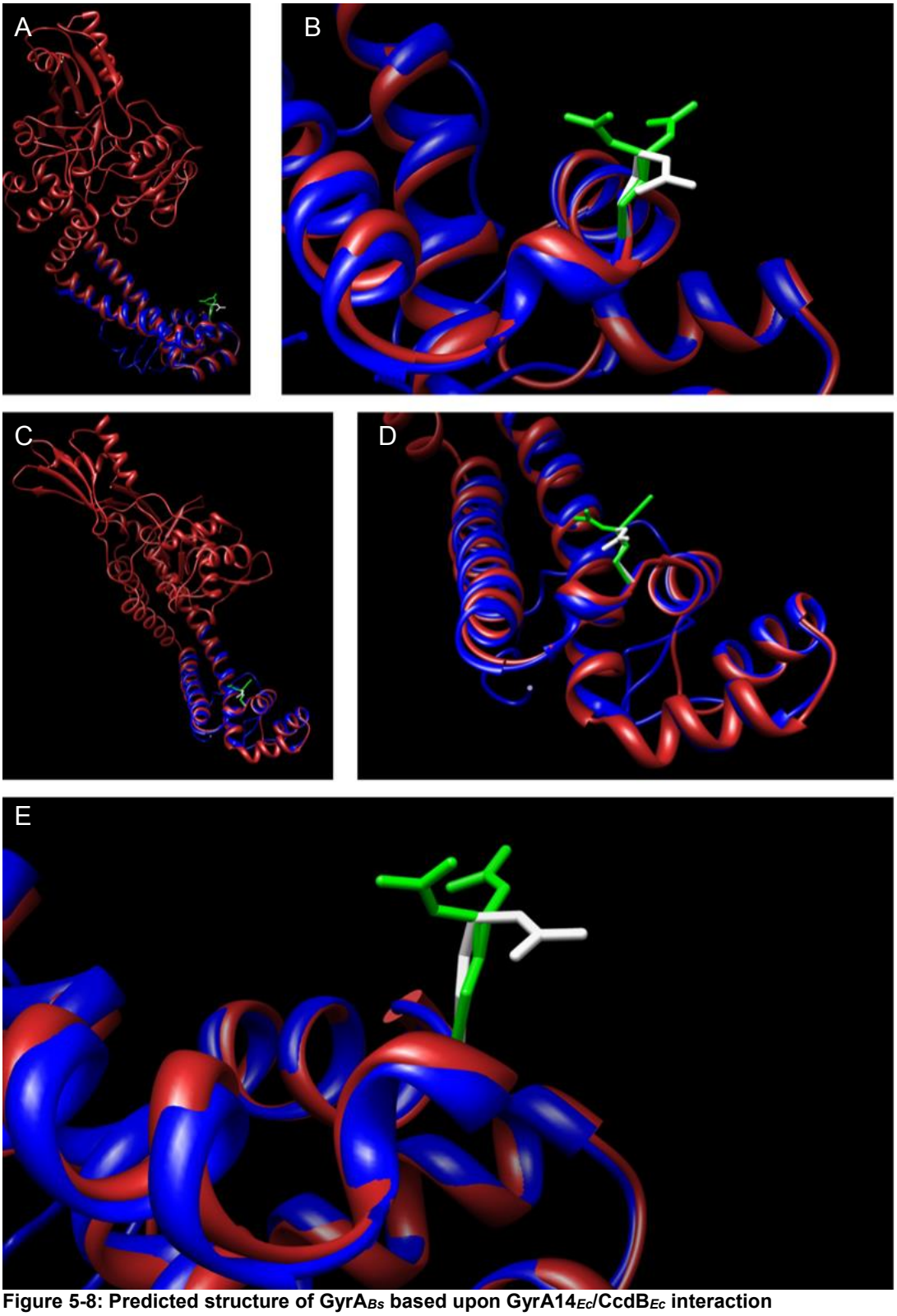

Figure 5-8: Predicted structure of GyrA$_{Bs}$ based upon GyrA14$_{Ec}$/CcdB$_{Ec}$ interaction

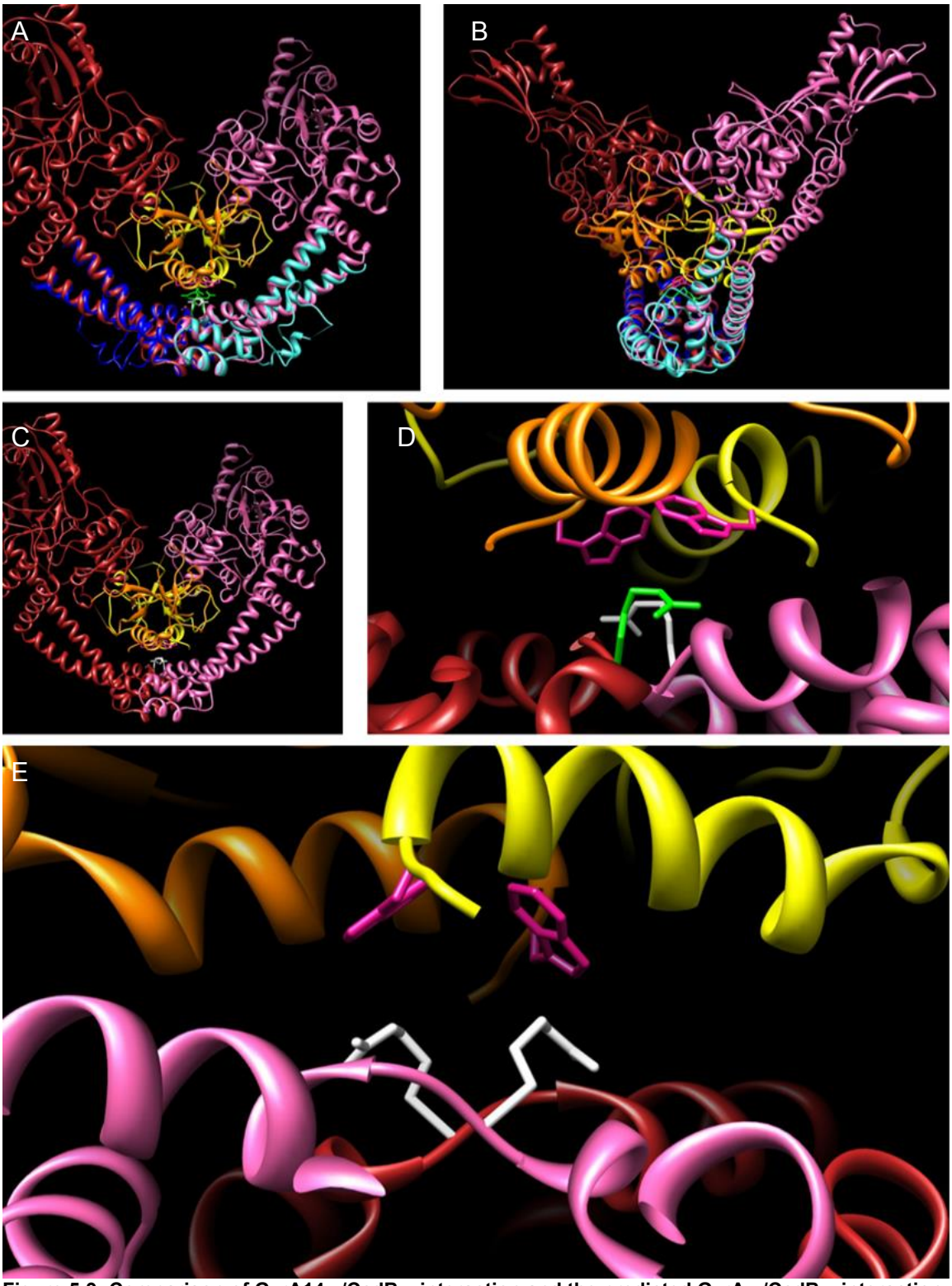

Figure 5-9: Comparison of GyrA14$_{Ec}$/CcdB$_{Ec}$ interaction and the predicted GyrA$_{Bs}$/CcdB$_{Ec}$ interaction

Based on the results shown in Figure 5-7 and Figure 5-8, it is likely that $GyrA_{Bs}$ is structured similarly to $GyrA14_{Ec}$ and interacts with $CcdB_{Ec}$ in a similar manner as does $GyrA_{Ec}$. When the predicted structure of a $GyrA_{Bs}$ monomer is overlaid on each of the $GyrA14_{Ec}$ chains in the determined structure of the $GyrA14_{Ec}/CcdB_{Ec}$ interaction (PDB: 1X75), the result is a likely structure of the $GyrA_{Bs}/CcdB_{Ec}$ interaction. Figure 5-9A shows such an overlay in the same orientation as Figure 5-6A. An identical color scheme is used for $GyrA14_{Ec}$ and $CcdB_{Ec}$; the two $GyrA_{Bs}$ chains are shown in red and pink, and the R428 residues are shown in white. Figure 5-9B shows the same structure as in a rotated 90° to show the spatial orientation of the large $GyrA_{Bs}$ chains pointing in opposite directions. Figure 5-9C shows the same hypothetical structure as in Figure 5-9A with the $GyrA14_{Ec}$ chains hidden; this structure represents the likely arrangement of $GyrA_{Bs}$ chains around the $CcdB_{Ec}$ dimer. Figure 5-9D shows a close-up view of the area of interaction between the $GyrA_{Bs}$ R428 residues and the $CcdB_{Ec}$ W99 residues; for this picture only, the foreground R428 is colored green to provide additional differentiation between the two R428 residues. Figure 5-9E is a rotated close-up view showing the proximity of the two $GyrA_{Bs}$ R428 residues to the $CcdB_{Ec}$ W99 residues. The flexible arginine side chains can rotate as necessary to interact with the N92, N95, and W99 side chains. The structural similarity between Figure 5-6E and Figure 5-9E is abundantly clear.

Based on the predicted similarity between $GyrA_{Bs}$ and $GyrA_{Ec}$, particularly with respect to the arginine residue critical for interaction with $CcdB_{Ec}$, it is

therefore expected that expression of $CcdB_{Ec}$ within vegetative *B. subtilis* cells will result in a cytotoxic response similar to that observed in *E. coli*. Naturally, prior to incorporating the $ccdB_{Ec}$ gene into the proposed strain and relying on its hypothesized cytotoxicity for the biological containment of the strain, the expected lethality of the $CcdB_{Ec}$ protein to *B. subtilis* would need to be experimentally verified *in vivo*.

5.3.3. Positive Regulatory Promoters

5.3.3.1. P_{xylA}

General Description The P_{xylA} promoter responds to the presence of the monosaccharide xylose. Its native context within the *B. subtilis* genome is as an inducible promoter that drives expression of the enzyme xylose isomerase, which converts D-xylose into D-xylulose. Xylose isomerase is a 445 aa protein encoded by the first gene of the *xylAB* operon. Just upstream of this operon (in the reverse direction) is the *xylR* gene, which encodes a transcriptional repressor of the P_{xylA} promoter. Only four nucleotides separate the P_{xylA} -10 region from the TSS of the *xylAB* operon. XylR binds to *xylO*, a 25 bp palindromic operator sequence beginning at +6 relative to the TSS and 73 bp upstream of the start codon.[381] The binding of XylR to *xylO* likely functions as a "roadblock".[382] Thus, an RNAP that attaches at the Shine-Dalgarno sequence will travel down the DNA strand for only a short distance before encountering the XylR repressor bound to

[381] P Kreuzer *et al.*, "Identification and sequence analysis of the *Bacillus subtilis* W23 *xylR* gene and *xyl* operator," *J Bacteriol*, 171(7), 1989.
[382] A Scheler and W Hillen, "Regulation of xylose utilization in *Bacillus licheniformis*: Xyl repressor-*xyl*-operator interaction studied by DNA modification protection and interference," *Mol Microbiol*, 13(3), 1994.

xylO, preventing further transcription from occurring. The XylR repressor interacts with xylose, resulting in a conformational change that causes detachment from the operator sequence, enabling transcription initiation.[383] (It should be noted that XylR is natively encoded by the *B. subtilis* genome. However, the efficient repression of P_{xylA} requires a greater level of XylR expression in order to minimize the leakiness of P_{xylA}.[384] This is the reason for supplementing the genome with a second copy of *xylR* on MARAcc.)

Optimization The sequence of *xylR* (BBa_K143036[385]) was obtained from the Registry of Standard Biological Parts (RSBP). The CAI of the wt *xylR* gene is 0.4277. Optimization using JCat resulted in the alteration of 253 nucleotides (24%) relative to the original sequence (Appendix 2). The CAI of the optimized *xylR* gene is 1.0. The proteins encoded by both the original and the optimized *xylR* genes are identical (Appendix 2).

5.3.3.2. AND Gate: [P_{BAD}/*supD* + P_{sal}/T7ptag] → P_{T7}

General Description As described in Chapter 5.1.2, synthetic biology allows the combining of individual biological parts into more complex devices that perform a specified biological function. These devices include molecular logic gates. Such gates are essentially artificial molecular microprocessors capable of "evaluating" several inputs and performing a "computation" that results in an

[383] S Stammen *et al.*, "High-yield intra- and extracellular protein production using *Bacillus megaterium*," *Appl Environ Microbiol*, 76(12), 2010.
[384] C Hirst, "Xylose operon regulatory protein (Part:BBa_K143036)," http://parts.igem.org/Part:BBa_K143036.
[385] Ibid.

output. These types of combinatorial logic calculations enable the development of highly customizable biological systems for a variety of specific purposes.

One such molecular logic gate is an AND gate, which is a logic gate that has two inputs and a single output. When both inputs equal 0, the processed output of an AND gate also equals 0. In the case where either input equals 0 and the other equals 1, the output equals 0. Only when both inputs are equal to 1 is the output of the AND gate equal to 1 (i.e., when both input A ***and*** input B equal 1, hence the name). These input and output combinations can be displayed in a logical table known as a truth table. The truth table for an AND gate is shown in Table 5-1.

Table 5-1: AND gate truth table

Input A	Input B	Output
0	0	0
0	1	0
1	0	0
1	1	1

A similarly behaving molecular AND gate can be created by "connecting" two independent inducible promoter systems (the inputs) to control a third inducible promoter system (the output). This type of connection requires that both of the products of the input regulons be present in order for the output promoter to be activated. When the inducer for both input promoters is not

present in the environment (i.e., input A and input B both equal 0), the protein regulated by the AND gate's output promoter is not produced (i.e., the output equals 0). If inducer A is present but inducer B is not, the protein regulated by the AND gate's output promoter is again not produced; the same holds true if inducer B is present but inducer A is not. Only when both inducer A and inducer B are present (i.e., both inputs equal 1) is the protein regulated by the AND gate's output promoter produced (i.e., the output equals 1).

Such a molecular AND gate was recently created in *E. coli* by Anderson, *et al*.[386] By mutating two of the codons in the gene encoding the RNAP of the T7 bacteriophage into amber stop codons (`tag`), a nonsense copy of the RNAP was created; this gene is referred to as *T7ptag*. However, the gene can be rescued by the concurrent expression of the amber suppressor gene *supD*, which encodes a non-standard tRNA. This tRNA has a serine attached to its aminoacyl end and recognizes the `tag` codon. Thus, if the amber suppressor tRNA is present in the cell, the amber stop codons in the *T7ptag* gene can be translated as serines resulting in a fully functional T7 RNAP. Because the constitutive P_{T7} promoter is not recognized by the *E. coli* RNAP, the T7 RNAP is required in order to initiate any transcription from the promoter. Anderson, *et al*. placed *supD* under the regulatory control of the P_{sal} promoter (which activates transcription in response to salicylate), the *T7ptag* gene under the regulatory control of the P_{BAD} promoter (which activates transcription in response to arabinose), and a reporter

[386] JC Anderson, CA Voigt, and AP Arkin, "Environmental signal integration by a modular AND gate," *Mol Syst Biol*, 3, 2007.

gene downstream of the P_{T7} promoter. Thus, a molecular AND gate was created: only when both salicylate and arabinose are present (i.e., both inputs equal 1) can the reporter protein regulated by the P_{T7} promoter be produced (i.e., the output equals 1). An adapted version of the system, with *supD* under the control of the P_{BAD} promoter and the *T7ptag* gene under the control of the P_{sal} promoter (i.e., input promoters switched), was recently determined to have better performance.[387]

If the CDS placed under the regulation of the [P_{BAD}/*supD* + P_{sal}/*T7ptag*] → P_{T7} AND gate is an essential gene, the cell will be unable to produce the essential protein unless both salicylate and arabinose are present. When neither salicylate nor arabinose are present in the environment (i.e., input A and input B both equal 0), the essential protein regulated by the P_{T7} promoter is not produced (i.e., the output equals 0). If salicylate is present but arabinose is not, the essential protein regulated by the P_{T7} promoter is again not produced; the same holds true if arabinose is present but salicylate is not. Only when both salicylate and arabinose are present (i.e., both inputs equal 1) is the essential protein regulated by the P_{T7} promoter produced (i.e., the output equals 1). Because the cells cannot survive without the proper expression of the essential protein, a system can be created where the viability of a bacterial culture is stringently dependent upon the presence of two specific chemicals in the culture environment.

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[387] G Zhang, "AND GATE AraC+SupD+Sal+RBS(J44001)+T7ptag (Part:BBa_K228260)," http://parts.igem.org/Part:BBa_K228260.

Optimization The [P_{BAD}/*supD* + P_{sal}/*T7ptag*] → P_{T7} AND gate (BBa_K228260) as constructed by the Peking University 2009 iGEM Team is a self-contained composite device which contains all the components necessary for the operation of the AND gate, including all the promoters and accessory proteins for activation and repression.[388] However, because the system was designed for expression in *E. coli*, the device will require some customization for efficient operation in *Bacillus subtilis*, in particular correction of codon bias.

The *araC*, *nahR*, and *T7ptag* genes were codon bias-corrected using JCat. The DNA sequence for the *araC* gene was obtained from the annotated full-genome sequence of *E. coli*, Strain MG1655.[389] The CAI of the wt *araC* gene is 0.3346. Optimization using JCat resulted in the alteration of 240 nucleotides (27%) relative to the original sequence (Appendix 2). The CAI of the optimized *araC* gene is 1.0. The proteins encoded by both the original and the optimized *araC* genes are identical (Appendix 2). The DNA sequence for the *nahR* gene was obtained from the composite genetic part consisting of *nahR* and the P_{sal} promoter (BBa_K228004[390]). The CAI of the wt *nahR* gene is 0.2782. Optimization using JCat resulted in the alteration of 231 nucleotides (26%) relative to the original sequence (Appendix 2). The CAI of the optimized *nahR*

[388] Ibid.
[389] National Center for Biotechnology Information (NCBI), "*Escherichia coli* str. K-12 substr. MG1655, complete genome (GenBank: U00096.2)," http://www.ncbi.nlm.nih.gov/nuccore/U00096.2.
[390] L Min, "NahR(reverse) - salicylate promoter (Part:BBa_K228004)," http://parts.igem.org/Part:BBa_K228004.

gene is 1.0. The proteins encoded by both the original and the optimized *nahR* genes are identical (Appendix 2).

The DNA sequence for the *T7ptag* gene was obtained from the RSBP (BBa_K228000[391]). The CAI of the wt *T7ptag* gene is 0.4660. Optimization using JCat resulted in the alteration of 519 nucleotides (19%) relative to the original sequence (Appendix 2). The CAI of the optimized *T7ptag* gene is 1.0. However, as was expected, the JCat optimization of *T7ptag* had the additional undesired effect of "optimizing" the `tag` amber stop codons into the `taa` ochre stop codons more frequently used in *B. subtilis*. This would be acceptable if in fact the goal was to have two active stop codons in those locations. However, mutation of these stop codons would uncouple the encoded T7 polymerase from the amber suppressor tRNA, leaving only a nonsense T7 RNAP to be produced (albeit highly efficiently). Thus, after the optimization of *T7ptag* with JCat, the two ochre `taa` stop codons in the returned sequence were manually edited back to `tag` (amber) stop codons ("reTAGged"). Because the equation for CAI excludes the initiation and stop codons[392], entering the reTAGged sequence back into JCat results in a returned CAI of 1.0. The proteins encoded by the original gene, the optimized *T7ptag*, and the reTAGged *T7ptag* are identical (Appendix 2).

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[391] L Min, "T7ptag(T7polymerase with amber mutation) (Part:BBa_K228000)," http://parts.igem.org/Part:BBa_K228000.

[392] A Grote *et al.*, "JCat: a novel tool to adapt codon usage of a target gene to its potential expression host."

5.3.4. Negative Regulatory Promoters

5.3.4.1. $P_{revTetR}$

General Description The $P_{revTetR}$ promoter responds to the presence of tetracycline or its analog, anhydrotetracycline (aTc). P_{Tet} contains an operator, *tetO*, which binds the repressor protein TetR. In the normal P_{Tet}/TetR system, TetR is bound to *tetO* in the absence of tetracycline, which prevents transcription from occurring. When tetracycline binds to TetR, the complex dissociates from *tetO*, allowing transcription to occur. Numerous mutants of the TetR repressor have been isolated in which the binding (and thus, the regulation) exhibited by the protein is reversed.[393] Because these variants function in a reverse manner, they are referred to as *revTetR* (reverse TetR). In a *revTetR* system, the RevTetR repressor is unable to bind to *tetO* in the absence of tetracycline, allowing transcription to occur. When tetracycline becomes available, RevTetR is able to bind to *tetO*, allowing transcription to occur.[394] One particularly effective variant is the $RevTetR^{r2}$ protein, which consists of just three mutations with respect to TetR: E15A, L17G, and L25V.[395]

It should be noted that only the TetR repressor in the system described above is mutated relative to the wild-type. The promoter and operator are unchanged, along with their respective functions: when a repressor protein is

[393] A Kamionka *et al.*, "Two mutations in the tetracycline repressor change the inducer anhydrotetracycline to a corepressor," *Nucleic Acids Res*, 32(2), 2004; O Scholz *et al.*, "Activity reversal of Tet repressor caused by single amino acid exchanges," *Mol Microbiol*, 53(3), 2004.
[394] A Kamionka, R Bertram, and W Hillen, "Tetracycline-dependent conditional gene knockout in *Bacillus subtilis*," *Appl Environ Microbiol*, 71(2), 2005.
[395] R Bertram *et al.*, "Phenotypes of combined *tet* repressor mutants for effector and operator recognition and allostery," *J Mol Microbiol Biotechnol*, 8(2), 2004; O Scholz *et al.*, "Activity reversal of Tet repressor caused by single amino acid exchanges."

bound to *tetO*, transcription from the promoter cannot occur, while transcription can occur when the repressor dissociates. The three amino acid mutations in RevTetR^{r2} have only altered the conditions under which the repressor binds to *tetO*. Thus, because the promoter is still wild-type, it is properly referred to as P_{Tet}. Despite the fact that only the repressor protein is different, use of the promoter name P_{Tet} might incorrectly imply that the genetic system is under positive regulatory (i.e., wild-type) control. Thus, the nomenclature used throughout this dissertation will refer to the promoter used in the P_{Tet}/RevTetR^{r2} system as $P_{revTetR\text{-}r2}$ (even though this name is technically incorrect) to differentiate its negative regulatory scheme from the positively regulated classical P_{Tet}/TetR system.

Optimization The CAI of the wt *revTetR^{r2}* gene is 0.3804. Optimization using JCat resulted in the alteration of 116 nucleotides (18%) relative to the original sequence (Appendix 2). The CAI of the optimized *revTetR^{r2}* gene is 1.0. The proteins encoded by both the original and the optimized *revTetR^{r2}* genes are identical (Appendix 2).

5.3.4.2. P_{glnRA}

General Description The P_{glnRA} promoter responds to the presence of the amino acid glutamine. Its native context within the *B. subtilis* genome is as an inducible promoter that drives expression of the enzyme glutamine synthetase (GS), which can convert glutamate into glutamine. GS in encoded by the second gene in the operon, *glnA*. The first gene in the operon, *glnR*, encodes a 135 aa

autorepressor that binds to two operator sites within the P_{glnRA} promoter. The second operator, *glnRAo*$_2$, entirely overlaps the -35 region of the promoter.[396] In the presence of glutamine, GS undergoes a conformational change into a feedback-inhibited form of GS (FBI-GS). FBI-GS then is able to bind to and stabilize a GlnR dimer, one of which binds to each of the operators, blocking access to regions critical for RNAP binding.[397]

Because GlnR is natively produced by *B. subtilis*, it is unnecessary to introduce the gene on any of the constructs.

5.3.4.3. NOT Gate: [P_{lial}/*cI*] → P_{cI}

General Description A NOT gate, also known as an inverter, is a logic gate that negates an input. If the input equals 0, the processed output of a NOT gate equals 1. The relatively simple truth table for a NOT gate is shown in Table 5-2. An inducible promoter-based molecular NOT gate behaves similarly. When the inducer is not present in the environment (i.e., the input equals 0), the protein regulated by the NOT gate's promoter is produced (i.e., the output equals 1). Conversely, when the inducer is present in the environment (i.e., the input equals 1), the protein regulated by the NOT gate's promoter is not produced (i.e., the output equals 0).

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[396] BR Belitsky, "Biosynthesis of Amino Acids of the Glutamate and Aspartate Families, Alanine, and Polyamines," in *Bacillus subtilis* and Its Closest Relatives: From Genes to Cells, Ed. AL Sonenshein, JA Hoch, and RM Losick (Washington, DC: ASM Press, 2002).

[397] AL Sonenshein, "Control of nitrogen metabolism by *Bacillus subtilis* glutamine synthetase," *Molecular Microbiology*, 68(2), 2008; LV Wray, Jr. and SH Fisher, "*Bacillus subtilis* GlnR contains an autoinhibitory C-terminal domain required for the interaction with glutamine synthetase," *Mol Microbiol*, 68(2), 2008.

Table 5-2: NOT gate truth table

Input A	Output
0	1
1	0

A combinatorial molecular NOT gate was designed using two promoter systems. The first is the P_{lial} system which is native to *B. subtilis*. The P_{lial} promoter is inhibited by the LiaR repressor protein. When the antibiotic bacitracin is present in the environment, the LiaR repressor undergoes a conformational change which causes it to detach from the P_{lial} operator, allowing transcription to occur. The second system is the P_{cI} system, which originates in the λ bacteriophage. The cI repressor protein binds to the operators in the P_{cI} promoter, preventing transcription. By placing the CDS of the cI repressor under the regulatory control of the P_{lial} promoter, the presence of the antibiotic bacitracin can cause transcription of *cI*, which in turn stops transcription of the CDS placed under the control of the P_{cI} promoter. Thus, a combinatorial molecular NOT gate is created.

If the CDS placed under the regulation of P_{cI} is a lethal gene, the cell will produce the lethal protein unless bacitracin is present. When bacitracin is not present in the environment (i.e., the input equals 0), the lethal gene regulated by the NOT gate's promoter is produced (i.e., the output equals 1). Conversely, when bacitracin is present in the environment (i.e., the input equals 1), the lethal gene regulated by the NOT gate's promoter is not produced (i.e., the output

equals 0). Because the cells cannot survive while expressing the lethal protein, a system can be created where the viability of a bacterial culture is dependent upon the presence of a specific chemical in the culture environment.

Because LiaR is natively produced by *B. subtilis*, it is unnecessary to introduce the gene on any of the constructs.

Optimization The DNA sequence for the *cI* gene was obtained from the annotated full-genome sequence of the λ bacteriophage (NC_001416.1).[398] The CAI of the wt *cI* gene is 0.3804. Optimization using JCat resulted in the alteration of 167 nucleotides (23%) relative to the original sequence (Appendix 2). The CAI of the optimized *cI* gene is 1.0. The proteins encoded by both the original and the optimized *cI* genes are identical (Appendix 2).

5.3.5. Ribosome Binding Sites (RBS)

The ribosome binding site (also known as the Shine-Dalgarno sequence in prokaryotes) is a region on the transcribed mRNA strand that is recognized by the ribosome. The ribosome binds and translation is initiated. The consensus sequence recognized by *B. subtilis* ribosomes is `aaaggagg`.[399] The efficiency of an RBS at initiating translation is dependent on its similarity to the consensus sequence. A variety of strong and weak RBS sequences will be used in this work, depending on the desired level of expression. For example, a strong RBS will be placed upstream of all CDS encoding lethal genes. This should provide

[398] National Center for Biotechnology Information (NCBI), "Enterobacteria phage lambda, complete genome (NCBI Reference Sequence: NC_001416.1)," http://www.ncbi.nlm.nih.gov/nuccore/9626243.
[399] EP Rocha, A Danchin, and A Viari, "Translation in *Bacillus subtilis*: roles and trends of initiation and termination, insights from a genome analysis," *Nucleic Acids Res*, 27(17), 1999.

for an efficient level of translation initiation from any mRNA transcripts that were transcribed. In theory, highly efficient translation should ensure that the requirement for transcriptional silencing of the lethal genes is met in order for cells of the strain to be viable.

However, it is possible that the strong RBS selected could be too efficient. Inducible promoters typically have a certain level of "leakiness," allowing a baseline level of transcription even in their repressed or inactivated state. This is true even for those considered highly stringent (i.e., low background transcription in the repressed or inactivated state). Thus, despite only a few mRNA transcripts being produced, an overly efficient RBS could result in the translation of sufficient numbers of lethal proteins as to be detrimental to cell viability. While *in silico* rational design provides a good starting point, determination of the ramifications of RBS efficiency of viability would require *in vivo* experimentation. If the RBS was indeed found to be too efficient, "down-designing" it for lower efficiency would similarly require testing of the new sequence *in vivo*.

5.3.6. Terminators

General Description Intrinsic termination of transcription (also known as rho-independent transcriptional termination) is a process by which the synthesis of a nascent mRNA strand is ceased. At the termination region, two inverted repeats separated by a short length of DNA. When the sequence is transcribed into mRNA, the two inverted repeats base pair, resulting in the formation of a structure known as a "stem-loop." This stem-loop causes the RNAP to pause

and detach, preventing transcription from continuing past the terminator sequence; the nascent mRNA is released from the active site of the RNAP. Intrinsic terminators typically have a termination efficiency (TE) that differs based on whether the sequence being transcribed is on the forward or reverse strand. Thus, the sequences for two high-TE terminators (one on either strand) can be juxtaposed to create a double terminator, ensuring that transcription is efficiently terminated regardless of which strand is being transcribed. Terminators for the synthetic constructs in this work were selected based on reported TE.

Two high-TE terminators were selected for combination. Dr. Drew Endy kindly supplied these parts (and many others) to the synthetic biology community via the BioBrick™ Public Agreement (BPA). This agreement allows for the free use of the contributed parts without the assertion of any intellectual property rights the contributor may hold with regard to the parts.[400] The first selected terminator, BBa_B1006 U10, has a reported mean cell TE of 99.42%.[401] The secondary structure formed by BBa_B1006 U10 in the forward direction is shown in Figure 5-10A, while the secondary structure formed in the reverse direction is shown in Figure 5-10B. The second selected terminator, ilvGEDA, has a reported mean cell TE of 98.95%.[402] The secondary structure formed by ilvGEDA in the forward direction is shown in Figure 5-11A, while the secondary structure formed in the forward direction is shown in Figure 5-11B.

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[400] Use of the sequences described herein is covered by The BioBrick™ User Agreement (v1.0) electronically signed by Marco A. Riojas. Agreement Timestamp: 2:35pm EDT, March 13, 2014.
[401] G Cambray *et al.*, "Measurement and modeling of intrinsic transcription terminators," *Nucleic Acids Res*, 41(9), 2013.
[402] Ibid.

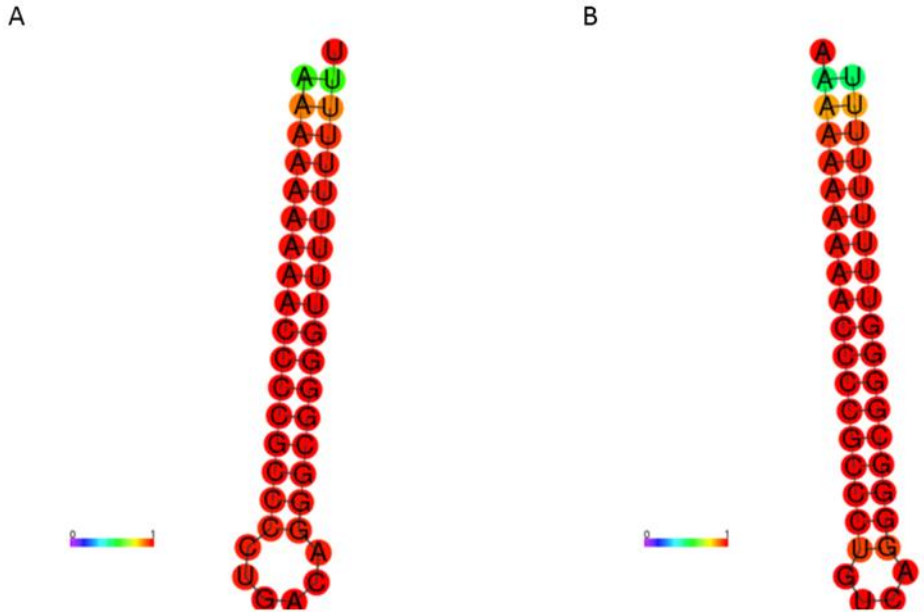

Figure 5-10: Terminator secondary structure of BBa_B1006 U10

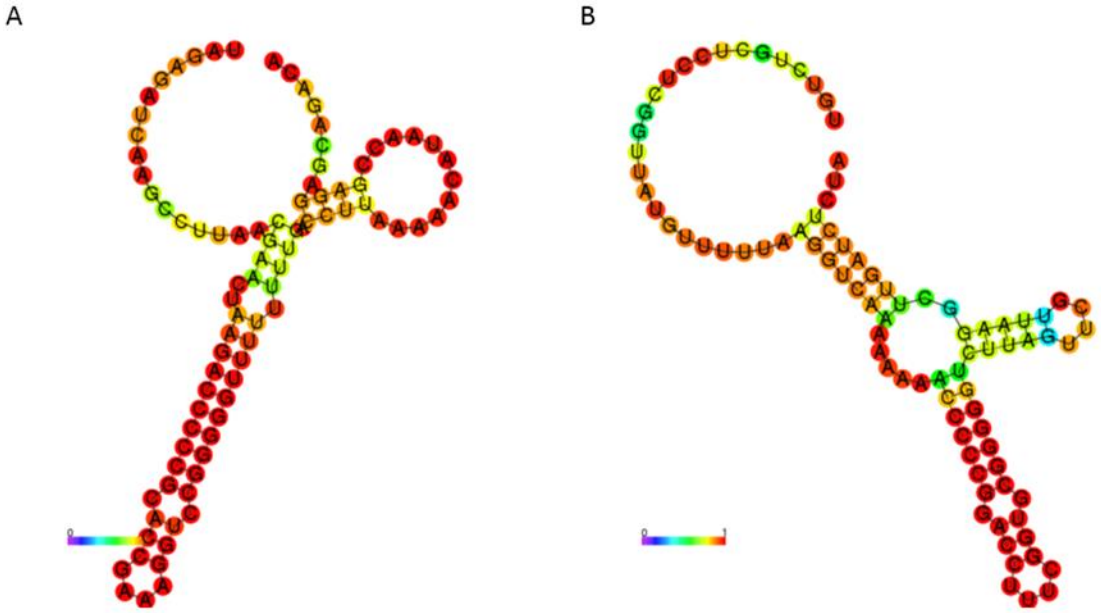

Figure 5-11: Terminator secondary structure of ilvGEDA

Optimization BBa_B1006 U10 and ilvGEDA were combined into a single bidirectional terminator called MARterm, which consists of the 41 bp BBa_B1006

U10 sequence in the forward direction, a 15 bp stretch of T nucleotides to provide a lengthy U-tail upon transcription, and the 89 bp ilvGEDA sequence in the reverse direction (Figure 5-12). The predicted secondary structure formed by MARterm in the forward direction is shown in Figure 5-13A, while the predicted secondary structure formed in the reverse direction is shown in Figure 5-13B.

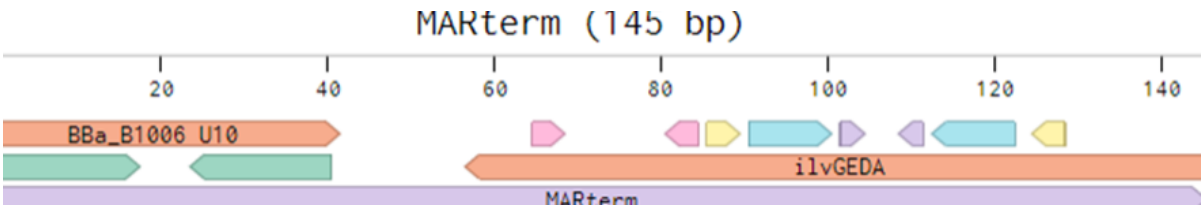

Figure 5-12: MARterm is comprised of BBa_B1006 U10 and ilvGEDA

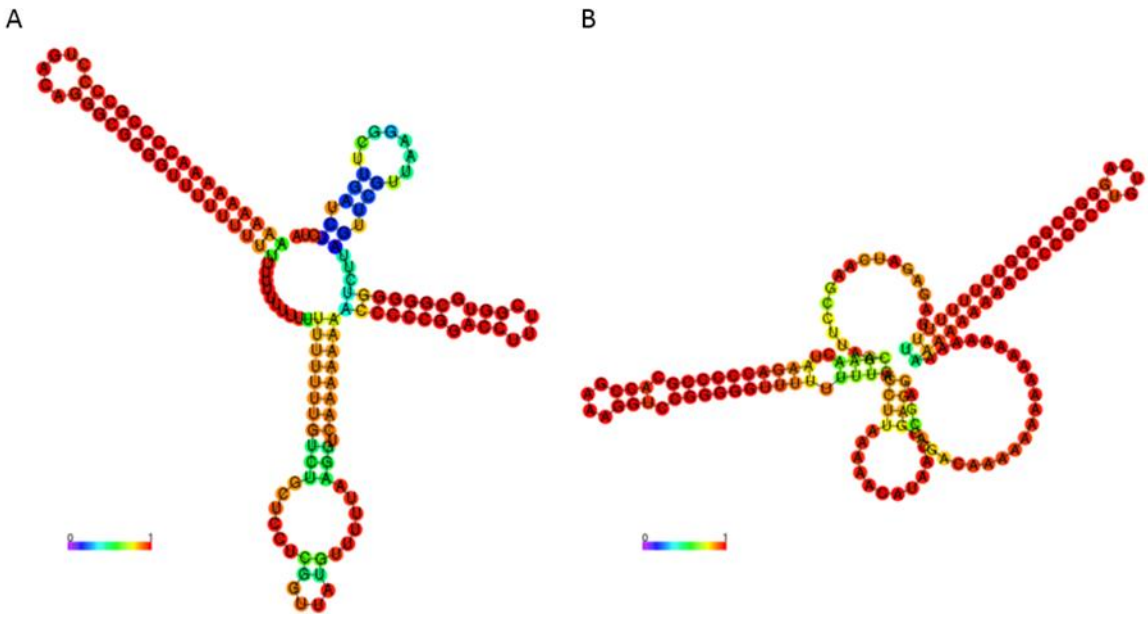

Figure 5-13: Predicted terminator secondary structure of MARterm

Assuming that the two individual terminators do not interact with each other, the TE of a double terminator can be predicted as a function of their individual TEs:

$$TE_{dbl} = 1 - (1 - TE_1)(1 - TE_2)$$

Equation 5-1: Termination efficiency of a double terminator[403]

However, because a terminator's TE may be different in the opposite direction, this equation is only valid if the two TE values are calculated for readthrough measured in the same direction. (Because the TE is normally measured and reported in the direction of the terminator's greatest activity, it can be assumed that TE > TE_{rev}.) Equation 5-1 can be modified to calculate the overall efficiency of a double terminator composed of two independent terminators, one in the forward direction and one in the reverse direction:

$$TE_{dbl} = 1 - (1 - TE_1)(1 - TE_{2rev}), \text{ where } \quad TE_{2rev} = \lim_{0 \to TE_2} x$$

Equation 5-2: Termination efficiency of a double terminator (for $TE_1 > TE_2$)

The limit-based TE_{2rev} calculation allows for the unknown contribution of a second terminator ranging between zero (i.e., the second terminator has no

[403] Ibid.

effect and thus TE_{dbl} is determined solely by TE_1 as if it were a single terminator) and TE_2 (i.e., TE_{2rev} = TE_2, and thus TE_{dbl} as the same value as if both terminators were in the same direction).

Based on Equation 5-2, the predicted value of TE_{dbl} for MARterm ranges between 99.42% and 99.99% in the forward direction and between 98.95% and 99.99% in the reverse direction. Thus, although experimental verification of TE_{dbl} is required to determine an accurate termination efficiency, it can be surmised that MARterm is a highly efficient bidirectional terminator that only allows between 0.01% and 1.05% readthrough in any direction.

5.3.7. Upstream & Downstream Homologous Regions

The USHR and the DSHR are sequences of DNA identical to sequences on the *B. subtilis* chromosome to facilitate homologous recombination at a specific location delineated by the sequence homologies. The USHR and DSHR should be of sufficient lengths to initiate homologous recombination. Previous research has shown the minimum efficiently processed segment (MEPS) in *E. coli* to be approximately 26 bp in length.[404] Additional research inserted PCR-generated ssDNA oligonucleotides into the *E. coli* chromosome using homologous regions of 44 bp at the upstream end and 43 bp at the downstream end.[405] In order to ensure an extremely high efficiency of recombination, the USHRs and DSHRs designed in this work are 150 bp in length. Because the

[404] P Shen and HV Huang, "Homologous recombination in *Escherichia coli*: dependence on substrate length and homology," *Genetics*, 112(3), 1986.
[405] HM Ellis *et al.*, "High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides," *Proc Natl Acad Sci U S A*, 98(12), 2001.

USHR and DSHR of each construct are specifically designed to be identical to the sequences at which insertion is desired, they essentially only exist as defined entities while on the construct. Once a given construct is inserted into the proper location, these regions “disappear” into the genome. The only net alteration occurs between (and not inclusive of) the USHR and DHSR.

5.3.8. Miscellaneous

5.3.8.1. Promoter Regulatory Genes

The typical mechanism of action for both positively- and negatively-regulated promoters involves an interaction with at least one other protein molecule, either an activator or a repressor. Thus, the proper functioning of these promoters is dependent on the presence of these proteins, which necessitates the pre-production of these proteins prior to the use of the promoters. (The regulatory proteins required for the proper activity of the specific promoter systems described in this dissertation can be found in Chapters 5.3.3 and 5.3.4.) The simplest way this can be accomplished is by placing the coding sequences for these genes under constitutive promoters in advance of any attempt to induce or repress transcription.

5.3.8.2. P_{veg}

In order to ensure sufficient quantities of promoter regulatory proteins and antibiotic resistance proteins, the genes that encode these proteins were placed under the control of a strong constitutive promoter. P_{veg} is a constitutive σ^A promoter with a fairly high level of downstream transcription initiation. The

sequence of the P_{veg} promoter (BBa_K143012[406]) was obtained from the Registry of Standard Biological Parts (RSBP).

5.3.8.3. P_{T7}/T7 RNA Polymerase (RNAP)

P_{T7} is a highly efficient promoter from the T7 bacteriophage. It is not recognized by most RNAPs other than the RNAP encoded by the T7 phage itself. Additionally, the T7 RNAP recognizes no promoters except P_{T7}. Because of these specificities, the P_{T7}/T7 RNAP system is referred to as "orthologous," meaning it can be expressed in an organism without affecting the organism's native regulatory systems or being affected by those same systems. This makes the P_{T7}/T7 RNAP system one of the most useful and frequently utilized in molecular biology. The specific version of the T7 RNAP used in this dissertation, the T7ptag RNAP with two amber stop codons, has already been described (see Chapter 5.3.3.2).

5.3.8.4. Antibiotic Resistance Gene (kan^R)

General Description An additional type of accessory gene is the antibiotic resistance gene (Ab^R). Such genes are necessary to select for the proper insertion of the constructs into the genome. The kanamycin resistance gene (kan^R) encodes a protein which enzymatically modifies the aminoglycoside antibiotic kanamycin. Aminoglycosides bind to the ribosome's 30S subunit, and causes misreading and/or premature termination of nascent polypeptide

[406] J Chappell, "Promoter veg: Constitutive Promoter for *B. subtilis* (Part:BBa_K143012)," http://parts.igem.org/Part:BBa_K143012.

elongation.[407] Because *Bacillus subtilis* is normally susceptible to kanamycin, introduction of the kan^R gene on a plasmid or construct can aid in the selection of successful transformants. Excision of the inserted kan^R from the genome after successful transformants have been selected allows for the reuse of the kan^R gene in subsequent transformations.

Optimization The DNA sequence of the kan^R gene (BBa_K389005[408]) was obtained from the RSBP. The CAI of the wt kan^R gene is 0.4382. Optimization using JCat resulted in the alteration of 173 nucleotides (21%) relative to the original sequence (Appendix 2). The CAI of the optimized kan^R gene is 1.0. The proteins encoded by both the original and the optimized kan^R genes are identical (Appendix 2).

5.3.8.5. Antibiotic Resistance Gene Excision

In order to avoid the creation of a strain with extreme antibiotic resistance (as described in Chapter 5.1.1), a system was sought which would allow the deletion or inactivation of the inserted Ab^R gene. One method which was entertained was the design of a second-stage construct which would interrupt the Ab^R ORF after it had served its purpose for selection of successful first-stage transformants. A second method is the use of naturally-occurring recombination systems. The *cre/lox* system was derived from the *E. coli*-infecting bacteriophage P1. The system uses a recombinase known as Cre to initiate a

[407] MP Mingeot-Leclercq, Y Glupczynski, and PM Tulkens, "Aminoglycosides: activity and resistance," *Antimicrob Agents Chemother*, 43(4), 1999.
[408] J Aretz, "Kanamycin resistance (Part:BBa_K389005)," http://parts.igem.org/Part:BBa_K389005.

recombination event between two 34 bp *loxP* sequences which are directional. A DNA sequence that is flanked by *loxP* sites is said to be "floxed." Cre recombination between two *loxP* sequences in opposite orientation causes an inversion of the floxed sequence and essentially recreates both *loxP* sites, leaving the inversion as the only net change. Cre recombination between two *loxP* sequences in the same orientation causes a deletion of the floxed sequence. Only a single *loxP* site which results from the recombination of a portion of each of the original *loxP* sites is left behind.[409] The relative simplicity and effectiveness of the *cre*/*lox* recombination system suggests it is an ideal method for the repeated excision of the inserted kan^R genes in the proposed work.

5.4. Results: *In Silico* Construction of Synthetic Constructs

For the purposes of selecting successful transformants, all the constructs described in this section were designed with a floxed kan^R gene regulated by the constitutive promoter P_{veg}.

5.4.1. MARLG1

Including the USHR and DSHR, the 4,195 bp lethal gene construct MARLG1 is comprised of 21 genetic parts, including three lethal genes downstream of three different negatively-regulated inducible promoters (Figure 5-14). The $ccdB_{Ec}$ gene is downstream of the $P_{revTetR}$ promoter, enabling the repression of transcription in the presence of aTc. The *spoIISA* gene is downstream of the P_{glnRA} promoter, enabling the repression of transcription in the

[409] A Nagy, "Cre recombinase: the universal reagent for genome tailoring," *Genesis*, 26(2), 2000.

presence of glutamine. The *lysB4* gene is downstream of the P_{cI} promoter under negative regulation via the [P_{lial}/*cI*] → P_{cI} NOT gate, enabling the repression of transcription in the presence of bacitracin. Because the RevTetR and cI transcriptional regulators are encoded by genes on the MARAcc cassette, the use of MARLG1 requires the use of MARAcc (described in Chapter 5.4.4) as well.

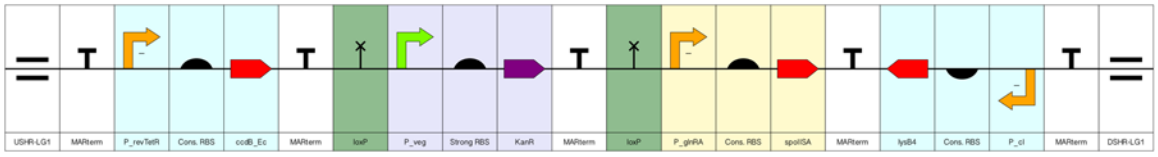

Figure 5-14: Design of MARLG1 cassette

The location chosen for insertion of the MARLG1 cassette was the region between the *groEL* gene and the *ydiM* gene. At 1,563 bp, the region between these two genes is the longest stretch of non-coding DNA in the *B. subtilis* genome. USHR-LG1 corresponds to bp 651,869..652,018 of the *B. subtilis* full genome sequence, while DSHR-LG1 corresponds to bp 653,251..653,400.[410] The full annotated sequence of MARLG1 in GenBank format is provided in Appendix 3. After the insertion of MARLG1, the kan^R module (including the P_{veg} promoter and RBS) can be excised by treatment with Cre recombinase, leaving

[410] National Center for Biotechnology Information (NCBI), "*Bacillus subtilis* subsp. *subtilis* str. 168 chromosome, complete genome (NCBI Reference Sequence: NC_000964.3)."

behind only a single *loxP* site. The final size of the remaining insertion cassette is 3,081 bp (Figure 5-15).

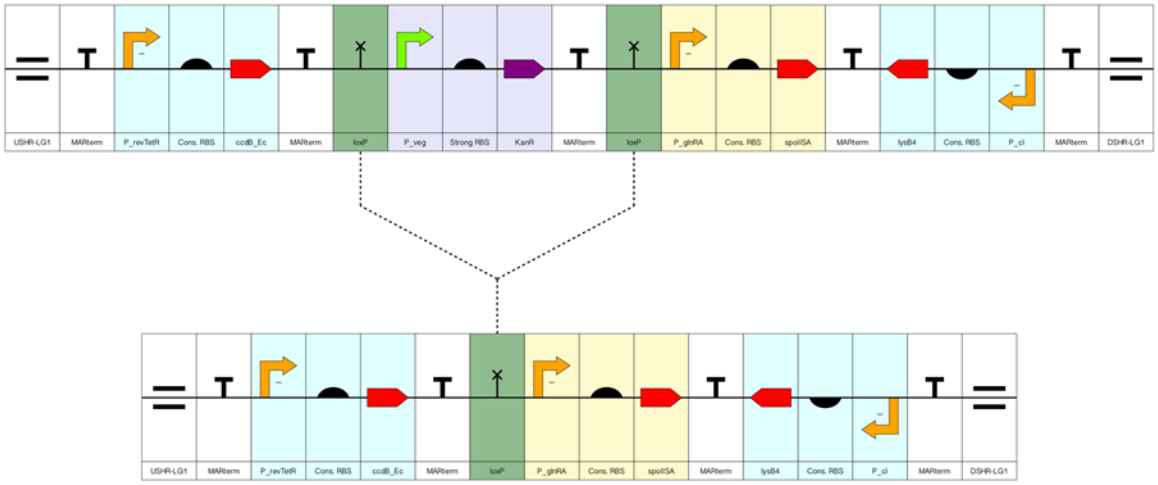

Figure 5-15: *cre*/*lox* excision of kan^R from MARLG1

5.4.2. MAREPR1

Including the USHR and DSHR, the 1,494 bp essential promoter replacement construct MAREPR1 is comprised of 9 genetic parts, including its primary part, the P_{T7} promoter (Figure 5-16). The full annotated sequence of MAREPR1 in GenBank format is provided in Appendix 3. Because P_{T7} is orthologous to *B. subtilis* and has been designed to be the output promoter of the [P_{BAD}/*supD* + P_{sal}/*T7ptag*] → P_{T7} AND gate, the use of MAREPR1 requires the use of the MARAND cassette (described in Chapter 5.4.5) as well.

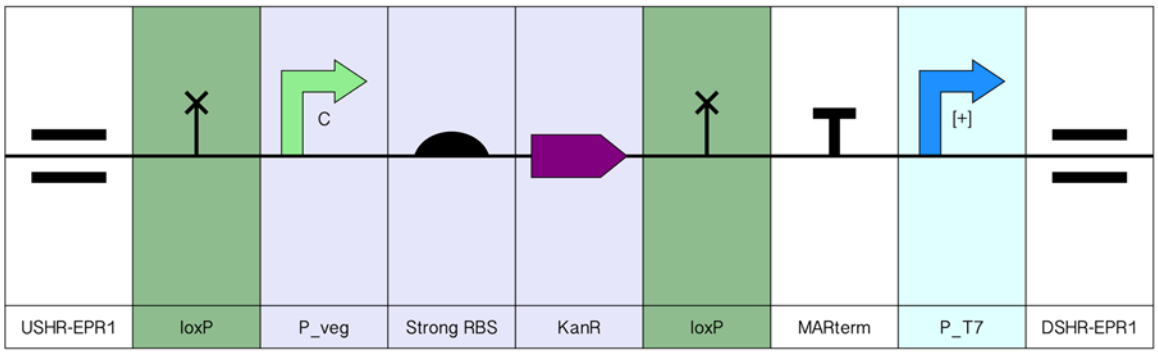

Figure 5-16: Design of MAREPR1 cassette

The location chosen for insertion of the MAREPR1 cassette was upstream of the essential *hbs* gene. The DSHR was designed to be identical to the RBS and CDS of *hbs*, such that only the promoter regulating the transcription of *hbs* was replaced. The 5' end of the DSHR begins 17 bp upstream of the *hbs* `atg` start codon, such that the native RBS is included. This should allow for essentially normal translational efficiency from mRNA. The remainder of the DSHR is identical to the first 133 bp of the *hbs* gene. The USHR was designed to be identical to the final 150 bp of *spoIVA*, the gene immediately upstream of *hbs* (Figure 5-17). (It should be unnecessary to ensure that the native *spoIVA* terminator remains intact. Because the insert contains the MARterm terminator, the deletion of the entire region between *spoIVA* and the hbs CDS will result in the replacement of the native terminator with MARterm. As *spoIVA* is monocistronic[411], the replacement of one terminator with a different one would be expected to have few, if any, effects on *B. subtilis* biology.) USHR-EPR1

[411] S Roels, A Driks, and R Losick, "Characterization of spoIVA, a sporulation gene involved in coat morphogenesis in *Bacillus subtilis*," *J Bacteriol*, 174(2), 1992.

corresponds to bp 2,386,344..2,386,195 of the *B. subtilis* full genome sequence, while DSHR-EPR1 corresponds to bp 2,385,838..2,385,689.[412] Together, the placement of the USHR at the end of *spoIVA* and the DSHR at the beginning of *hbs* should allow for the deletion of the entire native promoter region (minus the native RBS), including any uncharacterized features that may contribute to transcriptional regulation.

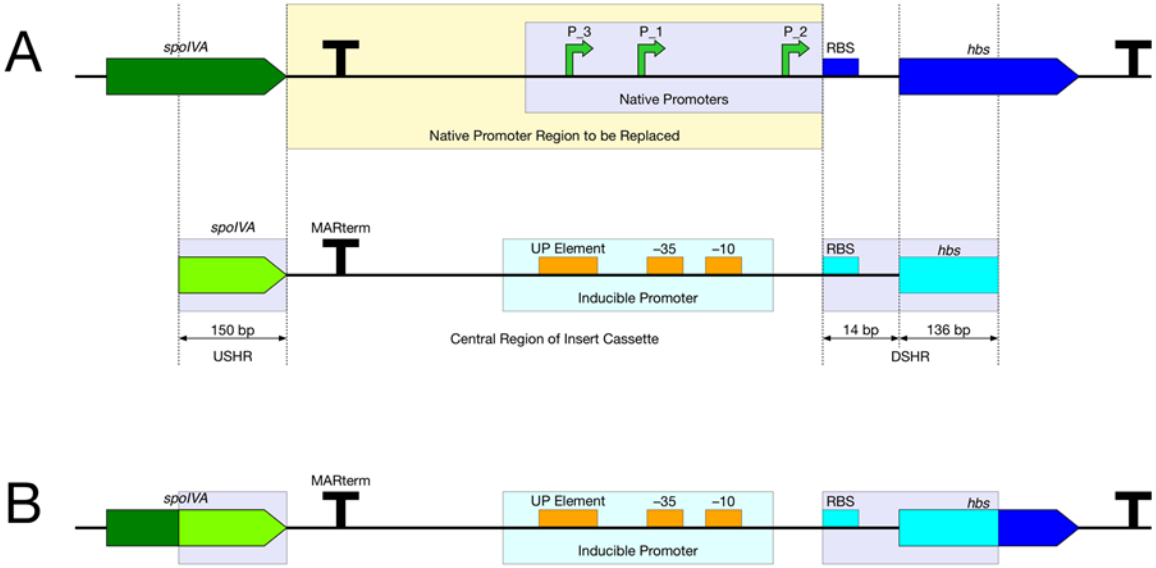

Figure 5-17: MAREPR1 insertion area between *spoIVA* and *hbs*

This placement of the P_{T7} promoter upstream of *hbs* enables the induction of transcription, and therefore the essential protein Hbsu, only in the presence of both arabinose and salicylate, which activate the proper transcription of the T7ptag RNAP. After the excision of the kan^R module from the inserted

[412] National Center for Biotechnology Information (NCBI), "*Bacillus subtilis* subsp. *subtilis* str. 168 chromosome, complete genome (NCBI Reference Sequence: NC_000964.3)."

sequence, the final size of the remaining insertion cassette is 525 bp (Figure 5-18).

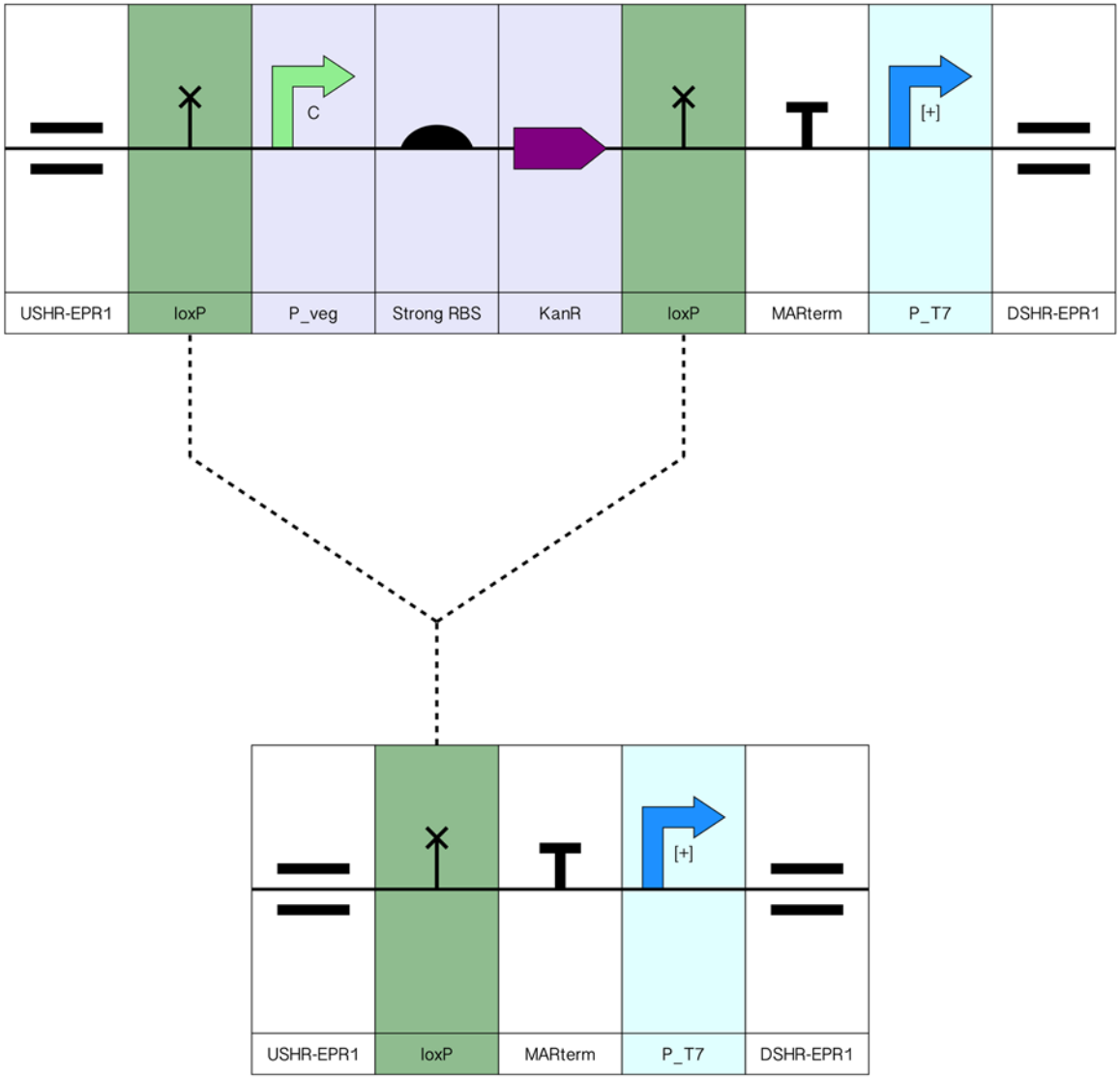

Figure 5-18: *cre/lox* excision of kan^R from MAREPR1

5.4.3. MAREPR2

Including the USHR and DSHR, the 1,530 bp essential promoter replacement construct MAREPR2 is comprised of 9 genetic parts, including its primary part, the P_{xylA} promoter (Figure 5-19). The full annotated sequence of MAREPR1 in GenBank format is provided in Appendix 3. Because P_{xylA} is regulated by the XylR repressor protein, the efficient use of MAREPR2 requires the use of the MARAcc cassette (described in Chapter 5.4.4) as well.

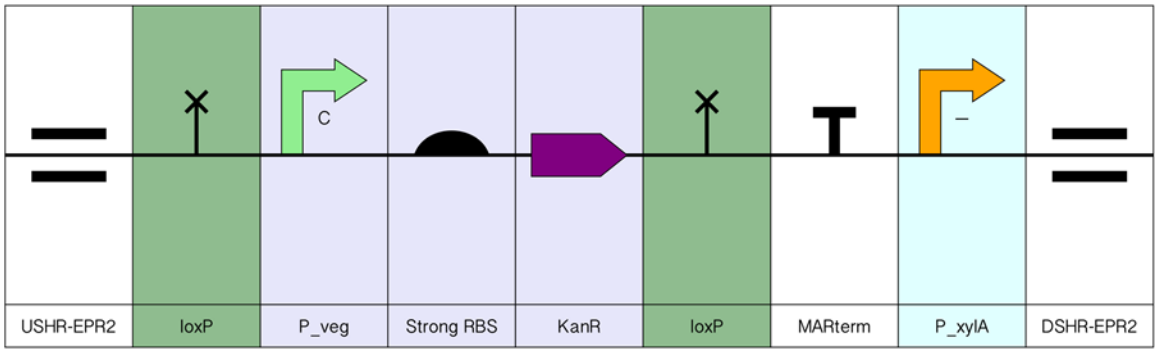

Figure 5-19: Design of MAREPR2 cassette

The location chosen for insertion of the MAREPR2 cassette was upstream of the essential *trxA* gene. The DSHR was designed to be identical to the RBS and CDS of *trxA*, such that only the promoter regulating the transcription of *trxA* was replaced. The 5' end of the DSHR begins 25 bp upstream of the *trxA* `atg` start codon. This region contains what appears to be a putative RBS: `AttGGAGG` (identity to the *B. subtilis* consensus RBS sequence is indicated by capital letters). Keeping this region intact such that the putative native RBS is included

should allow for essentially normal translational efficiency from mRNA. The remainder of the DSHR is identical to the first 125 bp of the *trxA* gene. The USHR was designed to be identical to the final 150 bp of *xsa*, the gene immediately upstream of *trxA* (Figure 5-20). (As described in the previous section, inclusion of a terminator just inside the USHR should allow the deletion of the native *xsa* terminator without repercussions.) USHR-EPR2 corresponds to bp 2,913,810..2,913,661 of the *B. subtilis* full genome sequence, while DSHR-EPR2 corresponds to bp 2,913,363..2,913,214.[413] Together, the placement of the USHR at the end of *xsa* and the DSHR at the beginning of *trxA* should allow for the deletion of the entire native promoter region (minus the native RBS), including any uncharacterized features that may contribute to transcriptional regulation.

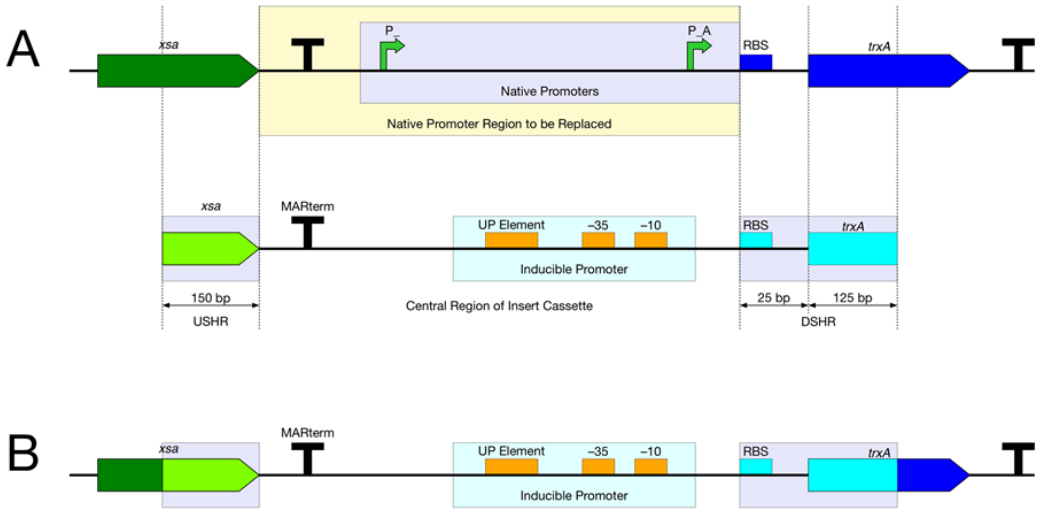

Figure 5-20: MAREPR2 insertion area between *xsa* and *trxA*

[413] Ibid.

This placement of the P_{xylA} promoter upstream of *trxA* enables the induction of transcription, and therefore the essential protein thioredoxin, only in the presence of xylose. After the excision of the kan^R module from the inserted sequence, the final size of the remaining insertion cassette is 561 bp (Figure 5-21).

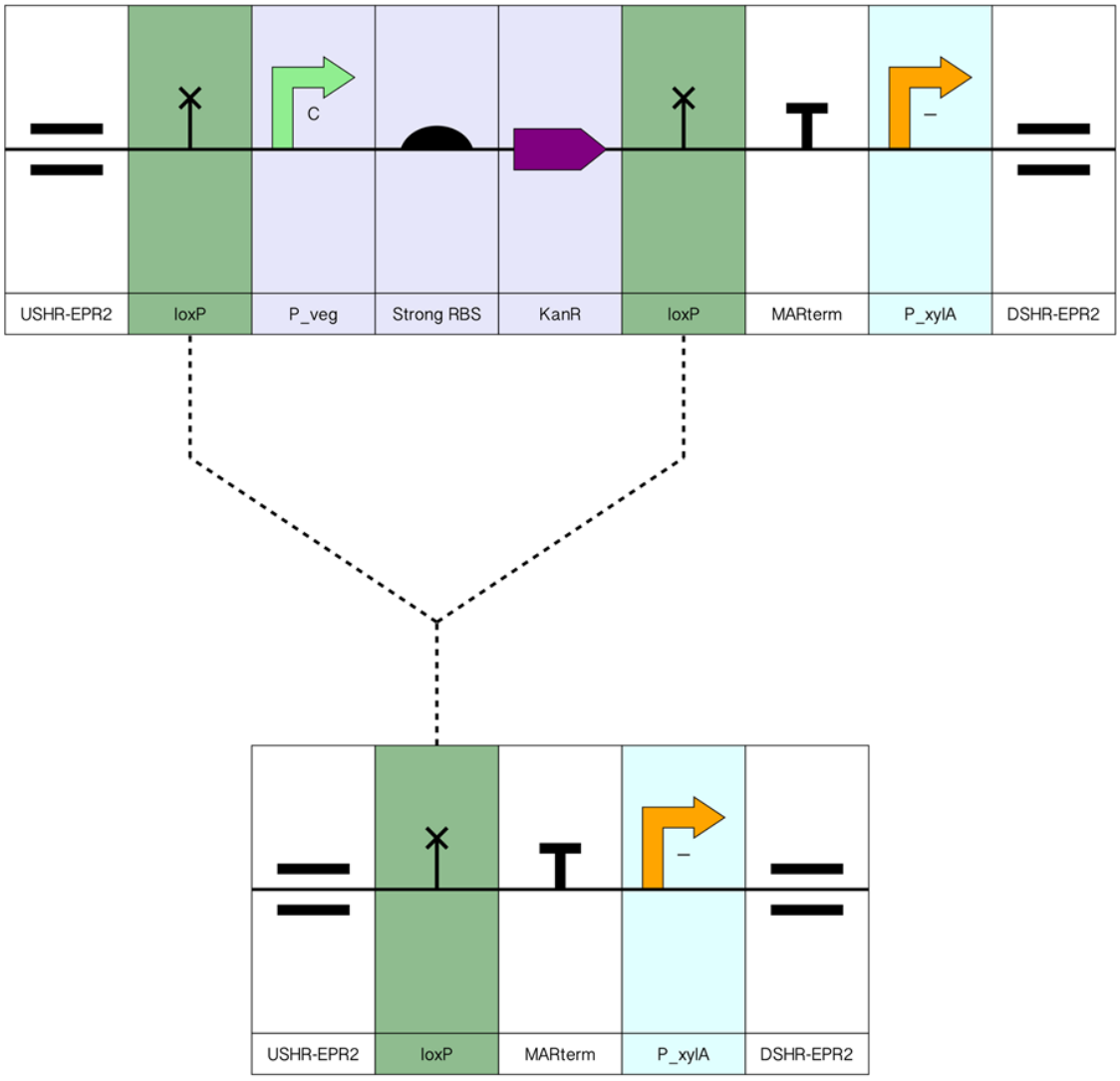

Figure 5-21: *cre/lox* excision of kan^R from MAREPR2

5.4.4. MARAcc

Including the USHR and DSHR, the 4,921 bp accessory construct MARAcc is comprised of 21 genetic parts, including its primary parts, the CDS of the RevTetR, XylR, and cI transcriptional regulators (Figure 5-22). The full annotated sequence of MARAcc in GenBank format is provided in Appendix 3. (As discussed in Chapter 5.3.3.1, the efficient repression of P_{xylA} requires a level of XylR expression greater than that available from the native *xylR* gene. Thus, a second copy of *xylR* is being introduced into the genome on MARAcc.)

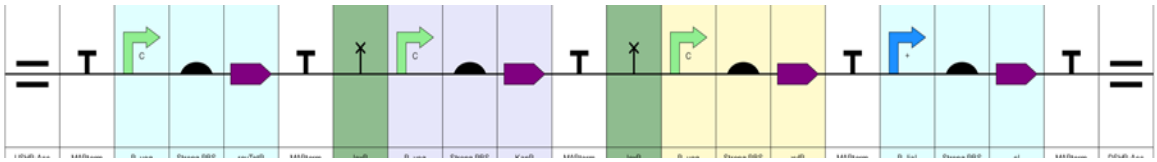

Figure 5-22: Design of MARAcc cassette

The location chosen for insertion of the MARAcc cassette was the region of the *spoIISAB* operon. The UHSR and DSHR were designed to delete the native *spoIISAB* operon, as described in Chapter 5.3.2.1. The USHR was designed to be identical to the 150 bp immediately upstream of (on the opposite strand) *spoIISB*. Keeping the region between *spoIISB* and *xlyA*, the gene immediately upstream of *spoIISB* (which should not be confused with the nearly identically-named *xylA* gene encoding xylose isomerase), intact should allow for any regulatory sequences (e.g., promoters) associated with *xylA* to be unaffected. The DSHR was designed to be identical to the final 150 bp of *pit*, the

gene immediately downstream of (on the opposite strand) *spoIISA* (Figure 5-23). USHR-Acc corresponds to bp 1,348,292..1,348,441 of the *B. subtilis* full genome sequence, while DSHR-Acc corresponds to bp 1,349,468..1,349,617.[414] After the excision of the kan^{R} module from the inserted sequence, the final size of the remaining insertion cassette is 3,807 bp (Figure 5-24).

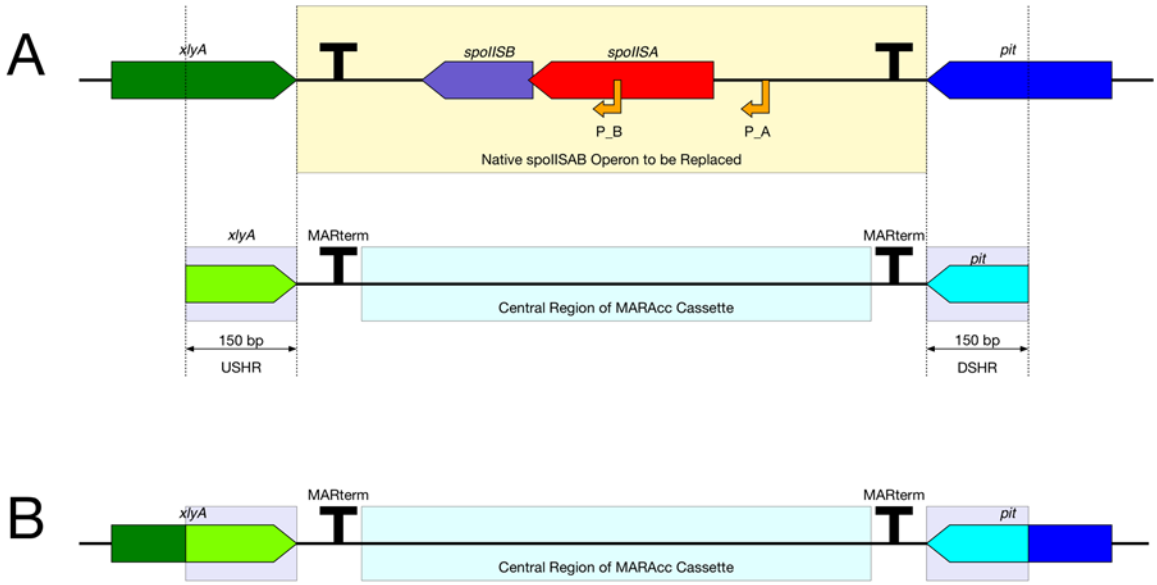

Figure 5-23: Deletion of *spoIISAB* by insertion of MARAcc

[414] Ibid.

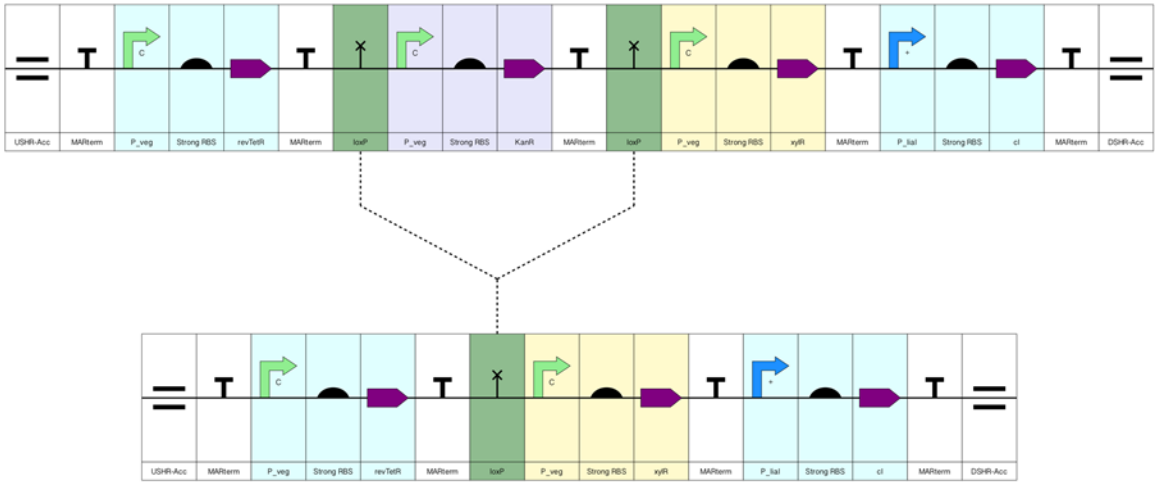

Figure 5-24: *cre*/*lox* excision of kan^{R} from MARAcc

5.4.5. MARAND

Including the USHR and DSHR, the 7,078 bp logic gate construct MARAND is comprised of 23 genetic parts, including the *T7ptag* gene encoding the amber nonsense mutations, the *supD* tRNA encoding the amber suppressor, and the CDS of the AraC and NahR transcriptional regulators (Figure 5-25). The full annotated sequence of MARAND in GenBank format is provided in Appendix 3.

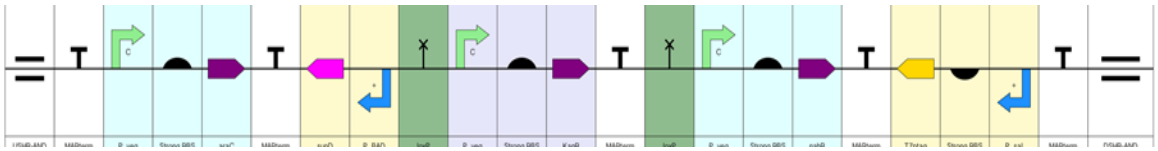

Figure 5-25: Design of MARAND cassette

The location chosen for insertion of the MARAND cassette was the *skin* (*sigK* intervening) element, a prophage-like element that has been shown to be

dispensable.[415] With a length of 4,758 bp, the nonessential gene *yqbO* is the longest in the *skin* element, making it an attractive target for such an insertion. USHR-AND corresponds to the final 150 bp of *yqbO*, 4,609..4,758 (in reverse orientation). DSHR-AND corresponds to the first 150 bp of *yqbO*, 1..150 (in reverse orientation). USHR-AND corresponds to bp 2,672,706..2,672,855 of the *B. subtilis* full genome sequence, while DSHR-AND corresponds to bp 2,677,314..2,677,463.[416] After the excision of the kan^R module from the inserted sequence, the final size of the remaining insertion cassette is 5,964 bp (Figure 5-26).

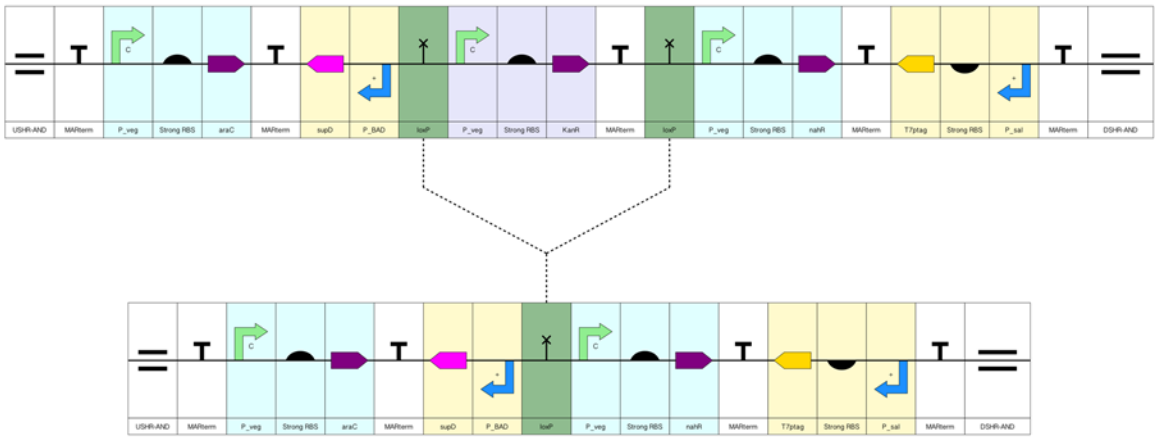

Figure 5-26: *cre/lox* excision of kan^R from MARAND

[415] B Kunkel, R Losick, and P Stragier, "The *Bacillus subtilis* gene for the development transcription factor sigma K is generated by excision of a dispensable DNA element containing a sporulation recombinase gene," *Genes Dev*, 4(4), 1990; H Westers *et al.*, "Genome engineering reveals large dispensable regions in *Bacillus subtilis*," *Mol Biol Evol*, 20(12), 2003.
[416] National Center for Biotechnology Information (NCBI), "*Bacillus subtilis* subsp. *subtilis* str. 168 chromosome, complete genome (NCBI Reference Sequence: NC_000964.3)."

5.4.6. *In Silico* Creation of Novel *B. subtilis* Strain

Together, the insertions and modifications described in Chapter 5.4 form a novel, inducible genetic network. Because of these regulatory dependencies and the fact that both lethal and essential genes are regulated, the genomic integration of the insertion cassettes requires serial transformations and deletions that must be performed in a specific order to ensure viability between insertions. For the purpose of tracking the creation of the novel strain through these various modifications, a nomenclatural scheme akin to software versioning has been adopted. Thus, the unmodified wild-type *B. subtilis* subsp. *subtilis*, strain 168 (e.g., ATCC® 23857™) has been designated Bs1.0. (A summary of these versions and their associated properties and requirements is presented in Table 5-3. The detailed description of the modifications follows.) The full network of inserts and regulatory interrelatedness is shown in Figure 5-27.

Table 5-3: Incremental construction of novel *B. subtilis* strain

Strain Version Number	Strain Characteristics	Lethal Gene Inducing Chemicals Required for Viability	Essential Gene Inducing Chemicals Required for Viability
Bs1.0	*B. subtilis* subsp. *subtilis*, Strain 168; *spoIISAB*$^{+}$	–	–
Bs1.1	MARAcc (+ kan^{R}); Δ*spoIISAB*	–	–
Bs1.2	MARAcc	–	–
Bs1.3	MARAND (+ kan^{R}), MARAcc	–	–
Bs1.4	MARAND, MARAcc	–	–
Bs1.5	MARLG1 (+ kan^{R}), MARAND, MARAcc	Bacitracin, aTc, glutamine	–
Bs1.6	MARLG1, MARAND, MARAcc	Bacitracin, aTc, glutamine	–
Bs1.7	MAREPR1 (+ kan^{R}), MARLG1, MARAND, MARAcc	Bacitracin, aTc, glutamine	Arabinose, salicylate
Bs1.8	MAREPR1, MARLG1, MARAND, MARAcc	Bacitracin, aTc, glutamine	Arabinose, salicylate
Bs1.9	MAREPR2 (+ kan^{R}), MAREPR1, MARLG1, MARAND, MARAcc	Bacitracin, aTc, glutamine	Arabinose, salicylate, xylose
Bs2.0	MAREPR2, MAREPR1, MARLG1, MARAND, MARAcc	Bacitracin, aTc, glutamine	Arabinose, salicylate, xylose

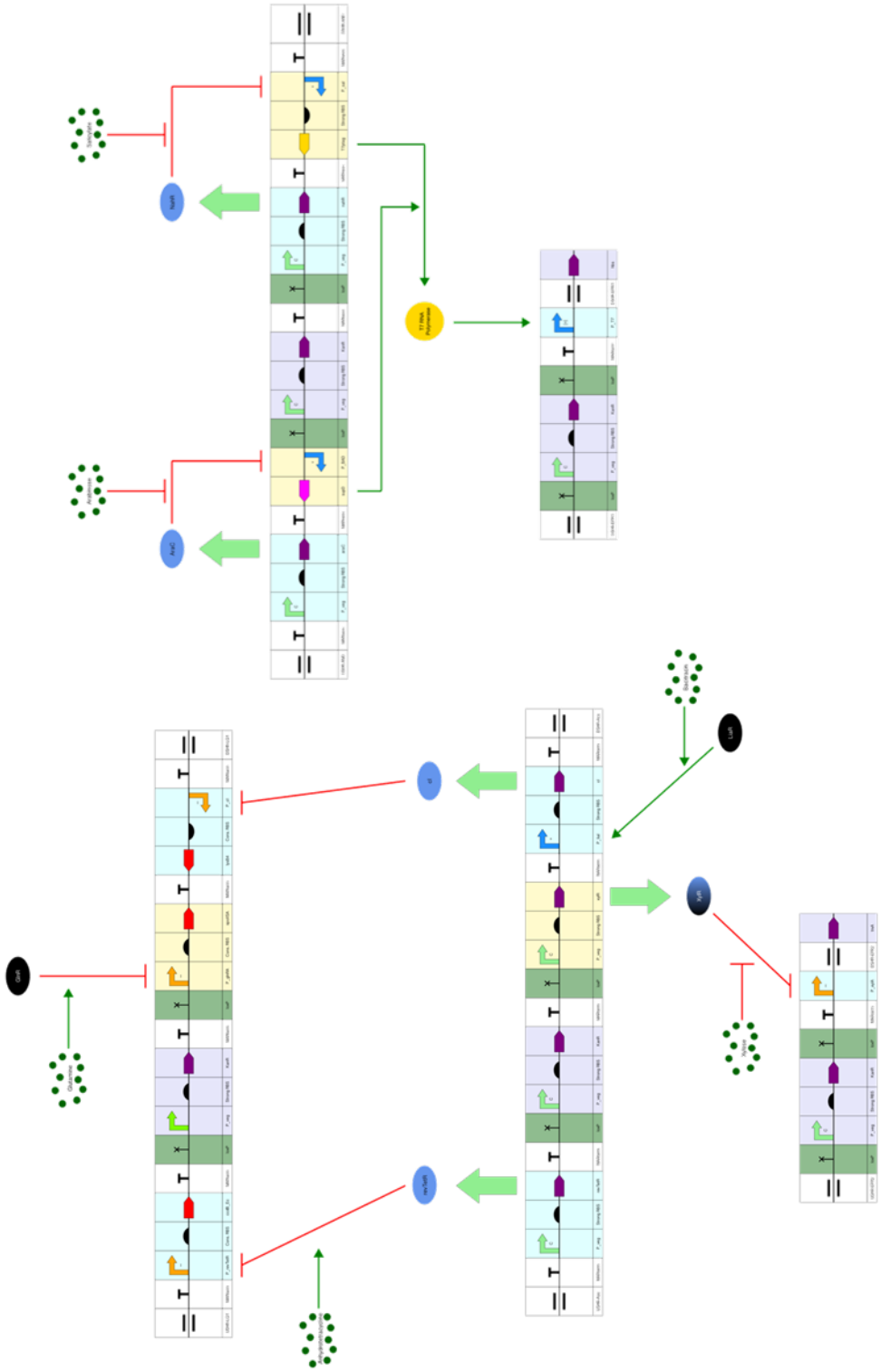

Figure 5-27: Designed genetic regulatory network
In this diagram of the full genetic circuitry, the proteins native to *B. subtilis* are colored black with white text, while those exogenous to *B. subtilis* are blue with black text.

The first cassette to be inserted into the *B. subtilis* genome is the MARAcc cassette since it contains three of the regulatory genes required for the use of the MARLG1 and MAREPR2 cassettes. Insertion of MARAcc into Bs1.0 results in Bs1.1. Selection of Bs1.1 cells containing the proper insertion is achieved by growth in media containing kanamycin (e.g., TSB + 50 μg/mL kan). After successful culturing, Bs1.1 can be treated with Cre recombinase, resulting in the excision of the kan^R module. Excision verification requires a two-step process that begins with growth of the Cre-treated culture on solid media without kanamycin (e.g., TSA). After colonies have been isolated, the antibiotic susceptibility to kanamycin can be determined via replica plating onto solid media containing kanamycin (e.g. TSA + 50 μg/mL kan). Selection and culturing of an antibiotic susceptible colony from the original kan^- plate establishes the Bs1.2 strain, which harbors the MARAcc cassette and has no non-native antibiotic resistance.

The second cassette to be inserted into the *B. subtilis* genome is the MARAND cassette since it contains the genetic parts that provide the functionality of the [P_{BAD}/*supD* + P_{sal}/*T7ptag*] → P_{T7} AND gate. Insertion of MARAND into Bs1.2 results in Bs1.3. Selection of Bs1.3 cells containing the proper insertion is achieved by growth in media containing kanamycin. After successful culturing, Bs1.3 can be treated with Cre recombinase, resulting in the excision of the kan^R module. Selection via replica plating and culturing of an antibiotic susceptible colony from the original kan^- plate establishes the Bs1.4

strain, which harbors both the MARAcc and MARAND cassettes and has no non-native antibiotic resistance.

Prior to the insertion of the third cassette, Bs1.4 should be cultured in media containing the required cofactors for the regulatory proteins that are encoded on the MARAcc and MARAND cassettes. Because the transcription process for the genes inserted will be begin immediately, the inclusion of lethal genes on the cassette should result in a lack of viability unless the transcription of these genes is prevented. Thus, having the required regulatory machinery in place and "primed" prior to the insertion of the lethal genes is essential. This requires growth of Bs1.4 in media containing bacitracin, aTc, and glutamine prior to transformation with MARLG1. Bacitracin interacts with the natively-produced LiaR, which activates the P_{lial} promoter, initiating the transcription of cI, the repressor of the P_{cI} promoter. The RevTetR protein encoded on MARAcc interacts with aTc, enabling it to bind to the operators of the $P_{revTetR}$ promoter. Glutamine interacts with the natively-produced GlnR, the repressor of the P_{glnRA} promoter. Although Bs1.4 does not yet contain any of these target promoters, this enables the repression of lethal gene transcription immediately upon the insertion of the promoters in the next transformation.

The third cassette to be inserted into the *B. subtilis* genome is the MARLG1 cassette containing the lethal genes *spoIISA*, *lysB4*, and $ccdB_{Ec}$. Insertion of MARLG1 into Bs1.4 results in Bs1.5. Selection of Bs1.5 cells containing the proper insertion is achieved by growth in media containing

kanamycin. After successful culturing, Bs1.5 can be treated with Cre recombinase, resulting in the excision of the kan^R module. Selection via replica plating and culturing of an antibiotic susceptible colony from the original kan^- plate establishes the Bs1.6 strain, which harbors the MARAcc, MARAND, and MARLG1 cassettes and has no non-native antibiotic resistance.

Prior to the insertion of the fourth cassette, Bs1.6 should be cultured in media containing the required cofactors to "prime" the regulatory systems used in the next cassette. As with the scenario described above, replacement of the native promoter of an essential gene with an inducible promoter requires "priming" of the transcription machinery prior to transformation. Because the transformed cell will attempt to begin the transcription process immediately, an orthologous promoter would otherwise be completely unreadable and result in an essential protein not being produced. This, of course, would result in a lack of viability that would prevent selection of successful transformants. Thus, the orthologous transcription machinery must already be in place prior to the transformation and available to begin transcription the instant the orthologous promoter is integrated upstream of the essential gene. In the case of the MAREPR1 cassette, this requires growth of Bs1.6 in media containing arabinose and salicylate.

Arabinose interacts with AraC (encoded on MARAND and constitutively transcribed by the P_{veg} promoter), which releases the repression of the P_{BAD} promoter, initiating the transcription of the *supD* amber suppressor. Salicylate

interacts with NahR (encoded on MARAND and constitutively transcribed by the P_{veg} promoter), which releases the repression of the P_{sal} promoter, initiating the transcription of the *T7ptag* gene. The presence of the amber suppressor during the translation of *T7ptag* ensures that complete, fully-functional T7 RNAP enzymes are produced, rather than the truncated nonsense proteins that would otherwise be produced.

The fourth cassette to be inserted into the *B. subtilis* genome is the MAREPR1 cassette containing the P_{cI} promoter. Insertion of MAREPR1 into Bs1.6 results in Bs1.7. Selection of Bs1.7 cells containing the proper insertion is achieved by growth in media containing kanamycin. After successful culturing, Bs1.7 can be treated with Cre recombinase, resulting in the excision of the kan^R module. Selection via replica plating and culturing of an antibiotic susceptible colony from the original kan^- plate establishes the Bs1.8 strain, which harbors the MARAcc, MARAND, MARLG1, and MAREPR1 cassettes and has no non-native antibiotic resistance.

Prior to the insertion of the fifth cassette, Bs1.8 should be cultured in media containing xylose, which interacts with XylR (encoded on MARAcc and constitutively transcribed by the P_{veg} promoter), preventing the repression of the P_{xylA} promoter. The fifth cassette to be inserted into the *B. subtilis* genome is the MAREPR2 cassette containing the P_{xylA} promoter. Insertion of MAREPR2 into Bs1.8 results in Bs1.9. Selection of Bs1.9 cells containing the proper insertion is achieved by growth in media containing kanamycin. After successful culturing,

Bs1.9 can be treated with Cre recombinase, resulting in the excision of the kan^R module. Selection via replica plating and culturing of an antibiotic susceptible colony from the original kan^- plate establishes the Bs2.0 strain.

The final product, Bs2.0, is the final construct, which harbors the MARAcc, MARAND, MARLG1, MAREPR1, and MAREPR2 cassettes and has no non-native antibiotic resistance. As shown in Table 5-3, the media required to ensure the viability of the Bs2.0 strain contains bacitracin, aTc, glutamine, arabinose, salicylate, and xylose. As an indication of its function, it has been given the name Simulant Induction Media (SIM).

6. DISCUSSION

The novel genetically-engineered strain of *Bacillus subtilis* described in Chapter 5 was designed with the intention of developing a system that could address the drawbacks of previous simulant systems described in Chapters 2, 3, and 4. This strain has the potential to be used in a wide variety of applications in research, industry, and biodefense in particular. After a discussion of potential technical pitfalls, this chapter describes some of these potential uses, and the possibility of this strain's application to dual-use research of concern.

6.1. Potential Pitfalls

The synthetic biology design described in Chapter 5 has been conducted entirely *in silico*. Thus, it is not definitively known if the methods as described will result in the successful creation of the strain or its functioning as intended. Three main pitfalls exist that could negatively affect the success of the *in vivo* development of the described novel strain.

First, the insertion of the design constructs could fail. This, however, would not be expected. The insertion of exogenous DNA into *Bacillus subtilis* via homologous recombination has been a commonly used technique for the genetic manipulation of this organism. For example, the *amyE* locus has been an insertion target via a double crossover homologous recombination event for

nearly three decades.[417] Because the overall mechanism that forms the technical basis for the insertion of the specific constructs has been extensively validated, its use as the mechanism for introducing the designed cassettes would not be expected to present difficulties. One uncertainty, however, is the extreme length of the cassettes to be inserted. The efficiency of homologous recombination decreases as a function of the insert length.[418] To compensate for this, the insert cassettes described in this work were designed with USHR and DSHR sequences 150 bp in length, much larger than the *E. coli* MEPS.[419] In *Xylella fastidiosa*, the efficiency of double-crossover homologous recombination has been shown to plateau at approximately 1 kb of flanking homology.[420] This could be true in *Bacillus subtilis* as well. Thus, one method to increase the recombination efficiency could be to extend the USHR and DSHR sequences on the insert cassettes further.

The second potential pitfall is the insertions having unforeseen secondary effects. The complex and highly interconnected nature of biological systems means that increasing or decreasing production of a particular gene could have effects on other systems with which that protein interacts. In some cases, a protein's interaction with other systems may not yet be known. In such a case,

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[417] S Hidenori and DJ Henner, "Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*," Gene, 43(1–2), 1986.
[418] SH Kung *et al.*, "Effects of DNA Size on Transformation and Recombination Efficiencies in *Xylella fastidiosa*," Appl Environ Microbiol, 79(5), 2013.
[419] P Shen and HV Huang, "Homologous recombination in *Escherichia coli*: dependence on substrate length and homology."
[420] SH Kung *et al.*, "Effects of DNA Size on Transformation and Recombination Efficiencies in *Xylella fastidiosa*."

replacing the native regulation of a gene with an inducible promoter could have unforeseen effects which could compromise the viability of the simulant strain. This is been mitigated to the greatest extent possible by using *Bacillus subtilis*, a very well-characterized bacterium, as the basis for creating the simulant strain. (Although *Bacillus atrophaeus* has historically been used as a simulant in dispersal studies, it is not as well-characterized, and its use as the basis upon which to design enhancements would introduce a much higher uncertainty for success.) Unfortunately, beyond this, the nature of unforeseen effects would require their experimental elucidation.

The third potential pitfall is related to the failure rate of promoter systems. According to a review of suicidal genetic elements, a small but significant fraction (10^{-3} – 10^{-6}) of bacteria always survive.[421] For example, a promoter system could be rendered inoperative by mutation of the operator sequence or a critical repressor protein. In the case of a hypothetical positively-regulated inducible promoter with a failure rate of 10^{-3}, nearly all the bacterial cells will be unable to transcribe the gene unless the inducing chemical is present. However, one in one thousand cells will be successful at initiating transcription despite the absence of the inducer. If this single hypothetical promoter is being used as a biological containment mechanism by regulation of the expression of an essential gene as proposed in Chapter 5, the 10^{-3} failure rate would allow in one bacterium in 1,000 to survive in the absence of the promoter's inducer. This would, of

[421] S Molin *et al.*, "Suicidal genetic elements and their use in biological containment of bacteria."

course, circumvent the entire purpose and design of the described simulant. Thus, the strain in Chapter 5 has been designed with multiple inducible and repressible promoters for redundant biological control.

Three positively-regulated promoters were used in the design of the strain: P_{xylA}, P_{BAD}, and P_{sal}, the latter two of which were used in combination in the design of the AND molecular logic gate [P_{BAD}/supD + P_{sal}/T7ptag] → P_{T7}. Three negatively-regulated promoters were used in the design of this strain: $P_{revTetR}$, P_{glnRA}, and P_{lial}, the latter of which was used in the design of the NOT molecular logic gate P_{lial}/cI] → P_{cI}. The overall failure probability of any number of systems can be determined using the equation

$$F_{\Pi} = \prod F_P,$$

Equation 6-1: Composite failure probability of multiple promoter system

where F_P is the failure rate inherent to any given promoter system, i.e., the frequency of an undesired transcriptional outcome (the transcription of a positively-regulated gene or non-transcription of a negatively-regulated gene) in the absence of the relevant inducing chemical.

Thus, for the six specific promoters in the current system, the overall failure probability can be calculated using the equation

$$F_{\Pi} = \left(F_{P_{xylA}}\right)\ \left(F_{P_{BAD}}\right)\ \left(F_{P_{sal}}\right)\ \left(F_{P_{revTetR}}\right)\ \left(F_{P_{glnRA}}\right)\ \left(F_{P_{liaI}}\right).$$

Equation 6-2: Composite failure probability of the designed six-promoter system

Assuming a hypothetical F_P of 10^{-3} (based on the highest failure probability as reported by Molin, *et al*.) for each of these six systems,

$$\begin{aligned} F_{\Pi} &= \left(F_{P_{xylA}}\right)\ \left(F_{P_{BAD}}\right)\ \left(F_{P_{sal}}\right)\ \left(F_{P_{revTetR}}\right)\ \left(F_{P_{glnRA}}\right)\ \left(F_{P_{liaI}}\right) \\ &= \left(10^{-3}\right)\ \left(10^{-3}\right)\ \left(10^{-3}\right)\ \left(10^{-3}\right)\ \left(10^{-3}\right)\ \left(10^{-3}\right) \\ &= 10^{-18} \end{aligned}.$$

Equation 6-3: Hypothetical solution to Equation 6-2

Thus, only one in 10^{18} (one quintillion) bacterial cells would be expected to have a simultaneous failure of ***all*** six promoter systems. In a bacterial culture with a typical concentration of 1×10^9 cells/mL, the volume of culture required to statistically result in a single viable cell is 1,000,000 L. Using the mean of the Molin, *et al*. failure rates ($F_P = 10^{-4.5}$) results in an $F_{\Pi} = 10^{-27}$, while using the least frequent of the cited failure rates ($F_P = 10^{-6}$) results in an $F_{\Pi} = 10^{-36}$.

Because the bacteria cannot survive if any of the essential proteins are missing or if any of the lethal proteins are produced, an overall undesirable outcome requires the simultaneous failure of all these systems. Theoretically, this six-promoter composite regulatory scheme should be able to withstand the complete failure of five different promoter systems. The remaining properly

functioning promoter should effect the desired transcriptional outcome (preventing the transcription of an essential gene or initiating transcription of a lethal gene) in the absence of the relevant inducing chemical. (More generally, an *n*-promoter scheme should be capable of functioning properly despite the failure of $n - 1$ promoter systems.)

One important additional point must be addressed: in the statistically unrealistic event that composite failure actually occurs, wild-type *Bacillus subtilis* is still a fairly safe bacterium which is ubiquitous in the environment and is Generally Regarded As Safe (GRAS) by the FDA. Thus, it is unlikely to cause infection in the majority of humans. Nevertheless, it can cause human infections (and has). However, given its ubiquity in nature (i.e., the number of potential encounters which could have resulted in infection), the statistical likelihood of such infections occurring is minimal. The impetus for this dissertation's work has been the belief that even this low probability of infection is too high for its general use as an open-air dispersal simulant. Thus, a complex artificial regulatory system with a statistically negligible probability of composite failure has been combined with an organism that, in the event composite failure actually occurs, has a statistically negligible probability of causing an infection. Although it is impossible to design a simulant system for which the probability of hazard is literally zero (i.e., ***absolutely*** impossible), the intention of this strain's design has been to reduce the probability of causing infection to ***essentially*** zero. To the

extent that theoretical predictions without *in vivo* verification allow, the presently designed system accomplishes this.

6.2. Applications

The most obvious practical application of the designed genetically-engineered bacterium is as a safer simulant for the spread of *B. anthracis* spores. (Indeed, it was rationally designed specifically with this goal in mind.) By growing these bacteria in the Simulant Induction Media (SIM) and allowing them to sporulate, the resulting bacteria would be in their spore form. The spores would germinate upon encountering a more hospitable, nutrient-containing environment. However, only spores that germinate in SIM that also induces expression of the essential proteins and silences expression of lethal proteins would survive and be capable of multiplication. All the other bacterial cells would die a rapid death through two different mechanisms (denial of essential proteins and production of lethal proteins). In the event such spores were to germinate in an otherwise growth-supporting environment (nutrient-rich, but lacking the inducing chemicals), the resultant vegetative cells would be unable to survive or reproduce. (See Figure 5-1 for a simplified graphical representation.)

Dispersal of these spores would scatter them across whatever environment is chosen. For example, if these spores were released inside a building's ventilation system (much as a terrorist might do with lethal *B. anthracis* spores), the spread of the spores could be analyzed, thereby indicating the areas most at risk from an infectious spore bioaerosol. This sampling could be

accomplished using numerous detection methods, for example, automated air samplers such as slit samplers equipped with agar plates of SIM. Which rooms the spores spread to and where the highest concentrations occur could be determined easily be examining and quantifying the growth of the bacteria on the media. Additional testing for presence of spores could be accomplished using swabs of surfaces and inoculating liquid SIM with the swabs; this would allow for testing of virtually any surface. Analysis of the dispersal could provide the basis for development of predictive dispersal models that are based on a realistic simulant of high fidelity to *B. anthracis*.

Generally speaking, this strain could be applied to a wide variety of different applications. Examples of potential defensive and research applications include:

- Release into the HVAC system of a critical infrastructure building to determine the dispersal patterns and speed of spread.
- Release of spores from inside a university or government laboratory, to test the nature of the threat to the public from a similar release with more pathogenic organisms.
- Release from an aerial vehicle to analyze outdoor dispersal patterns and the effects of various meteorological conditions.
- Testing the effectiveness of upgrades to the postal mail sorting infrastructure.

- Biological defense against microbes that cause less exotic diseases (such as bacterial respiratory diseases) that commonly infect people in buildings. The designed strain could be used to analyze and improve particle filtration or germicidal techniques.
- Testing of decontamination methods such as irradiation or sterilization.

Chapters 2 and 3 described the numerous drawbacks inherent to various dispersal models and historical experiments. These drawbacks provided the impetus for the creation of a safer biological simulant. Therefore, it is useful to return briefly to these models and simulant tests in order to reexamine them in the light of the novel simulant designed in this dissertation. This also helps highlight the possible specific defensive applications for which this strain could be used and how its use could improve on the previous methods.

As was mentioned by several of the research papers discussed in Chapter 2.2, the full-scale, open-air testing of the models described is not realistically feasible with existing simulants. However, the currently described simulant would prove an ideal solution for precisely this purpose. Spores of this simulant could be released from Metro stations in downtown Washington, DC, to examine their dispersal across the National Capital Region, as was modeled by Nicogossian, *et al*.[422] Releasing these spores on the first floor of a 50-story

[422] A Nicogossian, LA Schintler, and Z Boybeyi, "Modeling Urban Atmospheric Anthrax Spores Dispersion: Assessment of Health Impacts and Policy Implications."

building, as modeled by Reshetin and Regens[423], would allow their dispersal throughout the entire building to be studied. Introduction of these spores into a small office building staffed with people, similar to the scenario described by Sextro, *et al*.[424], would allow both the examination of the spores' dispersal properties and the effect of human activities on the patterns of dispersal. The analysis of deposition of these spores upon vertical surfaces such as those described by Lai and Nazaroff[425] would allow a refinement of such models.

The mathematical models described in Chapter 2.2 were all entirely hypothetical and were left unvalidated. Using this simulant in environments or under conditions such as those described in the cited research would allow for the validation of the respective mathematical models. Differences between the predictions of the models and the empirical results of the physical dispersal of this simulant could be examined and used to effect improvements of the models for greater accuracy.

This approach is similar to that utilized by most of the models described in Chapter 2.3, except that those research papers used physical (i.e., biologically nonviable) simulants and methods that are likely not accurately applicable to the dispersal of *B. anthracis* spores. Instead, this *B. subtilis* simulant can be used to directly simulate *B. anthracis* spores and more realistically model their dispersal. Rather than using artificial polystyrene beads with proteins bound to their

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[423] VP Reshetin and JL Regens, "Simulation modeling of anthrax spore dispersion in a bioterrorism incident."
[424] RG Sextro *et al.*, "Modelling the Spread of Anthrax in Buildings".
[425] ACK Lai and WW Nazaroff, "Modeling Indoor Particle Deposition from Turbulent Flow onto Smooth Surfaces."

surface, work such as that described by Farrell, *et al*.[426] could use spores of the currently described strain to test immunoassays with a more realistic simulant that has similar biosafety properties to BG Bugbeads.[427] Use of the novel designed spores would eliminates the need for constructing a scale physical model of a room as described by Thatcher, *et al*. (2004)[428], eliminating the incorporation of errors introduced by improper scaling. Further, it would eliminate the inaccuracies caused by improper construction of a computational model, as was seen in Finlayson, *et al*.[429] For simulation of bioaerosol dispersal, spores of this strain will be a higher-fidelity simulant than the olive oil/isopropanol mixture utilized by Thatcher, *et al*. (2002).[430]

The biological models described in Chapter 2.4 and most of the US government experiments described in Chapter 3 dispersed viable biological simulants, maximizing the fidelity of the simulant but simultaneously increasing the potential for pathogenicity to an unacceptable level. Using the novel simulant system described by this dissertation would allow the benefits of biological simulation to be maintained while simultaneously reducing the statistical risk of

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[426] S Farrell, HB Halsall, and WR Heineman, "*Bacillus globigii* bugbeads: a model simulant of a bacterial spore."

[427] Because the work of Farrell, *et al*. created BG Bugbeads using proteins from *B. atrophaeus* spores, the designed *B. subtilis* simulant strain might not be exactly interchangable. If the relevant similarities (e.g., the specific proteins upon which an immunoassay is based) between the two species are significant enough, the *B. subtilis* strain could be substituted for the BG Bugbeads. Otherwise, the general methodology described in Chapter 5 should be adaptable to the creation of a similar simulant strain based on *B. atrophaeus*.

[428] TL Thatcher *et al.*, "Pollutant dispersion in a large indoor space: Part 1 -- Scaled experiments using a water-filled model with occupants and furniture."

[429] EU Finlayson *et al.*, "Pollutant dispersion in a large indoor space. Part 2: Computational fluid dynamics predictions and comparison with a scale model experiment for isothermal flow."

[430] TL Thatcher *et al.*, "Effects of room furnishings and air speed on particle deposition rates indoors."

pathogenicity to essentially zero. In theory, any of the experiments described in Chapter 2.4 and Chapter 3 could be performed with a greater degree of safety and/or fidelity with the currently described strain than with the simulants used in those respective experiments.

6.3. Application to Dual-Use Research of Concern

Unfortunately, as with many technologies, this novel genetically-engineered strain has the potential to be used to conduct offensive rather than defensive research. For example, it could provide a state or non-state actor with the ability to safely test dissemination devices and conduct aerosol modeling with a high-fidelity *B. anthracis* simulant. However, a state or non-state actor that has made a decision to research, develop, or deploy offensive biological weapons has already made a deliberate choice to embark on a path that is, as stated by the Biological Weapons Convention (BWC), "repugnant to the conscience of mankind.[431]" Such an actor has displayed a lack of basic respect for human life and suffering that is the cornerstone of the BWC and the international cultural norms against such weapons and their use. Therefore, it seems likely that a state or non-state actor seeking to develop an offensive biological weapons capability would derive little added benefit by the use of this strain versus a wild-type *Bacillus* strain.

While infections from wild-type species such as *B. subtilis* and *B. thuringiensis* are rare, they are certainly possible and are occasionally fatal.

[431] "Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction," http://www.opbw.org/.

Eliminating that small percentage is the entire basis for designing the novel, strictly growth-regulated strain described in this dissertation. However, for an actor seeking to develop weapons that use biology to kill or incapacitate, one could imagine the wild-type strains are "safe enough" for simulant use. For example, the Iraqi BW program used *B. thuringiensis* as a simulant.[432] It seems unlikely that those seeking such an offensive capability would worry very much about a few collateral casualties that might result from such testing. For defensive work, however, the goal is clearly to protect people against biological weapons. Using the strain described by this dissertation would be far safer and more appropriate to researching protective strategies than using a potentially infectious wild-type simulant. Because the threshold of acceptable risks for defensive work is (appropriately) far lower, the difference between no casualties and one or two is much larger than it would be for the type of offensive BW simulation that could be conducted with this strain.

Although it seems unlikely that those seeking to engage in biological warfare or bioterrorism would bother with the use of the described strain rather than a wild-type strain for testing purposes, the most obvious way to reduce the risk this strain being used for offensive research would seem to be the restriction of its distribution.

[432] United Nations Monitoring, Verification, and Inspection Commission (UNMOVIC), "Unresolved Disarmament Issues: Iraq's Proscribed Weapons Programmes."

7. CONCLUSION

The dispersal of *Bacillus anthracis* spores for biological warfare or bioterrorism could result in mass casualties and is therefore a serious threat to US national security. The 2001 anthrax attacks dramatically illustrated just how much chaos can be caused by such an event. Because defending against such a dispersal requires an understanding of dispersal dynamics and effects, the modeling and simulation of bioaerosol dispersal is a critical component of biodefense research. Such simulations can be accomplished through the use of mathematical or computational models, physical simulations which disperse non-viable chemicals or particles, and the dispersal of biologically viable simulant organisms. In seeking to simulate the dispersal of *B. anthracis* spores, mathematical modeling suffers from questionable accuracy and a lack of flexibility, while physical simulation suffers from questionable fidelity. However, any naturally-occurring viable organism has the potential to be pathogenic, particularly among immunocompromised individuals. Thus, biological simulation may be the superior method in terms of accuracy and fidelity, but dispersing viable organisms, even ostensibly safe simulants, risks pathogenicity to exposed populations. Thus, the existing methods for modeling the dispersal of *B. anthracis* spores are insufficient or potentially hazardous.

In conducting research (both offensive and defensive) during its Cold War BW program, the US government dispersed biologically viable simulants, exposing human subjects (often unbeknownst to them) to these ostensibly safe but potentially pathogenic organisms. An analysis of these open-air tests illustrates three improvements to ensure that future biodefense research is conducted ethically: reduced pathogenicity of the biological simulant, obtaining informed consent (either standard or *de facto*) from potentially exposed populations, and ensuring that the unique concerns regarding military bioethics are addressed for any military service members who may be exposed. With specific regard to pathogenicity, if critical biodefense dispersal research is to continue, a safer biological simulant is required.

This work culminated in the *in silico* synthetic biology design of a strain of *Bacillus subtilis* with extremely stringent growth restrictions, which allow it to survive only in a specifically-defined, artificially-supplied chemical environment where growth is desired. This virtually eliminates the possibility of pathogenicity or viability where undesired, making this strain an ideal simulant for *Bacillus anthracis* spores and a valuable tool for the biodefense research community. On its own, this strain provides very few, if any, truly novel applications. What it does enable, however, is the ability to conduct such experimentation with a far greater margin of safety than is currently possible. Although this may be considered an incremental improvement, it is an important one. If biodefense

research is to continue (and it should), the novel *B. subtilis* simulant strain designed in this dissertation would allow the research to occur safely.

APPENDIX 1

DTC Test Number	63-1	63-1	63-4	63-2	64-1	64-5	64-6
Test Name	**Eager Belle, Phase I**	**Eager Belle II**	**Big Jack, Phase A**	**Autumn Gold**	**Errand Boy**	**Night Train**	**Yellow Leaf**
Testing Organization	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center
Test Dates	January, March 1963	February, March, June 1963	February 15 – March 15, 1963	May 3 – 31, 1963	September 6 – 17, 1963	November 30, 1963 – January 8, 1964	• February 1964 (Panama Canal Zone) • April – May 1966 (Hawaii)
Test Location	Testing was conducted in the Pacific Ocean, west of Oahu, Hawaii.	Testing was conducted in the Pacific Ocean, west of Oahu, Hawaii.	Near Fort Sherman Military Reservation, Panama Canal Zone	Pacific Ocean near the island of Oahu, Hawaii	Buoy X-9 in East Loch, Pearl Harbor, Oahu, Hawaii	Near Fort Greely, Alaska	• Fort Sherman Military Reservation, Panama Canal Zone (February 1964) • Island of Hawaii (April – May 1966)
Test Operations	To evaluate the effectiveness of selected protective devices in preventing penetration of a naval ship by a biological aerosol.	To study the downwind travel of biological aerosols.	To study the penetration of a jungle canopy by a biological aerosol generated by crosswind dissemination of a biological simulant from an elevated line source.	To determine the degree of ship penetration under three different material readiness conditions, by a simulant biological aerosol released from an operational weapon system; to estimate the magnitude and persistence of simulant biological aerosols retained after conducting an aircraft hose down procedure; to make observations on the performance of the Portichrome Selection Device and its components; and, to evaluate the M17 and Mark V protective masks.	To evaluate the effectiveness of various decontamination procedures for decontaminating exterior surfaces.	To obtain data on the downwind travel of a biological agent simulant under arctic conditions, when disseminated from the A/B 45Y-1 wet biological spray tank mounted on an operational aircraft and when carried from a tracked vehicle mounted dissemination device.	To measure burst height and cloud diffusion characteristics of the M143 bomblet when released into a jungle canopy.
Participating Services	• US Navy • Deseret Test Center personnel	• US Navy • Deseret Test Center personnel	• US Army • US Navy • US Air Force • Deseret Test Center personnel	• US Navy • US Marine Corps • Deseret Test Center personnel	• US Army • US Navy • US Air Force • Deseret Test Center personnel	• US Army • US Air Force • Deseret Test Center personnel	Deseret Test Center personnel
Units and Ships Involved	• USS *George Eastman* (YAG-39)	• USS *George Eastman* (YAG 39) • USS *Granville S. Hall* (YAG 40) • USS *Carpenter* (DD 825) • USS *Navarro* (APA 215) • USS *Tioga County* (LST 1185) • Marine Medium Helicopter Squadron 161	• VMA 225, Marine Aircraft Group 14	• USS *Navarro* (APA 215) • USS *Tioga County* (LST 1158) • USS *Carpenter* (DD 825) • USS *Hoel* (DDG 13) • USS *Granville S. Hall* (YAG 40) • Marine Air Group 13 • 1st Marine Brigade	• USS *George Eastman* (YAG-39)	Not identified	Not identified
Dissemination Procedures	Biological tracer released from an E-2 biological disseminator	Biological tracer released as a line source generated by Aero 14B spray tanks mounted on A-4 series jet attack aircraft.	Sprayed from US Air Force A/B45Y-1 and US Navy Aero 14B spray tanks center mounted on Marine A-4 aircraft.	In each trial, two A-4B aircraft, each equipped with two modified Aero 14 B spray tanks, disseminated tracer *Bacillus atrophaeus* (BG).	Control sample patches impregnated with known number of *Bacillus atrophaeus* microorganisms were set out in the ship's zone being decontaminated.	Biological simulant *Bacillus atrophaeus* was released from an A/B45Y-1 spray tank carried on an F-105 or F-100 aircraft. In surface trials, *Bacillus atrophaeus* was disseminated from the rear of a moving, tracked vehicle. Fluorescent particles were released from contractor-flown aircraft (Aero Commander - yellow particles and Cessna 180 - green particles).	M143 bomblets statically detonated above jungle canopy.
Pathogenic/Toxic Agents	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Biological Simulants	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG) (Hawaii)
Chemical Tracers	n/a	n/a	Zinc cadmium sulfide (FP)	n/a	n/a	Zinc cadmium sulfide	Tiara (Panama Canal Zone and Hawaii)
Ancillary Testing	• Mk V protective masks • M17 protective masks	Particle-size analyzer under development	Not identified	• Mk V protective masks • M17 protective masks	Not identified	Not identified	Not identified
Decontamination	Not identified	Not identified	Not identified	Air wash and hose down decontamination procedures	Teams disseminated betapropriolactone when decontaminating each zone; a standard dissemination time of 80 minutes was employed in all zones.	Not identified	Not identified

DTC Test Number	64-4	66-8	65-3	65-1	65-6	66-6	66-13
Test Name	**Shady Grove**	**West Side, Phase II**	**West Side I**	**Copper Head**	**Big Tom**	**Scarlet Sage**	**Half Note**
Testing Organization	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center
Test Dates	• Phase A, May 1964 • Phase B, February 12 – March 15, 1965 • Phase C, October 5 – 14, 1965 • Phase D, March 22 – April 3, 1965	January 5 – March 7, 1965	January 8 – February 21, 1965	January 24 – February 25, 1965	May – June 1965	February 9 – March 4, 1966	August 18 – September 30 1966
Test Location	• Phase A: Pacific Ocean near the island of Oahu, Hawaii. • Phases B and D: Pacific Ocean near Johnston Island. • Phase C: Eglin Air Force Base, Florida	Great Plains Region of Central Canada, north and east of the Suffield Experimental Station, southern Alberta Province, and into southwestern Saskatchewan	Tanana Valley of central Alaska near Fort Greely	Atlantic Ocean, off the coast of Newfoundland, Canada	Oahu, Hawaii, and surrounding waters and airspace	Testing was conducted in the Pacific Ocean, off San Diego, California.	In the Pacific Ocean off the coast of Hawaii, approximately 80 nautical miles south-southwest of Oahu.
Test Operations	Four phases of testing were conducted. Test operations included both aerial and surface releases of agents and tracer material. • Phase A served as a preliminary check of all test procedures prior to conducting the pathogenic agent phases. • Phase B obtained decay and infectivity data for *Francisella tularensis* (UL). • Phase C obtained estimates of dissemination efficiencies for the Aero 14B/A-4 weapon system. • Phase D obtained decay data for *Coxiella burnetti* (OU). Fluorescent particles (FP) were released in each phase to obtain meteorological data.	To evaluate the area coverage capability of an airborne dry agent dissemination system when operated in a frigid environment.	To evaluate the A/B 45Y-4 dry agent disseminator in a frigid environment.	To evaluate aerosol penetration into an operational ship and biological cloud travel in a frigid environment.	To evaluate the feasibility of a biological attack against an island complex and to evaluate doctrine and tactics for delivery of such an attack.	To test the Shipboard Toxicological Operational Protective System (STOPS), a BW tracer was disseminated upwind of the ship.	To determine biological decay rates of *Escherichia coli* and *Serratia marcescens* in a marine environment.
Participating Services	• US Navy • US Marine Corps • US Air Force • Deseret Test Center personnel	• US Air Force • Deseret Test Center personnel	• US Army • US Air Force • Deseret Test Center personnel	• US Navy • US Marine Corps • Deseret Test Center personnel	• US Navy • US Marine Corps • US Air Force • Deseret Test Center personnel	• US Navy • Deseret Test Center personnel	• US Navy • Deseret Test Center personnel
Units and Ships Involved	• USS *Granville S. Hall* (YAG-40) • Army Light Tugs 2080, 2081, 2085, 2086, and 2087 • Marine Aviation Group 13 • Patrol Squadron Four • Patrol Squadron Six • AEWBARONPAC Detachment	Not identified	• Selected members of the 171st Infantry Brigade (Mechanized)	• USS *Power* (DD 839)	• USS *Granville S. Hall* (YAG-40) • USS *Carbonero* (SS-337)	• USS *Herbert J. Thomas* (DD-833)	• USS *George Eastman* (YAG-39) • USS *Granville S. Hall* (YAG-40) • USS *Carbonero* (SS 337) • Army light tugs 2080, 2081, 2085, 2086, and 2087, all staffed by USN personnel
Dissemination Procedures	Agent and tracer material were disseminated from Aero 14B spray tanks mounted on A-4 aircraft and from the Multihead E-2 Disseminator mounted on an Army Light Tug	Simulant and tracer material sprayed from an AB 45-Y4 powdered agent disseminator mounted on an F-105 aircraft. Tracer material was also disseminated above and below the inversion layer using an EW-2 disseminator mounted on a contractor-operated JHC-47 aircraft.	Tracer material sprayed from an A/B 45Y-4 disseminator tank mounted on an F-105D aircraft	*Bacillus atrophaeus* (BG) was disseminated from an Aero 14B spray tank mounted on an A-4 aircraft. A contractor C-47 aircraft attempted congruent releases of fluorescent particles (FP).	Liquid *Bacillus atrophaeus* was disseminated from an Aero 14B spray tank mounted on a US Navy A-4 aircraft; dry *Bacillus atrophaeus* was disseminated from an A/B Y45-4 spray tank mounted on a US Air Force F-105 aircraft. *Bacillus atrophaeus* was also released from a specially equipped fleet submarine using a submarine-biological-disseminator.	An aerosolized slurry of *Bacillus atrophaeus* (BG) was released from a point source located approximately 500 meters upwind of the target vessel.	Sprayed from A-4 aircraft equipped with Aero 14B spray tanks and released from a fleet submarine specially equipped with a submarine-biological-disseminator.
Pathogenic/Toxic Agents	• *Coxiella burnetti* (OU) • *Francisella tularensis* (UL)	n/a	n/a	n/a	n/a	n/a	n/a
Biological Simulants	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	• *Bacillus atrophaeus* (BG) • *Escherichia coli* • *Serratia marcescens*
Chemical Tracers	Zinc cadmium sulfide (FP)	Zinc cadmium sulfide (FP)	Zinc cadmium sulfide (FP)	Zinc cadmium sulfide (FP)	Zinc cadmium sulfide (FP)	n/a	• Calcofluor (fluorescent brightner 28) • Zinc cadmium sulfide (FP)
Ancillary Testing	Not identified	Not identified	Not identified	Exterior deck wash-down system	Not identified	Not identified	Not identified
Decontamination	Not identified	Not identified	Not identified	Betapropriolactone	Not identified	Not identified	Not identified

DTC Test Number	67-7	67-8	67-6	68-71	69-31	68-50	69-75
Test Name	**Red Cloud**	**Watch Dog**	**Blue Tango**	**Folded Arrow**	**n/a**	**Speckled Start**	**n/a**
Testing Organization	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center
Test Dates	November 1966 – February 1967	Summer 1967	January 18 – March 1, 1968	April – May 1968	August 19 – September 4, 1968	September and October 1968	October 31 – December 1, 1968
Test Location	Tanana Valley of central Alaska, near Fort Greely	Delta Creek area of central Alaska, near Fort Greely	Island of Hawaii	Oahu, Hawaii and surrounding waters	Testing was conducted in the Pacific Ocean, off the coast of San Diego, California	Testing was conducted at Eniwetok Atoll, Marshall Islands.	In the vicinity of Yeehaw Junction, Florida
Test Operations	To obtain biological decay rates on *Francisella tularensis* (wet and dry form), *Escherichia coli* , and *Serratia marcesens* in a sub-zero overland environment.	To obtain biological decay rates on *Francisella tularensis* (wet and dry form), *Escherichia coli* , and *Serratia marcesens* in a summer temperate environment.	To determine the decay rates of *Serratia marcescens* and *Escherichia coli* aerosols when released at ground level into a tropical rain forest environment, and when released from above the canopy of a tropical rain forest. To evaluate environmental factors affecting the decay of *Serratia marcescens* and *Escherichia coli* .	To study over-ocean downwind travel of a biological aerosol material when disseminated from a submarine-biological system and to demonstrate the submarine weapon system capability to carry out an effective biological attack against an island complex; and a naval port facility.	To test the Shipboard Toxicological Operational Protective System (STOPS) using *Bacillus atrophaeus* , a nonpathogenic biological aerosol.	The F-4/AB45Y-4/PG2 weapon system disseminated an aerosol over a 40-50 kilometer downwind grid, encompassing a segment of the Eniwetok Atoll and an array of five light tugs.	To investigate the effectiveness of the F-4/A/B 45Y-2/TX weapon system to reduce wheat crop yields in selected geographic areas.
Participating Services	• US Army • Deseret Test Center personnel	• US Army • Deseret Test Center personnel	• US Army • US Air Force • Deseret Test Center personnel	• US Navy • US Marine Corps • Deseret Test Center personnel	• US Navy • Deseret Test Center personnel	• US Army • US Navy • US Air Force • Deseret Test Center personnel	• US Air Force • Deseret Test Center personnel
Units and Ships Involved	Not identified	Not identified	Not identified	• USS *Carbonero* (SS-337) • USS *Granville S. Hall* (YAG-40) • Five Army light tugs	• USS *Herbert J. Thomas* (DD-833)	• USS *Granville S. Hall* (YAG-40) • Five Army light tugs • 4533rd Tactical Test Squadron • 33rd Tactical Fighter Wing (F-4E aircraft)	Not identified
Dissemination Procedures	M143 bomblets were projected from a tower-mounted gun into a wintertime spruce forest simulating an operational drop. E26 and M32 dissemination devices were also used to disseminate aerosols for biological decay rate measurements.	Not identified	Dissemination in all trials was from E2-type nozzles with suitable pressurizing equipment. Above canopy releases were made from a 32-meter tower using equipment and procedures similar to ground-release trials.	*Bacillus atrophaeus* was disseminated from a fleet submarine using a submarine-biological-disseminator.	MARS generator to disseminate MAAPCF "swift boats" for *Bacillus atrophaeus* (BG) dissemination	Aerial-delivered aerosolized agent and agent tracers	TX was sprayed from an A/B 45Y-2 spray tank mounted on an F-4 aircraft.
Pathogenic/Toxic Agents	• *Francisella tularensis* (wet) (TT) • *Francisella tularensis* (dry) (ZZ)	• *Francisella tularensis* (wet) (TT) • *Francisella tularensis* (dry) (ZZ)	n/a	n/a	n/a	Staphylococcal enterotoxin, Type B	*Puccinia graminis* var. *tritici* (TX)
Biological Simulants	• *Bacillus atrophaeus* (BG) • *Escherichia coli* • *Serratia marcescens*	• *Bacillus atrophaeus* (BG) • *Escherichia coli* • *Serratia marcescens*	• *Bacillus atrophaeus* (BG) • *Escherichia coli* • *Serratia marcescens*	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	*Bacillus atrophaeus* (BG)	n/a
Chemical Tracers	n/a	n/a	Zinc cadmium sulfide (FP)	n/a	n/a	Uranine dye (sodium fluorescein)	n/a
Ancillary Testing	Not identified	Not identified	Not identified	Not identified	Not identified	Not identified	Not identified
Decontamination	Not identified	Not identified	Not identified	• Calcium hypochlorite • Betapropriolactone	Not identified	Not identified	Not identified

DTC Test Number	69-32	70-73	70-74	73-30
Test Name	**n/a**	**n/a**	**n/a**	**n/a**
Testing Organization	US Army Deseret Test Center	US Army Deseret Test Center	US Army Deseret Test Center	Dugway Proving Ground, Utah
Test Dates	April 30 – June 28, 1969	July – December 1970	August 1972 – January 1973	February – June 1973
Test Location	Testing was conducted at sea southwest of the Hawaiian Islands.	Dugway Proving Ground, Utah	Dugway Proving Ground, Utah	Dugway Proving Ground, Utah
Test Operations	To examine the effect of solar radiation on the viability of aerosolized *Serratia marcescens* and *Escherichia coli* after being aerially disseminated in a temperate marine environment during time periods about sunrise and sunset.	DTC Test 70-73 examined potential secondary aerosol hazards to friendly troops following a biological agent attack. The types of biological attack simulated in this study were (a) a liquid filled bomblet point source, (b) an aerial liquid spray line source, and (c) a surface deposition with dry biological spores.	A mixed slurry of *Serratia marcescens* and *Bacillus atrophaeus* was used in each of 38 trials. The mixture was disseminated from a collison atomizer and the resulting aerosol was passed over stainless steel microfilaments wound on a series of 40 individual frames. In the viability decay study the frames were removed at selected intervals and exposed to one of six test conditions. Biological assay was conducted to determine the number of *Serratia marcescens* -*Bacillus atrophaeus* organisms per time period. The biological decay-variability information was produced from those data.	The microfilament technique trials: "charged" aerosol particles passed over microfilaments wound on stainless steel frames with a portion of the aerosol particles impacting on the microfilaments. An E2 disseminator at the center of the Dugway Proving Ground West Vertical grid released free-floating aerosol particles.
Participating Services	• US Army • US Navy • US Air Force • Deseret Test Center personnel	• Deseret Test Center personnel	• Deseret Test Center personnel	• Life Sciences Laboratory personnel, Dugway Proving Ground, Utah
Units and Ships Involved	• USS *Granville S. Hall* (YAG-40) • Five Army light tugs • VC-1 (previously designated VU-1, Utility Squadron One) the Blue Aiis (Blue Warriors) Squadron, stationed at Barbers Point, Hawaii, provided a Navy A-4C as a disseminator aircraft. • Patrol Squadron Six (PATRON SIX), Fleet Air Wing Two, provided two P3V Orion aircraft as airborne command posts.	Not identified	None identified	Not identified
Dissemination Procedures	Releases were made from two Aero 14B spray tanks wing mounted on an A-4C aircraft. *Bacillus atrophaeus* (BG) with fluorescent tracer suspension (calcofluor) was released from one tank while either *Serratia marcescens* or *Escherichia coli* was simultaneously released from the other.	Liquid *Bacillus atrophaeus* (BG) was dispersed by an explosive test fixture or by a vehicle mounted generator. Dry BG was manually deposited with a gravity test fixture at an area designated for road deposit trials. Zinc cadmium sulfide (FP) was disseminated with the BG.	The mixture was disseminated from a collison atomizer in the Controlled Environmental Mobile Facility (a closed system) at Dugway Proving Ground, Utah.	For the conventional aerosols trials an E2 disseminator was used.
Pathogenic/Toxic Agents	n/a	n/a	n/a	n/a
Biological Simulants	• *Bacillus atrophaeus* (BG) • *Escherichia coli* • *Serratia marcescens*	*Bacillus atrophaeus* (BG)	• *Bacillus atrophaeus* (BG) • *Serratia marcescens*	• *Bacillus atrophaeus* (BG) • *Serratia marcescens* • T-3 coliphage
Chemical Tracers	Calcofluor (fluorescent brightner 28)	Zinc cadmium sulfide (FP)	n/a	n/a
Ancillary Testing	Not identified	Not identified	Not identified	Not identified
Decontamination	Not identified	Not identified	Not identified	Not identified

APPENDIX 2

***araC* Optimization**

```
CLUSTAL O(1.2.1) multiple sequence alignment

araC            atggctgaagcgcaaaatgatcccctgctgccgggatactcgtttaacgcccatctggtg
araC_JCat       atggctgaagctcaaaacgatcctcttcttcctggctactctttcaacgctcatcttgtt
                *********** ***** ***** ** ** ** ** ***** ** ***** ***** **

araC            gcgggtttaacgccgattgaggccaacggttatctcgatttttttatcgaccgaccgctg
araC_JCat       gctggccttacacctatcgaagctaacggctaccttgatttcttcatcgatcgtcctctt
                ** **  * ** ** ** ** ** ***** ** ** ***** ** ***** ** ** **

araC            ggaatgaaaggttatattctcaatctcaccattcgcggtcaggggtggtgaaaaatcag
araC_JCat       ggcatgaaaggctacatccttaaccttacaatccgtggccaaggcgttgttaaaaaccaa
                ** ******** ** ** ** ** ** ** ** ** ** ** ** ** ** ***** **

araC            ggacgagaatttgtctgccgaccgggtgatattttgctgttcccgccaggagagattcat
araC_JCat       ggccgtgaattcgtttgccgtcctggcgatatccttcttttccctcctggcgaaatccat
                ** ** ***** ** ***** ** ** *****  * ** ***** ** ** ** ** ***

araC            cactacggtcgtcatccggaggctcgcgaatggtatcaccagtgggtttactttcgtccg
araC_JCat       cattacggccgtcatcctgaagctcgtgaatggtaccatcaatgggtttacttccgtcct
                ** ***** ******** ** ***** ******** ** ** *********** *****

araC            cgcgcctactggcatgaatggcttaactggccgtcaatatttgccaatacgggtttcttt
araC_JCat       cgtgcttactggcatgaatggcttaactggccttctatcttcgctaacacaggcttcttc
                ** ** ************************** ** ** ** ** ** ** ** *****

araC            cgcccggatgaagcgcaccagccgcatttcagcgacctgtttgggcaaatcattaacgcc
araC_JCat       cgtcctgatgaagctcatcaacctcatttctctgatcttttcggccaaatcatcaacgct
                ** ** ******** ** ** ** ******   ** ** ** ** ******** *****

araC            gggcaaggggaagggcgctattcggagctgctggcgataaatctgcttgagcaattgtta
araC_JCat       ggccaaggcgaaggccgttactctgaacttcttgctatcaaccttcttgaacaacttctt
                ** ***** ***** ** ** ** ** ** ** ** ** ** ** ***** *** *  *

araC            ctgcggcgcatggaagcgattaacgagtcgctccatccaccgatggataatcgggtacgc
araC_JCat       cttcgtcgtatggaagctatcaacgaatctcttcatcctcctatggataaccgtgttcgt
                ** ** ** ******** ** ***** ** ** ***** ** ******** ** ** **

araC            gaggcttgtcagtacatcagcgatcacctggcagacagcaattttgatatcgccagcgtc
araC_JCat       gaagcttgccaatacatctctgatcatcttgctgattctaacttcgatatcgcttctgtt
                ** ***** ** ******   ***** ** ** **    ** ** ********    **

araC            gcacagcatgtttgcttgtcgccgtcgcgtctgtcacatcttttccgccagcagttaggg
araC_JCat       gctcaacatgtttgcctttctccttctcgtctttctcatcttttccgtcaacaacttggc
                ** ** ********* * ** ** ** ***** ** ********** ** **  * **
```

```
araC           attagcgtcttaagctggcgcgaggaccaacgcattagtcaggcgaagctgcttttgagc
araC_JCat      atctctgttctttcttggcgtgaagatcaacgtatctctcaagctaaacttcttctttct
               **    **  *    ***** ** ** ***** **   *** ** ** ** *** *

araC           actacccggatgcctatcgccaccgtcggtcgcaatgttggttttgacgatcaactctat
araC_JCat      acaacacgtatgcctatcgctacagttggccgtaacgttggcttcgatgatcaactttac
               ** ** ** ********** ** ** ** ** ** ***** ** ** ******** **

araC           ttctcgcgagtatttaaaaaatgcaccggggccagcccgagcgagtttcgtgccggttgt
araC_JCat      ttctctcgtgttttcaaaaaatgcacaggcgcttctccttctgaattccgtgctggctgc
               ***** ** ** ** ********** ** **    **    ** ** ***** ** **

araC           gaagaaaaagtgaatgatgtagccgtcaagttgtcataa
araC_JCat      gaagaaaaagttaacgatgttgctgttaaactttcttaa
               ********** ** ***** ** ** **  * ** ***

CAI-Value of the original sequence:      0. 3346467659458933
CAI-Value of the optimized sequence:     1.0



CLUSTAL O(1.2.1) multiple sequence alignment

araC           MAEAQNDPLLPGYSFNAHLVAGLTPIEANGYLDFFIDRPLGMKGYILNLTIRGQGVVKNQ
araC_JCat      MAEAQNDPLLPGYSFNAHLVAGLTPIEANGYLDFFIDRPLGMKGYILNLTIRGQGVVKNQ
               ************************************************************

araC           GREFVCRPGDILLFPPGEIHHYGRHPEAREWYHQWVYFRPRAYWHEWLNWPSIFANTGFF
araC_JCat      GREFVCRPGDILLFPPGEIHHYGRHPEAREWYHQWVYFRPRAYWHEWLNWPSIFANTGFF
               ************************************************************

araC           RPDEAHQPHFSDLFGQIINAGQGEGRYSELLAINLLEQLLLRRMEAINESLHPPMDNRVR
araC_JCat      RPDEAHQPHFSDLFGQIINAGQGEGRYSELLAINLLEQLLLRRMEAINESLHPPMDNRVR
               ************************************************************

araC           EACQYISDHLADSNFDIASVAQHVCLSPSRLSHLFRQQLGISVLSWREDQRISQAKLLLS
araC_JCat      EACQYISDHLADSNFDIASVAQHVCLSPSRLSHLFRQQLGISVLSWREDQRISQAKLLLS
               ************************************************************

araC           TTRMPIATVGRNVGFDDQLYFSRVFKKCTGASPSEFRAGCEEKVNDVAVKLS*
araC_JCat      TTRMPIATVGRNVGFDDQLYFSRVFKKCTGASPSEFRAGCEEKVNDVAVKLS*
               **************************************************
```

ccdB$_{Ec}$ Optimization

```
CLUSTAL O(1.2.1) multiple sequence alignment

ccdB_Ec         atgcagtttaaggtttacacctataaaagagagagccgttatcgtctgtttgtggatgta
ccdB_Ec_JCat    atgcaattcaaagtttacacatacaaacgtgaatctcgttaccgtcttttcgttgatgtt
                ***** ** ** ******** ** *** * **    ***** ***** ** ** *****

ccdB_Ec         cagagtgatattattgacacgcccgggcgacggatggtgatccccctggccagtgcacgt
ccdB_Ec_JCat    caatctgatatcatcgatacacctggccgtcgtatggttatccctcttgcttctgctcgt
                **   ****** ** ** ** ** ** ** ** ***** ***** ** **   *** ***

ccdB_Ec         ctgctgtcagataaagtctcccgtgaactttacccggtggtgcatatcggggatgaaagc
ccdB_Ec_JCat    cttctttctgataaagtttctcgtgaactttaccctgttgttcatatcggcgatgaatct
                ** ** ** ******** ** ************** ** ** ******** ******

ccdB_Ec         tggcgcatgatgaccaccgatatggccagtgtgccggtctccgttatcggggaagaagtg
ccdB_Ec_JCat    tggcgtatgatgacaacagatatggcttctgttcctgtttctgttatcggcgaagaagtt
                ***** ******** ** ********   *** ** ** ** ******** ********

ccdB_Ec         gctgatctcagccaccgcgaaaatgacatcaaaaacgccattaacctgatgttctgggga
ccdB_Ec_JCat    gctgatctttctcatcgtgaaaacgatatcaaaaacgctatcaaccttatgttctggggc
                ********    ** ** ***** ** *********** ** ***** ***********

ccdB_Ec         atataa
ccdB_Ec_JCat    atctaa
                ** ***

CAI-Value of the original sequence:      0.31874048632321195
CAI-Value of the optimized sequence:     1.0



CLUSTAL O(1.2.1) multiple sequence alignment

CcdB_Ec         MQFKVYTYKRESRYRLFVDVQSDIIDTPGRRMVIPLASARLLSDKVSRELYPVVHIGDES
CcdB_Ec_JCat    MQFKVYTYKRESRYRLFVDVQSDIIDTPGRRMVIPLASARLLSDKVSRELYPVVHIGDES
                ************************************************************

CcdB_Ec         WRMMTTDMASVPVSVIGEEVADLSHRENDIKNAINLMFWGI*
CcdB_Ec_JCat    WRMMTTDMASVPVSVIGEEVADLSHRENDIKNAINLMFWGI*
                ******************************************
```

***cI* Optimization**

```
CLUSTAL O(1.2.1) multiple sequence alignment

cI           atgagcacaaaaaagaaaccattaacacaagagcagcttgaggacgcacgtcgccttaaa
cI_JCat      atgtctacaaaaaaaaaacctcttacacaagaacaacttgaagatgctcgtcgtcttaaa
             ***   ******** *****  * ******** ** ***** ** ** ***** ******

cI           gcaatttatgaaaaaaagaaaaatgaacttggcttatcccaggaatctgtcgcagacaag
cI_JCat      gctatctacgaaaaaaaaaaaaacgaacttggcctttctcaagaatctgttgctgataaa
             ** ** ** ******** ***** ********* * ** ** ******** ** ** **

cI           atggggatggggcagtcaggcgttggtgctttatttaatggcatcaatgcattaaatgct
cI_JCat      atgggcatgggccaatctggcgttggcgctcttttcaacggcatcaacgctcttaacgct
             ***** ***** ** ** ******** *** * ** ** ******** **  * ** ***

cI           tataacgccgcattgcttgcaaaaattctcaaagttagcgttgaagaatttagcccttca
cI_JCat      tacaacgctgctcttcttgctaaaatccttaaagtttctgttgaagaattctctccttct
             ** ***** **  * ***** ***** ** ******   ***********    *****

cI           atcgccagagaaatctacgagatgtatgaagcggttagtatgcagccgtcacttagaagt
cI_JCat      atcgctcgtgaaatctacgaaatgtacgaagctgtttctatgcaaccttctcttcgttct
             *****  * *********** ***** ***** ***  ****** ** ** *** *   *

cI           gagtatgagtaccctgttttttctcatgttcaggcagggatgttctcacctgagcttaga
cI_JCat      gaatacgaataccctgttttctctcatgttcaagctggcatgttctctcctgaacttcgt
             ** ** ** *********** *********** ** ** ******** ***** *** *

cI           acctttaccaaaggtgatgcggagagatgggtaagcacaaccaaaaaagccagtgattct
cI_JCat      acattcacaaaaggcgatgctgaacgttgggtttctacaacaaaaaaagcttctgattct
             ** ** ** ***** ***** **  * *****    ***** ********   *******

cI           gcattctggcttgaggttgaaggtaattccatgaccgcaccaacaggctccaagccaagc
cI_JCat      gctttctggcttgaagttgaaggcaactctatgacagctcctacaggctctaaaccttct
             ** *********** ******** ** ** ***** ** ** ******** ** **

cI           tttcctgacggaatgttaattctcgttgaccctgagcaggctgttgagccaggtgatttc
cI_JCat      ttccctgatggcatgcttatccttgttgatcctgaacaagctgttgaacctggcgatttc
             ** ***** ** *** * ** ** ***** ***** ** ******** ** ** ******

cI           tgcatagccagacttgggggtgatgagtttaccttcaagaaactgatcagggatagcggt
cI_JCat      tgcatcgctcgtcttggcggcgatgaattcacattcaaaaaacttatccgtgattctggc
             ***** **  * ***** ** ***** ** ** ***** ***** *** * ***   **

cI           caggtgtttttacaaccactaaacccacagtacccaatgatcccatgcaatgagagttgt
cI_JCat      caagttttccttcaacctcttaaccctcaataccctatgatcccttgcaacgaatcttgc
             ** ** **  * ***** ** ***** ** ***** ******** ***** **   ***

cI           tccgttgtggggaaagttatcgctagtcagtggcctgaagagacgtttggctga
cI_JCat      tctgttgttggcaaagttatcgcttctcaatggcctgaagaaacattcggctaa
             ** ***** ** *************  *** ************ ** ** **** *

CAI-Value of the original sequence:      0.4165948291720712
CAI-Value of the optimized sequence:     1.0
```

```
CLUSTAL O(1.2.1) multiple sequence alignment

cI           MSTKKKPLTQEQLEDARRLKAIYEKKKNELGLSQESVADKMGMGQSGVGALFNGINALNA
cI_JCat      MSTKKKPLTQEQLEDARRLKAIYEKKKNELGLSQESVADKMGMGQSGVGALFNGINALNA
             ************************************************************

cI           YNAALLAKILKVSVEEFSPSIAREIYEMYEAVSMQPSLRSEYEYPVFSHVQAGMFSPELR
cI_JCat      YNAALLAKILKVSVEEFSPSIAREIYEMYEAVSMQPSLRSEYEYPVFSHVQAGMFSPELR
             ************************************************************

cI           TFTKGDAERWVSTTKKASDSAFWLEVEGNSMTAPTGSKPSFPDGMLILVDPEQAVEPGDF
cI_JCat      TFTKGDAERWVSTTKKASDSAFWLEVEGNSMTAPTGSKPSFPDGMLILVDPEQAVEPGDF
             ************************************************************

cI           CIARLGGDEFTFKKLIRDSGQVFLQPLNPQYPMIPCNESCSVVGKVIASQWPEETFG*
cI_JCat      CIARLGGDEFTFKKLIRDSGQVFLQPLNPQYPMIPCNESCSVVGKVIASQWPEETFG*
             ************************************************************
```

kan^{R} Optimization

```
CLUSTAL O(1.2.1) multiple sequence alignment

kanR           atgattgaacaagatggattgcacgcaggttctccggccgcttgggtggagaggctattc
kanR_JCat      atgatcgaacaagatggccttcatgctggctctcctgctgcttgggttgaacgtcttttc
               ***** ***********  * ** ** ** ***** ** ******** **  * ** ***

kanR           ggctatgactgggcacaacagacaatcggctgctctgatgccgccgtgttccggctgtca
kanR_JCat      ggctacgattgggctcaacaaacaatcggctgctctgatgctgctgttttccgtctttct
               ***** ** ***** ***** ******************** ** ** ***** ** **

kanR           gcgcaggggcgcccggttctttttgtcaagaccgacctgtccggtgccctgaatgaactg
kanR_JCat      gctcaaggccgtcctgttcttttcgttaaaacagatctttctggcgctcttaacgaactt
               ** ** ** ** ** ******** ** ** ** ** ** ** ** ** ** ** *****

kanR           caagacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgcagctgtg
kanR_JCat      caagatgaagctgctcgtctttcttggcttgctacaacaggcgttccttgcgctgctgtt
               ***** ** ** ** ** ** ** ***** ** ** ** ************** *****

kanR           ctcgacgttgtcactgaagcgggaagggactggctgctattgggcgaagtgccggggcag
kanR_JCat      cttgatgttgttacagaagctggccgtgattggcttcttcttggcgaagttcctggccaa
               ** ** ***** ** ***** **  * ** ***** **  * ******** ** ** **

kanR           gatctcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgcaatg
kanR_JCat      gatcttctttcttctcatcttgctcctgctgaaaaagtttctatcatggctgatgctatg
               ***** ** ** ***** ********** ** ***** ** ************** ***

kanR           cggcggctgcatacgcttgatccggctacctgcccattcgaccaccaagcgaaacatcgc
kanR_JCat      cgtcgtcttcatacacttgatcctgctacatgccctttcgatcatcaagctaaacatcgt
               ** ** ** ***** ******** ***** ***** ***** ** ***** ********

kanR           atcgagcgagcacgtactcggatggaagccggtcttgtcgatcaggatgatctggacgaa
kanR_JCat      atcgaacgtgctcgtacacgtatggaagctggccttgttgatcaagatgatcttgatgaa
               ***** ** ** ***** ** ******** ** ***** ***** ******** ** ***

kanR           gagcatcaggggctcgcgccagccgaactgttcgccaggctcaaggcgagcatgcccgac
kanR_JCat      gaacatcaaggccttgctcctgctgaacttttcgctcgtcttaaagcttctatgcctgat
               ** ***** ** ** ** ** ** ***** *****  * ** ** **    ***** **

kanR           ggcgaggatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaat
kanR_JCat      ggcgaagatcttgttgttacacatggcgatgcttgccttcctaacatcatggttgaaaac
               ***** ***** ** ** ** ********** *** * ** ** ******** *****

kanR           ggccgcttttctggattcatcgactgtggccggctgggtgtggcggaccgctatcaggac
kanR_JCat      ggccgtttctctggcttcatcgattgcggccgtcttggcgttgctgatcgttaccaagat
               ***** ** ***** ******** ** ***** ** ** ** ** ** ** ** ** **

kanR           atagcgttggctacccgtgatattgctgaagagcttggcggcgaatgggctgaccgcttc
kanR_JCat      atcgctcttgctacacgtgatatcgctgaagaacttggcggcgaatgggctgatcgtttc
               ** **  * ***** ******** ******** ****************** ** ***

kanR           ctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttctatcgccttctt
kanR_JCat      cttgttctttacggcatcgctgctcctgattctcaacgtatcgctttctaccgtcttctt
               ** ** ******** ***** ***** ***** ** ** ***** ***** ** ******

kanR           gacgagttcttctga
kanR_JCat      gatgaattcttctaa
               ** ** ******* *
```

```
CAI-Value of the original sequence:      0.3467943049171567
CAI-Value of the optimized sequence:     1.0


CLUSTAL O(1.2.1) multiple sequence alignment

KanR            MIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNEL
KanR_JCat       MIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNEL
                ************************************************************

KanR            QDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHLAPAEKVSIMADAM
KanR_JCat       QDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHLAPAEKVSIMADAM
                ************************************************************

KanR            RRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKASMPD
KanR_JCat       RRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKASMPD
                ************************************************************

KanR            GEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWADRF
KanR_JCat       GEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWADRF
                ************************************************************

KanR            LVLYGIAAPDSQRIAFYRLLDEFF*
KanR_JCat       LVLYGIAAPDSQRIAFYRLLDEFF*
                *************************
```

***lysB4* Optimization**

```
CLUSTAL O(1.2.1) multiple sequence alignment

lysB4           atggcaatggcattacaaactttaatcgacaaggcgaaccgtaaattgaacgtttctggt
lysB4_JCat      atggctatggctcttcaaacacttatcgataaagctaaccgtaaacttaacgtttctggc
                ***** *****  * *****  * ***** ** ** ********* * ***********

lysB4           atgcgtaaggacgtagcagaccgtacccgcgctgtcattacacaaatgcatgcacaaggt
lysB4_JCat      atgcgtaaagatgttgctgatcgtacacgtgctgttatcacacaaatgcatgctcaaggc
                ******** ** ** ** ** ***** ** ***** ** ************** *****

lysB4           atttatatctgtgtagcacaaggtttccgttcgtttgctgaacagaacgctttatacgcg
lysB4_JCat      atctacatctgcgttgctcaaggcttccgttctttcgctgaacaaaacgctctttacgct
                ** ** ***** ** ** ***** ******** ** ******** ****** * *****

lysB4           caaggtcgtactaaaccgggtagcatcgtaacaaatgcacgaggcggacaatcgaaccac
lysB4_JCat      caaggccgtacaaaacctggctctatcgttacaaacgctcgtggcggccaatctaaccat
                ***** ***** ***** **    ***** ***** ** ** ***** ***** *****

lysB4           aactacggagtagcggtagacttatgcttgtacacacaagacggttctgacgttatctgg
lysB4_JCat      aactacggcgttgctgttgatctttgcctttacacacaagatggctctgatgttatctgg
                ******** ** ** ** **  * *** * ********** ** ***** *********

lysB4           acagttgaaggtaatttccgtaaggttatcgcagcaatgaaagcacaaggcttcaaatgg
lysB4_JCat      acagttgaaggcaacttccgtaaagttatcgctgctatgaaagctcaaggcttcaaatgg
                *********** ** ******** ******** ** ******** ***************

lysB4           ggcggagattgggtttcatttaaagattaccctcactttgaattgtacgatgtagtaggc
lysB4_JCat      ggcggcgattgggtttctttcaaagattaccctcatttcgaactttacgatgttgttggc
                ***** *********** ** ************** ** *** * ******** ** ***

lysB4           ggacaaaaaccacctgcggataatggcggtgcagtagataacggtggaggctctggtagc
lysB4_JCat      ggccaaaaacctcctgctgataacggcggcgctgttgataacggcggcggctctggctct
                ** ******** ***** ***** ***** ** ** ******** ** ********

lysB4           acaggcggttctggcggaggaagtacaggaggtggctctacaggtggaggttacgattct
lysB4_JCat      acaggcggctctggcggcggctctacaggcggcggctctacaggcggcggctacgattct
                ******** ******** **   ****** ** *********** ** ** *********

lysB4           agctggtttacaaaagagactggtactttcgtaacaaatacttcaatcaaattacgtaca
lysB4_JCat      tcttggttcacaaaagaaacaggcacattcgttacaaacacatctatcaaacttcgtaca
                   ***** ******** ** ** ** ***** ***** ** ** ****** * ******

lysB4           gcaccattcacaagtgcagacgtaatcgctacacttccggctggttctccagttaactac
lysB4_JCat      gctcctttcacatctgctgatgttatcgctacacttcctgctggctctcctgttaactac
                ** ** ******  *** ** ** ************** ***** ***** *********

lysB4           aatggcttcggtatcgaatatgatggttacgtttggattcgtcaaccacgtagcaatggt
lysB4_JCat      aacggcttcggcatcgaatacgatggctacgtttggatccgtcaacctcgttctaacggc
                ** ******** ******** ***** *********** ******** ***   ** **

lysB4           tacggctatcttgctacaggtgaatctaaaggcggaaaacgtcagaactactggggtacg
lysB4_JCat      tacggctaccttgctacaggcgaatctaaaggcggcaaacgtcaaaactactggggcaca
                ******** *********** ************** ******** *********** **

lysB4           ttcaaataa
lysB4_JCat      ttcaaataa
                *********
```

```
CAI-Value of the original sequence:      0.6925778600099153
CAI-Value of the optimized sequence:     1.0


CLUSTAL O(1.2.1) multiple sequence alignment

LysB4           MAMALQTLIDKANRKLNVSGMRKDVADRTRAVITQMHAQGIYICVAQGFRSFAEQNALYA
LysB4_JCat      MAMALQTLIDKANRKLNVSGMRKDVADRTRAVITQMHAQGIYICVAQGFRSFAEQNALYA
                ************************************************************

LysB4           QGRTKPGSIVTNARGGQSNHNYGVAVDLCLYTQDGSDVIWTVEGNFRKVIAAMKAQGFKW
LysB4_JCat      QGRTKPGSIVTNARGGQSNHNYGVAVDLCLYTQDGSDVIWTVEGNFRKVIAAMKAQGFKW
                ************************************************************

LysB4           GGDWVSFKDYPHFELYDVVGGQKPPADNGGAVDNGGGSGSTGGSGGGSTGGGSTGGGYDS
LysB4_JCat      GGDWVSFKDYPHFELYDVVGGQKPPADNGGAVDNGGGSGSTGGSGGGSTGGGSTGGGYDS
                ************************************************************

LysB4           SWFTKETGTFVTNTSIKLRTAPFTSADVIATLPAGSPVNYNGFGIEYDGYVWIRQPRSNG
LysB4_JCat      SWFTKETGTFVTNTSIKLRTAPFTSADVIATLPAGSPVNYNGFGIEYDGYVWIRQPRSNG
                ************************************************************

LysB4           YGYLATGESKGGKRQNYWGTFK*
LysB4_JCat      YGYLATGESKGGKRQNYWGTFK*
                **********************
```

***nahR* Optimization**

```
CLUSTAL O(1.2.1) multiple sequence alignment

nahR           atggaactgcgtgacctggatttaaacctgctggtggtgttcaaccagttgctggtcgac
nahR_JCat      atggaacttcgtgatcttgatcttaaccttcttgttgttttcaaccaacttcttgttgat
               ******** ***** ** *** * ***** ** ** ** ********  * ** ** **

nahR           agacgcgtctctatcactgcggagaacctgggcctgacccagcctgccgtgagcaatgcg
nahR_JCat      cgtcgtgtttctatcacagctgaaaaccttggccttacacaacctgctgtttctaacgct
                * ** ** ******** ** ** ***** ***** ** ** ***** **    ** **

nahR           ctgaaacgcctgcgcacctcgctacaggacccactcttcgtgcgcacacatcagggaatg
nahR_JCat      cttaaacgtcttcgtacatctcttcaagatcctcttttcgttcgtacacatcaaggcatg
               ** ***** ** ** ** ** ** ** ** ** ** ***** ** ******** ** ***

nahR           gaacccacaccctatgccgcgcatctggccgagcccgtcacttcggccatgcacgcactg
nahR_JCat      gaacctacaccttacgctgctcatcttgctgaacctgttacatctgctatgcatgctctt
               ***** ***** ** ** ** ***** ** ** ** ** ** ** ** ***** ** **

nahR           cgcaacgccctacagcaccatgaaagcttcgatccgctgaccagcgagcgtaccttcacc
nahR_JCat      cgtaacgctcttcaacatcatgaatctttcgatcctcttacatctgaacgtacattcaca
               ** ***** ** ** ** ******   ******** ** **    ** ***** *****

nahR           ctggccatgaccgacattggcgagatctacttcatgccgcggctgatggatgtgctggct
nahR_JCat      cttgctatgacagatatcggcgaaatctacttcatgcctcgtcttatggatgttcttgct
               ** ** ***** ** ** ***** ************** ** ** ******** ** ***

nahR           caccaggcccccaattgcgtgatcagtacggtgcgcgacagttcgatgagcctgatgcag
nahR_JCat      catcaagctcctaactgcgttatctctacagttcgtgattcttctatgtctcttatgcaa
               ** ** ** ** ** ***** ***  *** ** ** **   *** ***   ** *****

nahR           gccttgcagaacggaaccgtggacttggccgtgggcctgcttcccaatctgcaaactggc
nahR_JCat      gctcttcaaaacggcacagttgatcttgctgttggccttcttcctaaccttcaaacaggc
               **  * ** ***** ** ** **  * ** ** ***** ***** ** ** ***** ***

nahR           ttctttcagcgccggctgctccagaatcactacgtgtgcctatgtcgcaaggaccatcca
nahR_JCat      ttcttccaacgtcgtcttcttcaaaaccattacgtttgcctttgccgtaaagatcatcct
               ***** ** ** ** ** ** ** ** ** ***** ***** ** ** ** ** *****

nahR           gtcacccgcgaacccctgactctggagcgcttctgttcctacggccacgtgcgtgtcatc
nahR_JCat      gttacacgtgaacctcttacacttgaacgtttctgctcttacggccatgttcgtgttatc
               ** ** ** ***** ** ** ** ** ** ***** ** ******** ** ***** ***

nahR           gccgctggcaccggccacggcgaggtggacacgtacatgacacgggtcggcatccggcgc
nahR_JCat      gctgctggcacaggccatggcgaagttgatacatacatgacacgtgttggcatccgtcgt
               ** ******** ***** ***** ** ** ** ************ ** ******** **

nahR           gacatccgtctggaagtgccgcacttcgccgccgttggccacatcctccagcgcaccgat
nahR_JCat      gatatccgtcttgaagttcctcatttcgctgctgttggccatatccttcaacgtacagat
               ** ******** ***** ** ** ***** ** ******** ***** ** ** ** ***

nahR           ctgctcgccactgtgccgatacgtttagccgactgctgcgtggagcccttcggcctaagc
nahR_JCat      cttcttgctacagttcctatccgtcttgctgattgctgcgttgaacctttcggcctttct
               ** ** ** ** ** ** ** *** * ** ** ******** ** ** ********

nahR           gccttgccgcacccagtcgtcttgcctgaaatagccatcaacatgttctggcatgcgaag
nahR_JCat      gctcttcctcatcctgttgttcttcctgaaatcgctatcaacatgttctggcatgctaaa
               **  * ** ** ** ** **  * ******** ** ********************
```

```
nahR            taccacaaggacctagccaatatttggttgcggcaactgatgtttgacctgtttacggat
nahR_JCat       taccataaagatcttgctaacatctggcttcgtcaacttatgttcgatcttttcacagat
                ***** ** ** ** ** ** ** *** * ** ***** ***** ** ** ** ** ***

nahR            tga
nahR_JCat       taa
                * *

CAI-Value of the original sequence:      0.27815302026898114
CAI-Value of the optimized sequence:     1.0



CLUSTAL O(1.2.1) multiple sequence alignment

nahR            MELRDLDLNLLVVFNQLLVDRRVSITAENLGLTQPAVSNALKRLRTSLQDPLFVRTHQGM
nahR_JCat       MELRDLDLNLLVVFNQLLVDRRVSITAENLGLTQPAVSNALKRLRTSLQDPLFVRTHQGM
                ************************************************************

nahR            EPTPYAAHLAEPVTSAMHALRNALQHHESFDPLTSERTFTLAMTDIGEIYFMPRLMDVLA
nahR_JCat       EPTPYAAHLAEPVTSAMHALRNALQHHESFDPLTSERTFTLAMTDIGEIYFMPRLMDVLA
                ************************************************************

nahR            HQAPNCVISTVRDSSMSLMQALQNGTVDLAVGLLPNLQTGFFQRRLLQNHYVCLCRKDHP
nahR_JCat       HQAPNCVISTVRDSSMSLMQALQNGTVDLAVGLLPNLQTGFFQRRLLQNHYVCLCRKDHP
                ************************************************************

nahR            VTREPLTLERFCSYGHVRVIAAGTGHGEVDTYMTRVGIRRDIRLEVPHFAAVGHILQRTD
nahR_JCat       VTREPLTLERFCSYGHVRVIAAGTGHGEVDTYMTRVGIRRDIRLEVPHFAAVGHILQRTD
                ************************************************************

nahR            LLATVPIRLADCCVEPFGLSALPHPVVLPEIAINMFWHAKYHKDLANIWLRQLMFDLFTD
nahR_JCat       LLATVPIRLADCCVEPFGLSALPHPVVLPEIAINMFWHAKYHKDLANIWLRQLMFDLFTD
                ************************************************************

nahR            *
nahR_JCat       *
                *
```

revTetR^{r2} **Optimization**

```
CLUSTAL O(1.2.1) multiple sequence alignment

revTetR         atgtcaagacttgataaatcaaaagttattaattcagcacttgcacttggaaatgaagtt
revTetR_JCat    atgtctcgtcttgataaatctaaagttatcaactctgctcttgctcttggcaacgaagtt
                *****  * *********** ******** ** ** ** ***** ***** ** ******

revTetR         ggaattgaaggagttacaacaagaaaacttgcacaaaaacttggagttgaacaaccgaca
revTetR_JCat    ggcatcgaaggcgttacaacacgtaaacttgctcaaaaacttggcgttgaacaacctaca
                ** ** ***** ********* * ******** *********** *********** ***

revTetR         ctttattggcatgttaaaaataaaagagcacttcttgatgcacttgcagttgaaattctt
revTetR_JCat    ctttactggcatgttaaaaacaaacgtgctcttcttgatgctcttgctgttgaaatcctt
                ***** *************** *** * ** *********** ***** ******** ***

revTetR         gcaagacatcatgattattcacttccggcagcaggagaatcatggcaatcatttcttaga
revTetR_JCat    gctcgtcatcatgattactctcttcctgctgctggcgaatcttggcaatctttccttcgt
                **  * *********** ** ***** ** ** ** ***** ******** ** *** *

revTetR         aataatgcaatgtcatttagaagagcacttcttagatatagagatggagcaaaagttcat
revTetR_JCat    aacaacgctatgtctttccgtcgtgctcttcttcgttaccgtgatggcgctaaagttcat
                ** ** ** ***** **  *  * ** ****** * **  * ***** ** *********

revTetR         cttggaacaagaccggatgaaaaacaatatgatacagttgaaacacaacttagatttatg
revTetR_JCat    cttggcacacgtcctgatgaaaaacaatacgatacagttgaaacacaacttcgtttcatg
                ***** *** * ** ************** ********************** * ** ***

revTetR         acagaaaatggattttcacttagagatggactttatgcaatttcagcagtttcacatttt
revTetR_JCat    acagaaaacggcttctctcttcgtgatggcctttacgctatctctgctgtttctcatttc
                ******** ** ** ** *** * ***** ***** ** ** ** ** ***** *****

revTetR         acacttggagcagttcttgaacaacaagaacatacagcagcacttacagatagaccggca
revTetR_JCat    acacttggcgctgttcttgaacaacaagaacatacagctgctcttacagatcgtcctgct
                ******** ** ************************** ** ********* * ** **

revTetR         gcaccggatgaaaatcttccgccgcttcttagagaagcacttcaaattatggattcagat
revTetR_JCat    gctcctgatgaaaaccttcctcctcttcttcgtgaagctcttcaaatcatggattctgat
                ** ** ******** ***** ** ****** * ***** ******** ******** ***

revTetR         gatggagaacaagcatttcttcatggacttgaatcacttattagaggatttgaagttcaa
revTetR_JCat    gatggcgaacaagctttccttcatggccttgaatctcttatccgtggcttcgaagttcaa
                ***** ******** ** ******** ******** *****  * ** ** *********

revTetR         cttacagcacttcttcaaattgttggaggagataaacttattattccgttttgc
revTetR_JCat    cttacagctcttcttcaaatcgttggcggcgataaacttatcatccctttctgc
                ******** ************ ***** ** ************ ** ** ** ***

CAI-Value of the original sequence:     0.6737319798271094
CAI-Value of the optimized sequence:    1.0



CLUSTAL O(1.2.1) multiple sequence alignment

RevTetR         MSRLDKSKVINSALALGNEVGIEGVTTRKLAQKLGVEQPTLYWHVKNKRALLDALAVEIL
RevTetR_JCat    MSRLDKSKVINSALALGNEVGIEGVTTRKLAQKLGVEQPTLYWHVKNKRALLDALAVEIL
                ************************************************************
```

```
RevTetR          ARHHDYSLPAAGESWQSFLRNNAMSFRRALLRYRDGAKVHLGTRPDEKQYDTVETQLRFM
RevTetR_JCat     ARHHDYSLPAAGESWQSFLRNNAMSFRRALLRYRDGAKVHLGTRPDEKQYDTVETQLRFM
                 ************************************************************

RevTetR          TENGFSLRDGLYAISAVSHFTLGAVLEQQEHTAALTDRPAAPDENLPPLLREALQIMDSD
RevTetR_JCat     TENGFSLRDGLYAISAVSHFTLGAVLEQQEHTAALTDRPAAPDENLPPLLREALQIMDSD
                 ************************************************************

RevTetR          DGEQAFLHGLESLIRGFEVQLTALLQIVGGDKLIIPFC
RevTetR_JCat     DGEQAFLHGLESLIRGFEVQLTALLQIVGGDKLIIPFC
                 ***************************************
```

The sequences highlighted in green indicate the locations of the E15A, L17G, and L25V mutations that alter the functionality of the TetR protein, resulting in revTetR. (See Chapter 5.3.4.1.) This shows that the revTetR mutation is conserved after optimization.

***spoIISA* Optimization**

```
CLUSTAL O(1.2.1) multiple sequence alignment

spoIISA         atggttttattctttcagatcatggtctggtgcatcgtggccggactggggttatacgtg
spoIISA_JCat    atggttcttttcttccaaatcatggtttggtgcatcgttgctggccttggcctttacgtt
                ****** * ***** ** ******** ********** ** ** ** **  * *****

spoIISA         tatgccacgtggcgtttcgaagcgaaggtcaaagaaaaaatgtccgccattcggaaaact
spoIISA_JCat    tacgctacatggcgtttcgaagctaaagttaaagaaaaaatgtctgctatccgtaaaaca
                ** ** ** ************** ** ** ************** ** ** ** *****

spoIISA         tggtatttgctgtttgttctgggcgctatggtatactggacatatgagcccacttcccta
spoIISA_JCat    tggtaccttcttttcgttcttggcgctatggtttactggacatacgaacctacatctctt
                *****  * ** ** ***** ********** ********** ** ** ** ** **

spoIISA         tttacccactgggaacggtatctcattgtcgcagtcagttttgctttgattgatgctttt
spoIISA_JCat    ttcacacattgggaacgttaccttatcgttgctgtttctttcgctcttatcgatgctttc
                ** ** ** ******** ** ** ** ** ** **   *** *** * ** ********

spoIISA         atcttcttaagtgcatatgtcaaaaaactggccggcagcgagcttgaaacagacacaaga
spoIISA_JCat    atcttcctttctgcttacgttaaaaaacttgctggctctgaacttgaaacagatacacgt
                ****** *   *** ** ** ******** ** ***   ** ********** *** *

spoIISA         gaaattcttgaagaaaacaacgaaatgctccacatgtatctcaatcggctgaaaacatac
spoIISA_JCat    gaaatccttgaagaaaacaacgaaatgcttcatatgtaccttaaccgtcttaaaacatac
                ***** ********************** ** ***** ** ** ** ** *********

spoIISA         caatacctattgaaaaacgaaccgatccatgtttattatggaagtatagatgcttatgct
spoIISA_JCat    caataccttcttaaaaacgaacctatccatgtttactacggctctatcgatgcttacgct
                ********  * ********** ********** ** **   *** ******** ***

spoIISA         gaaggtattgataagctgctgaaaacctatgctgataaaatgaacttaacggcttctctt
spoIISA_JCat    gaaggcatcgataaacttcttaaaacatacgctgataaaatgaaccttacagcttctctt
                ***** ** ***** ** ** ***** ** *************** * ** *********

spoIISA         tgccactattcgacacaggctgataaagaccggttaaccgagcatatggatgatccggca
spoIISA_JCat    tgccattactctacacaagctgataaagatcgtcttacagaacatatggatgatcctgct
                ***** ** ** ***** ********** **  * ** ** ************** **

spoIISA         gatgtacaaacacggctcgatcgaaaggatgtttattacgaccaatacggaaaagtggtt
spoIISA_JCat    gatgttcaaacacgtcttgatcgtaaagatgtttactacgatcaatacggcaaagttgtt
                ***** ******** ** ***** ** ******** ***** ******** ***** ***

spoIISA         ctcatccctttaccatcgagacacagaactatgtcatcaagctgacgtctgacagcatt
spoIISA_JCat    cttatccctttcacaatcgaaacacaaaactacgttatcaaacttacatctgattctatc
                ** ******** ** ***** ***** ***** ** ***** ** ** *****    **

spoIISA         gtcacggaatttgattatttgctatttacgtcattaacgagcatatatgatttggtgctg
spoIISA_JCat    gttacagaattcgattaccttcttttcacatctcttacatctatctacgatcttgttctt
                ** ** ***** *****  * ** ** ** **  * **    ** ** *** * ** **

spoIISA         ccaattgaggaggaaggtgaaggataa
spoIISA_JCat    cctatcgaagaagaaggcgaaggctaa
                ** ** ** ** ***** ***** ***

CAI-Value of the original sequence:      0.3803765799066098
CAI-Value of the optimized sequence:     1.0
```

```
CLUSTAL O(1.2.1) multiple sequence alignment

SpoIISA         MVLFFQIMVWCIVAGLGLYVYATWRFEAKVKEKMSAIRKTWYLLFVLGAMVYWTYEPTSL
SpoIISA_JCat    MVLFFQIMVWCIVAGLGLYVYATWRFEAKVKEKMSAIRKTWYLLFVLGAMVYWTYEPTSL
                ************************************************************

SpoIISA         FTHWERYLIVAVSFALIDAFIFLSAYVKKLAGSELETDTREILEENNEMLHMYLNRLKTY
SpoIISA_JCat    FTHWERYLIVAVSFALIDAFIFLSAYVKKLAGSELETDTREILEENNEMLHMYLNRLKTY
                ************************************************************

SpoIISA         QYLLKNEPIHVYYGSIDAYAEGIDKLLKTYADKMNLTASLCHYSTQADKDRLTEHMDDPA
SpoIISA_JCat    QYLLKNEPIHVYYGSIDAYAEGIDKLLKTYADKMNLTASLCHYSTQADKDRLTEHMDDPA
                ************************************************************

SpoIISA         DVQTRLDRKDVYYDQYGKVVLIPFTIETQNYVIKLTSDSIVTEFDYLLFTSLTSIYDLVL
SpoIISA_JCat    DVQTRLDRKDVYYDQYGKVVLIPFTIETQNYVIKLTSDSIVTEFDYLLFTSLTSIYDLVL
                ************************************************************

SpoIISA         PIEEEGEG*
SpoIISA_JCat    PIEEEGEG*
                *********
```

***T7ptag* Optimization**

```
CLUSTAL O(1.2.1) multiple sequence alignment

T7ptag          atgaccatgattaccgtgcactagaataccattaacattgctaagaacgacttctctgac
T7ptag_JCat     atgacaatgatcacagttcattaaaacacaatcaacatcgctaaaaacgatttctctgat
T7ptag_reTAG    atgacaatgatcacagttcattagaacacaatcaacatcgctaaaaacgatttctctgat
                ***** ***** ** ** ** ** ** ** ** ***** ***** ***** ********

T7ptag          atcgaactggctgctatcccgttcaacactctggctgaccattacggtgagcgtttagct
T7ptag_JCat     atcgaacttgctgctatccctttcaacacacttgctgatcattacggcgaacgtcttgct
T7ptag_reTAG    atcgaacttgctgctatccctttcaacacacttgctgatcattacggcgaacgtcttgct
                ******** *********** ******** ** ***** ******** ** *** * ***

T7ptag          cgcgaacagttggcccttgagcatgagtcttacgagatgggtgaagcacgcttccgcaag
T7ptag_JCat     cgtgaacaacttgctcttgaacatgaatcttacgaaatgggcgaagctcgtttccgtaaa
T7ptag_reTAG    cgtgaacaacttgctcttgaacatgaatcttacgaaatgggcgaagctcgtttccgtaaa
                ** *****  * ** ***** ***** ******** ***** ***** ** ***** **

T7ptag          atgtttgagcgtcaacttaaagctggtgaggttgcggataacgctgccgccaagcctctc
T7ptag_JCat     atgttcgaacgtcaacttaaagctggcgaagttgctgataacgctgctgctaaacctctt
T7ptag_reTAG    atgttcgaacgtcaacttaaagctggcgaagttgctgataacgctgctgctaaacctctt
                ***** ** ***************** ** ***** *********** ** ** *****

T7ptag          atcactaccctactccctaagatgattgcacgcatcaacgactggtttgaggaagtgaaa
T7ptag_JCat     atcacaacacttcttcctaaaatgatcgctcgtatcaacgattggttcgaagaagttaaa
T7ptag_reTAG    atcacaacacttcttcctaaaatgatcgctcgtatcaacgattggttcgaagaagttaaa
                ***** ** ** ** ***** ***** ** ** ******** ***** ** ***** ***

T7ptag          gctaagcgcggcaagcgcccgacagccttccagttcctgtaggaaatcaagccggaagcc
T7ptag_JCat     gctaaacgtggcaaacgtcctacagctttccaattccttttagaaatcaaacctgaagct
T7ptag_reTAG    gctaaacgtggcaaacgtcctacagctttccaattccttttaggaaatcaaacctgaagct
                ***** ** ***** ** ** ***** ***** ***** ** ******** ** *****

T7ptag          gtagcgtacatcaccattaagaccactctggcttgcctaaccagtgctgacaatacaacc
T7ptag_JCat     gttgcttacatcacaatcaaaacaacacttgcttgccttacatctgctgataacacaaca
T7ptag_reTAG    gttgcttacatcacaatcaaaacaacacttgcttgccttacatctgctgataacacaaca
                ** ** ******** ** ** ** ** ** ******** **   ****** ** *****

T7ptag          gttcaggctgtagcaagcgcaatcggtcgggccattgaggacgaggctcgcttcggtcgt
T7ptag_JCat     gttcaagctgttgcttctgctatcggccgtgctatcgaagatgaagctcgtttcggccgt
T7ptag_reTAG    gttcaagctgttgcttctgctatcggccgtgctatcgaagatgaagctcgtttcggccgt
                ***** ***** **    ** ***** ** ** ** ** ** ** ***** ***** ***

T7ptag          atccgtgaccttgaagctaagcacttcaagaaaaacgttgaggaacaactcaacaagcgc
T7ptag_JCat     atccgtgatcttgaagctaaacatttcaaaaaaaacgttgaagaacaacttaacaaacgt
T7ptag_reTAG    atccgtgatcttgaagctaaacatttcaaaaaaaacgttgaagaacaacttaacaaacgt
                ******** *********** ** ***** *********** ******** ***** **

T7ptag          gtagggcacgtctacaagaaagcatttatgcaagttgtcgaggctgacatgctctctaag
T7ptag_JCat     gttggccatgtttacaaaaaagctttcatgcaagttgttgaagctgatatgctttctaaa
T7ptag_reTAG    gttggccatgtttacaaaaaagctttcatgcaagttgttgaagctgatatgctttctaaa
                ** ** ** ** ***** ***** ** *********** ** ***** ***** *****

T7ptag          ggtctactcggtggcgaggcgtggtcttcgtggcataaggaagactctattcatgtagga
T7ptag_JCat     ggccttcttggcggcgaagcttggtcttcttggcataaagaagattctatccatgttggc
T7ptag_reTAG    ggccttcttggcggcgaagcttggtcttcttggcataaagaagattctatccatgttggc
                ** ** ** ** ***** ** ******** ******** ***** ***** ***** **
```

```
T7ptag          gtacgctgcatcgagatgctcattgagtcaaccggaatggttagcttacaccgccaaaat
T7ptag_JCat     gttcgttgcatcgaaatgcttatcgaatctacaggcatggtttctcttcatcgtcaaaac
T7ptag_reTAG    gttcgttgcatcgaaatgcttatcgaatctacaggcatggtttctcttcatcgtcaaaac
                ** ** ******** ***** ** ** ** ** ** ******    * ** ** *****

T7ptag          gctggcgtagtaggtcaagactctgagactatcgaactcgcacctgaatacgctgaggct
T7ptag_JCat     gctggcgttgttggccaagattctgaaacaatcgaacttgctcctgaatacgctgaagct
T7ptag_reTAG    gctggcgttgttggccaagattctgaaacaatcgaacttgctcctgaatacgctgaagct
                ******** ** ** ***** ***** ** ******** ** ************** ***

T7ptag          atcgcaacccgtgcaggtgcgctggctggcatctctccgatgttccaaccttgcgtagtt
T7ptag_JCat     atcgctacacgtgctggcgctcttgctggcatctctcctatgttccaaccttgcgttgtt
T7ptag_reTAG    atcgctacacgtgctggcgctcttgctggcatctctcctatgttccaaccttgcgttgtt
                ***** ** ***** ** ** ** ************** ***************** ***

T7ptag          cctcctaagccgtggactggcattactggtggtggctattgggctaacggtcgtcgtcct
T7ptag_JCat     cctcctaaaccttggacaggcatcacaggcggcggctactgggctaacggccgtcgtcct
T7ptag_reTAG    cctcctaaaccttggacaggcatcacaggcggcggctactgggctaacggccgtcgtcct
                ******** ** ***** ***** ** ** ** ***** *********** *********

T7ptag          ctggcgctggtgcgtactcacagtaagaaagcactgatgcgctacgaagacgtttacatg
T7ptag_JCat     cttgctcttgttcgtacacattctaaaaaagctcttatgcgttacgaagatgtttacatg
T7ptag_reTAG    cttgctcttgttcgtacacattctaaaaaagctcttatgcgttacgaagatgtttacatg
                ** ** ** ** ***** **   *** ***** ** ***** ******** *********

T7ptag          cctgaggtgtacaaagcgattaacattgcgcaaaacaccgcatggaaaatcaacaagaaa
T7ptag_JCat     cctgaagtttacaaagctatcaacatcgctcaaaacacagcttggaaaatcaacaaaaaa
T7ptag_reTAG    cctgaagtttacaaagctatcaacatcgctcaaaacacagcttggaaaatcaacaaaaaa
                ***** ** ******** ** ***** ** ******** ** ************** ***

T7ptag          gtcctagcggtcgccaacgtaatcaccaagtggaagcattgtccggtcgaggacatccct
T7ptag_JCat     gttcttgctgttgctaacgttatcacaaaatggaaacattgccctgttgaagatatccct
T7ptag_reTAG    gttcttgctgttgctaacgttatcacaaaatggaaacattgccctgttgaagatatccct
                ** ** ** ** ** ***** ***** ** ***** ***** ** ** ** ** ******

T7ptag          gcgattgagcgtgaagaactcccgatgaaaccggaagacatcgacatgaatcctgaggct
T7ptag_JCat     gctatcgaacgtgaagaacttcctatgaaacctgaagatatcgatatgaaccctgaagct
T7ptag_reTAG    gctatcgaacgtgaagaacttcctatgaaacctgaagatatcgatatgaaccctgaagct
                ** ** ** ************ ** ******** ***** ***** ***** ***** ***

T7ptag          ctcaccgcgtggaaacgtgctgccgctgctgtgtaccgcaaggacaaggctcgcaagtct
T7ptag_JCat     cttacagcttggaaacgtgctgctgctgctgtttaccgtaaagataaagctcgtaaatct
T7ptag_reTAG    cttacagcttggaaacgtgctgctgctgctgtttaccgtaaagataaagctcgtaaatct
                ** ** ** *************** ******** ***** ** ** ** ***** ** ***

T7ptag          cgccgtatcagccttgagttcatgcttgagcaagccaataagtttgctaaccataaggcc
T7ptag_JCat     cgtcgtatctctcttgaattcatgcttgaacaagctaacaaattcgctaaccataaagct
T7ptag_reTAG    cgtcgtatctctcttgaattcatgcttgaacaagctaacaaattcgctaaccataaagct
                ** *******   ***** *********** ***** ** ** ** *********** **

T7ptag          atctggttcccttacaacatggactggcgcggtcgtgtttacgctgtgtcaatgttcaac
T7ptag_JCat     atctggttcccttacaacatggattggcgtggccgtgtttacgctgtttctatgttcaac
T7ptag_reTAG    atctggttcccttacaacatggattggcgtggccgtgtttacgctgtttctatgttcaac
                ************************ ***** ** *************** ** *********

T7ptag          ccgcaaggtaacgatatgaccaaaggactgcttacgctggcgaaaggtaaaccaatcggt
T7ptag_JCat     cctcaaggcaacgatatgacaaaaggccttcttacacttgctaaaggcaaacctatcggc
T7ptag_reTAG    cctcaaggcaacgatatgacaaaaggccttcttacacttgctaaaggcaaacctatcggc
                ** ***** *********** ***** ** ***** ** ** ***** ***** *****
```

```
T7ptag          aaggaaggttactactggctgaaaatccacggtgcaaactgtgcgggtgtcgataaggtt
T7ptag_JCat     aaagaaggctactactggcttaaaatccatggcgctaactgcgctggcgttgataaagtt
T7ptag_reTAG    aaagaaggctactactggcttaaaatccatggcgctaactgcgctggcgttgataaagtt
                ** ***** *********** ******** ** ** ***** ** ** ** ***** ***

T7ptag          ccgttccctgagcgcatcaagttcattgaggaaaaccacgagaacatcatggcttgcgct
T7ptag_JCat     cctttccctgaacgtatcaaattcatcgaagaaaaccatgaaaacatcatggcttgcgct
T7ptag_reTAG    cctttccctgaacgtatcaaattcatcgaagaaaaccatgaaaacatcatggcttgcgct
                ** ******** ** ***** ***** ** ******** ** ******************

T7ptag          aagtctccactggagaacacttggtgggctgagcaagattctccgttctgcttccttgcg
T7ptag_JCat     aaatctcctcttgaaaacacatggtgggctgaacaagattctcctttctgcttccttgct
T7ptag_reTAG    aaatctcctcttgaaaacacatggtgggctgaacaagattctcctttctgcttccttgct
                ** ***** ** ** ***** *********** *********** **************

T7ptag          ttctgctttgagtacgctggggtacagcaccacggcctgagctataactgctcccttccg
T7ptag_JCat     ttctgcttcgaatacgctggcgttcaacatcatggcctttcttacaactgctctcttcct
T7ptag_reTAG    ttctgcttcgaatacgctggcgttcaacatcatggcctttcttacaactgctctcttcct
                ******** ** ******** ** ** ** ** *****    ** ******** *****

T7ptag          ctggcgtttgacgggtcttgctctggcatccagcacttctccgcgatgctccgagatgag
T7ptag_JCat     cttgctttcgatggctcttgctctggcatccaacatttctctgctatgcttcgtgatgaa
T7ptag_reTAG    cttgctttcgatggctcttgctctggcatccaacatttctctgctatgcttcgtgatgaa
                ** ** ** ** ** ***************** ** ***** ** ***** ** *****

T7ptag          gtaggtggtcgcgcggttaacttgcttcctagtgaaaccgttcaggacatctacgggatt
T7ptag_JCat     gttggcggccgtgctgttaaccttcttccttctgaaacagttcaagatatctacggcatc
T7ptag_reTAG    gttggcggccgtgctgttaaccttcttccttctgaaacagttcaagatatctacggcatc
                ** ** ** ** ** ****** * ******  ****** ***** ** ******** **

T7ptag          gttgctaagaaagtcaacgagattctacaagcagacgcaatcaatgggaccgataacgaa
T7ptag_JCat     gttgctaaaaaagttaacgaaatccttcaagctgatgctatcaacggcacagataacgaa
T7ptag_reTAG    gttgctaaaaaagttaacgaaatccttcaagctgatgctatcaacggcacagataacgaa
                ******** ***** ***** ** ** ***** ** ** ***** ** ** *********

T7ptag          gtagttaccgtgaccgatgagaacactggtgaaatctctgagaaagtcaagctgggcact
T7ptag_JCat     gttgttacagttacagatgaaaacacaggcgaaatctctgaaaaagttaaacttggcaca
T7ptag_reTAG    gttgttacagttacagatgaaaacacaggcgaaatctctgaaaaagttaaacttggcaca
                ** ***** ** ** ***** ***** ** ************ ***** ** ** *****

T7ptag          aaggcactggctggtcaatggctggcttacggtgttactcgcagtgtgactaagcgttca
T7ptag_JCat     aaagctcttgctggccaatggcttgcttacggcgttacacgttctgttacaaaacgttct
T7ptag_reTAG    aaagctcttgctggccaatggcttgcttacggcgttacacgttctgttacaaaacgttct
                ** ** ** ***** ******** ******** ***** **   *** ** ** *****

T7ptag          gtcatgacgctggcttacgggtccaaagagttcggcttccgtcaacaagtgctggaagat
T7ptag_JCat     gttatgacacttgcttacggctctaaagaattcggcttccgtcaacaagttcttgaagat
T7ptag_reTAG    gttatgacacttgcttacggctctaaagaattcggcttccgtcaacaagttcttgaagat
                ** ***** ** ******** ** ***** ******************** ** ******

T7ptag          accattcagccagctattgattccggcaagggtctgatgttcactcagccgaatcaggct
T7ptag_JCat     acaatccaacctgctatcgattctggcaaaggccttatgttcacacaacctaaccaagct
T7ptag_reTAG    acaatccaacctgctatcgattctggcaaaggccttatgttcacacaacctaaccaagct
                ** ** ** ** ***** ***** ***** ** ** ******** ** ** ** ** ***
```

```
T7ptag          gctggatacatggctaagctgatttgggaatctgtgagcgtgacggtggtagctgcggtt
T7ptag_JCat     gctggctacatggctaaacttatctgggaatctgtttctgttacagttgttgctgctgtt
T7ptag_reTAG    gctggctacatggctaaacttatctgggaatctgtttctgttacagttgttgctgctgtt
                ***** *********** ** ** ***********    ** ** ** ** ***** ***

T7ptag          gaagcaatgaactggcttaagtctgctgctaagctgctggctgctgaggtcaaagataag
T7ptag_JCat     gaagctatgaactggcttaaatctgctgctaaacttcttgctgctgaagttaaagataaa
T7ptag_reTAG    gaagctatgaactggcttaaatctgctgctaaacttcttgctgctgaagttaaagataaa
                ***** ************** *********** ** ** ******** ** ********

T7ptag          aagactggagagattcttcgcaagcgttgcgctgtgcattgggtaactcctgatggtttc
T7ptag_JCat     aaaacaggcgaaatccttcgtaaacgttgcgctgttcattgggttacacctgatggcttc
T7ptag_reTAG    aaaacaggcgaaatccttcgtaaacgttgcgctgttcattgggttacacctgatggcttc
                ** ** ** ** ** ***** ** *********** ******** ** ******** ***

T7ptag          cctgtgtggcaggaatacaagaagcctattcagacgcgcttgaacctgatgttcctcggt
T7ptag_JCat     cctgtttggcaagaatacaaaaaacctatccaaacacgtcttaaccttatgttccttggc
T7ptag_reTAG    cctgtttggcaagaatacaaaaaacctatccaaacacgtcttaaccttatgttccttggc
                ***** ***** ******** ** ***** ** ** **  * ***** ******** **

T7ptag          cagttccgcttacagcctaccattaacaccaacaaagatagcgagattgatgcacacaaa
T7ptag_JCat     caattccgtcttcaacctacaatcaacacaaacaaagattctgaaatcgatgctcataaa
T7ptag_reTAG    caattccgtcttcaacctacaatcaacacaaacaaagattctgaaatcgatgctcataaa
                ** *****  * ** ***** ** ***** *********   ** ** ***** ** ***

T7ptag          caggagtctggtatcgctcctaactttgtacacagccaagacggtagccaccttcgtaag
T7ptag_JCat     caagaatctggcatcgctcctaacttcgttcattctcaagatggctctcatcttcgtaaa
T7ptag_reTAG    caagaatctggcatcgctcctaacttcgttcattctcaagatggctctcatcttcgtaaa
                ** ** ***** ************** ** **    ***** **    ** ********

T7ptag          actgtagtgtgggcacacgagaagtacggaatcgaatcttttgcactgattcacgactcc
T7ptag_JCat     acagttgtttgggctcatgaaaaatacggcatcgaatctttcgctcttatccatgattct
T7ptag_reTAG    acagttgtttgggctcatgaaaaatacggcatcgaatctttcgctcttatccatgattct
                ** ** ** ***** ** ** ** ***** *********** ** ** ** ** ** **

T7ptag          ttcggtaccattccggctgacgctgcgaacctgttcaaagcagtgcgcgaaactatggtt
T7ptag_JCat     ttcggcacaatccctgctgatgctgctaacctttcaaagctgttcgtgaaacaatggtt
T7ptag_reTAG    ttcggcacaatccctgctgatgctgctaacctttcaaagctgttcgtgaaacaatggtt
                ***** ** ** ** ***** ***** ***** ******** ** ** ***** ******

T7ptag          gacacatatgagtcttgtgatgtactggctgatttctacgaccagttcgctgaccagttg
T7ptag_JCat     gatacatacgaatcttgcgatgttcttgctgatttctacgatcaattcgctgatcaactt
T7ptag_reTAG    gatacatacgaatcttgcgatgttcttgctgatttctacgatcaattcgctgatcaactt
                ** ***** ** ***** ***** ** ************** ** ******** **  *

T7ptag          cacgagtctcaattggacaaaatgccagcacttccggctaaaggtaacttgaacctccgt
T7ptag_JCat     catgaatctcaacttgataaaatgcctgctcttcctgctaaaggcaaccttaaccttcgt
T7ptag_reTAG    catgaatctcaacttgataaaatgcctgctcttcctgctaaaggcaaccttaaccttcgt
                ** ** ****** * ** ******** ** ***** ******** *** * ***** ***

T7ptag          gacatcttagagtcggacttcgcgttcgcataa
T7ptag_JCat     gatatccttgaatctgatttcgctttcgcttaa
T7ptag_reTAG    gatatccttgaatctgatttcgctttcgcttaa
                ** *** * ** ** ** ***** ***** ***

CAI-Value of the original sequence:      0.46596750257418346
CAI-Value of the optimized sequence:     1.0
CAI-Value of the reTAGed sequence:       1.0
```

The **tag** sequences highlighted in red for the T7ptag gene (Line 1) indicate the stop codons critical for the proper control of the AND gate using the amber suppressor. The a sequences highlighted in green for T7ptag_JCat (Line 2) indicate the nucleotides that were codon "optimized" by JCat by replacing the **tag** stop codon with the **taa** stop more common in *B. subtilis*. Because this would circumvent the intended activity of the T7ptag gene, the T7ptag_reTAG sequence (Line 3) shows the g nucleotides that were manually changed back to reconstruct the **tag** stop codon. (See Chapter 5.3.3.2.)

Entering the reTAGged sequence back into JCat results in a returned CAI of 1.0. This is because the equation for CAI excludes the initiation and stop codons.[433]

```
CLUSTAL O(1.2.1) multiple sequence alignment

T7ptag          MTMITVH*NTINIAKNDFSDIELAAIPFNTLADHYGERLAREQLALEHESYEMGEARFRK
T7ptag_JCat     MTMITVH*NTINIAKNDFSDIELAAIPFNTLADHYGERLAREQLALEHESYEMGEARFRK
T7ptag_reTAG    MTMITVH*NTINIAKNDFSDIELAAIPFNTLADHYGERLAREQLALEHESYEMGEARFRK
                ************************************************************

T7ptag          MFERQLKAGEVADNAAAKPLITTLLPKMIARINDWFEEVKAKRGKRPTAFQFL*EIKPEA
T7ptag_JCat     MFERQLKAGEVADNAAAKPLITTLLPKMIARINDWFEEVKAKRGKRPTAFQFL*EIKPEA
T7ptag_reTAG    MFERQLKAGEVADNAAAKPLITTLLPKMIARINDWFEEVKAKRGKRPTAFQFL*EIKPEA
                ************************************************************

T7ptag          VAYITIKTTLACLTSADNTTVQAVASAIGRAIEDEARFGRIRDLEAKHFKKNVEEQLNKR
T7ptag_JCat     VAYITIKTTLACLTSADNTTVQAVASAIGRAIEDEARFGRIRDLEAKHFKKNVEEQLNKR
T7ptag_reTAG    VAYITIKTTLACLTSADNTTVQAVASAIGRAIEDEARFGRIRDLEAKHFKKNVEEQLNKR
                ************************************************************

T7ptag          VGHVYKKAFMQVVEADMLSKGLLGGEAWSSWHKEDSIHVGVRCIEMLIESTGMVSLHRQN
T7ptag_JCat     VGHVYKKAFMQVVEADMLSKGLLGGEAWSSWHKEDSIHVGVRCIEMLIESTGMVSLHRQN
T7ptag_reTAG    VGHVYKKAFMQVVEADMLSKGLLGGEAWSSWHKEDSIHVGVRCIEMLIESTGMVSLHRQN
                ************************************************************

T7ptag          AGVVGQDSETIELAPEYAEAIATRAGALAGISPMFQPCVVPPKPWTGITGGGYWANGRRP
T7ptag_JCat     AGVVGQDSETIELAPEYAEAIATRAGALAGISPMFQPCVVPPKPWTGITGGGYWANGRRP
T7ptag_reTAG    AGVVGQDSETIELAPEYAEAIATRAGALAGISPMFQPCVVPPKPWTGITGGGYWANGRRP
                ************************************************************

T7ptag          LALVRTHSKKALMRYEDVYMPEVYKAINIAQNTAWKINKKVLAVANVITKWKHCPVEDIP
T7ptag_JCat     LALVRTHSKKALMRYEDVYMPEVYKAINIAQNTAWKINKKVLAVANVITKWKHCPVEDIP
T7ptag_reTAG    LALVRTHSKKALMRYEDVYMPEVYKAINIAQNTAWKINKKVLAVANVITKWKHCPVEDIP
                ************************************************************

T7ptag          AIEREELPMKPEDIDMNPEALTAWKRAAAAVYRKDKARKSRRISLEFMLEQANKFANHKA
T7ptag_JCat     AIEREELPMKPEDIDMNPEALTAWKRAAAAVYRKDKARKSRRISLEFMLEQANKFANHKA
T7ptag_reTAG    AIEREELPMKPEDIDMNPEALTAWKRAAAAVYRKDKARKSRRISLEFMLEQANKFANHKA
                ************************************************************

T7ptag          IWFPYNMDWRGRVYAVSMFNPQGNDMTKGLLTLAKGKPIGKEGYYWLKIHGANCAGVDKV
T7ptag_JCat     IWFPYNMDWRGRVYAVSMFNPQGNDMTKGLLTLAKGKPIGKEGYYWLKIHGANCAGVDKV
T7ptag_reTAG    IWFPYNMDWRGRVYAVSMFNPQGNDMTKGLLTLAKGKPIGKEGYYWLKIHGANCAGVDKV
                ************************************************************
```

[433] A Grote *et al.*, "JCat: a novel tool to adapt codon usage of a target gene to its potential expression host."

```
T7ptag          PFPERIKFIEENHENIMACAKSPLENTWWAEQDSPFCFLAFCFEYAGVQHHGLSYNCSLP
T7ptag_JCat     PFPERIKFIEENHENIMACAKSPLENTWWAEQDSPFCFLAFCFEYAGVQHHGLSYNCSLP
T7ptag_reTAG    PFPERIKFIEENHENIMACAKSPLENTWWAEQDSPFCFLAFCFEYAGVQHHGLSYNCSLP
                ************************************************************

T7ptag          LAFDGSCSGIQHFSAMLRDEVGGRAVNLLPSETVQDIYGIVAKKVNEILQADAINGTDNE
T7ptag_JCat     LAFDGSCSGIQHFSAMLRDEVGGRAVNLLPSETVQDIYGIVAKKVNEILQADAINGTDNE
T7ptag_reTAG    LAFDGSCSGIQHFSAMLRDEVGGRAVNLLPSETVQDIYGIVAKKVNEILQADAINGTDNE
                ************************************************************

T7ptag          VVTVTDENTGEISEKVKLGTKALAGQWLAYGVTRSVTKRSVMTLAYGSKEFGFRQQVLED
T7ptag_JCat     VVTVTDENTGEISEKVKLGTKALAGQWLAYGVTRSVTKRSVMTLAYGSKEFGFRQQVLED
T7ptag_reTAG    VVTVTDENTGEISEKVKLGTKALAGQWLAYGVTRSVTKRSVMTLAYGSKEFGFRQQVLED
                ************************************************************

T7ptag          TIQPAIDSGKGLMFTQPNQAAGYMAKLIWESVSVTVVAAVEAMNWLKSAAKLLAAEVKDK
T7ptag_JCat     TIQPAIDSGKGLMFTQPNQAAGYMAKLIWESVSVTVVAAVEAMNWLKSAAKLLAAEVKDK
T7ptag_reTAG    TIQPAIDSGKGLMFTQPNQAAGYMAKLIWESVSVTVVAAVEAMNWLKSAAKLLAAEVKDK
                ************************************************************

T7ptag          KTGEILRKRCAVHWVTPDGFPVWQEYKKPIQTRLNLMFLGQFRLQPTINTNKDSEIDAHK
T7ptag_JCat     KTGEILRKRCAVHWVTPDGFPVWQEYKKPIQTRLNLMFLGQFRLQPTINTNKDSEIDAHK
T7ptag_reTAG    KTGEILRKRCAVHWVTPDGFPVWQEYKKPIQTRLNLMFLGQFRLQPTINTNKDSEIDAHK
                ************************************************************

T7ptag          QESGIAPNFVHSQDGSHLRKTVVWAHEKYGIESFALIHDSFGTIPADAANLFKAVRETMV
T7ptag_JCat     QESGIAPNFVHSQDGSHLRKTVVWAHEKYGIESFALIHDSFGTIPADAANLFKAVRETMV
T7ptag_reTAG    QESGIAPNFVHSQDGSHLRKTVVWAHEKYGIESFALIHDSFGTIPADAANLFKAVRETMV
                ************************************************************

T7ptag          DTYESCDVLADFYDQFADQLHESQLDKMPALPAKGNLNLRDILESDFAFA*
T7ptag_JCat     DTYESCDVLADFYDQFADQLHESQLDKMPALPAKGNLNLRDILESDFAFA*
T7ptag_reTAG    DTYESCDVLADFYDQFADQLHESQLDKMPALPAKGNLNLRDILESDFAFA*
                **************************************************
```

The sequences highlighted in red indicate the locations of the stop codons introduced into the T7 polymerase gene to create *T7ptag*.

***xylR* Optimization**

```
CLUSTAL O(1.2.1) multiple sequence alignment

xylR           atgactggattaaataaatcaactgtctcatcacaggtaaacacgttaatgaaagaaagt
xylR_JCat      atgacaggccttaacaaatctacagtttcttctcaagttaacacacttatgaaagaatct
               ***** **  * ** ***** ** ** ** ** ** ** *****  * *********  *

xylR           atggtatttgaaataggtcaaggacaatcaagtggcggaagaagacctgtcatgcttgtt
xylR_JCat      atggttttcgaaatcggccaaggccaatcttctggcggccgtcgtcctgttatgcttgtt
               ***** ** ***** ** ***** *****   ******  *  * ***** *********

xylR           tttaataaaaaggcaggatactccgttggaatagatgttggtgtggattatattaatggc
xylR_JCat      ttcaacaaaaaagctggctactctgttggcatcgatgttggcgttgattacatcaacggc
               ** ** ***** ** ** ***** ***** ** ******** ** ***** ** ** ***

xylR           attttaacagaccttgaaggaacaatcgttcttgatcaataccgccatttggaatccaat
xylR_JCat      atccttacagatcttgaaggcacaatcgttcttgatcaataccgtcatcttgaatctaac
               **  * ***** ******** ********************** *** * ***** **

xylR           tctccagaaataacgaaagacattttgattgatatgattcatcactttattacgcaaatg
xylR_JCat      tctcctgaaatcacaaaagatatccttatcgatatgatccatcatttcatcacacaaatg
               ***** ***** ** ***** **  * ** ******** ***** ** ** ** ******

xylR           ccccaatctccgtacgggtttattggtataggtatttgcgtgcctggactcattgataaa
xylR_JCat      cctcaatctccttacggcttcatcggcatcggcatctgcgttcctggccttatcgataaa
               ** ******** ***** ** ** ** ** ** ** ***** ***** ** ** ******

xylR           gatcaaaaaattgttttcactccgaactccaactggagagatattgacttaaaatcttcg
xylR_JCat      gatcaaaaaatcgttttcacacctaactctaactggcgtgatatcgatcttaaatcttct
               *********** ******** ** ***** ****** * ***** **  * ********

xylR           atacaagagaagtacaatgtgtctgtttttattgaaaatgaggcaaatgctggcgcatat
xylR_JCat      atccaagaaaaatacaacgtttctgttttcatcgaaaacgaagctaacgctggcgcttac
               ** ***** ** ***** ** ******** ** ***** ** ** ** ******** **

xylR           ggagaaaaactatttggagctgcaaaaaatcacgataacattatttacgtaagtatcagc
xylR_JCat      ggcgaaaaacttttcggcgctgctaaaaaccatgataacatcatctacgtttctatctct
               ** ******** ** ** ***** ***** ** ******** ** *****   ****

xylR           acaggaatagggatcggtgttattatcaacaatcatttatatagaggagtaagcggcttc
xylR_JCat      acaggcatcggcatcggcgttatcatcaacaaccatctttaccgtggcgtttctggcttc
               ***** ** ** ***** ***** ******** *** * **  * ** **    ******

xylR           tctggagaaatgggacatatgacaatagactttaatggtcctaaatgcagttgcggaaac
xylR_JCat      tctggcgaaatgggccatatgacaatcgatttcaacggccctaaatgctcttgcggcaac
               ***** ******** *********** ** ** ** ** *********  ****** ***

xylR           cgaggatgctgggaattgtatgcttcagagaaggctttattaaaatctcttcagaccaaa
xylR_JCat      cgtggctgctgggaactttacgcttctgaaaaagctcttcttaaatctcttcaaacaaaa
               ** ** ********* * ** ***** ** ** *** *  * *********** ** ***

xylR           gagaaaaaactgtcctatcaagatatcataaacctcgcccatctgaatgatatcggaacc
xylR_JCat      gaaaaaaaactttcttaccaagatatcatcaaccttgctcatcttaacgatatcggcaca
               ** ******** ** ** *********** ***** ** ***** ** ******** **

xylR           ttaaatgcattacaaaattttggattctatttaggaataggccttaccaatattctaaat
xylR_JCat      cttaacgctcttcaaaacttcggcttctaccttggcatcggccttacaaacatccttaac
                * ** **  * ***** ** ** *****  * ** ** ******** ** ** ** **
```

```
xylR            actttcaacccacaagccgtaattttaagaaatagcataattgaatcgcatcctatggtt
xylR_JCat       acattcaaccctcaagctgttatccttcgtaactctatcatcgaatctcatcctatggtt
                ** ******** ***** ** **  *  * **    ** ** ***** ***********

xylR            ttaaattcaatgagaagtgaagtatcatcaagggtttattcccaattaggcaatagctat
xylR_JCat       cttaactctatgcgttctgaagtttcttctcgtgtttactctcaacttggcaactcttac
                 * ** ** *** *   ****** ** **  * ***** ** *** * *****    **

xylR            gaattattgccatcttccttaggacagaatgcaccggcattaggaatgtcctccattgtg
xylR_JCat       gaacttcttccttcttctcttggccaaaacgctcctgctcttggcatgtcttctatcgtt
                *** *  * ** *****  * ** ** ** ** ** **  * ** ***** ** ** **

xylR            attgatcattttctggacatgattacaatgtaataa
xylR_JCat       atcgatcatttccttgatatgatcacaatgtaataa
                ** ******** ** ** ***** ************

CAI-Value of the original sequence:      0.4277108875925065
CAI-Value of the optimized sequence:     1.0



CLUSTAL O(1.2.1) multiple sequence alignment

xylR            MTGLNKSTVSSQVNTLMKESMVFEIGQGQSSGGRRPVMLVFNKKAGYSVGIDVGVDYING
xylR_JCat       MTGLNKSTVSSQVNTLMKESMVFEIGQGQSSGGRRPVMLVFNKKAGYSVGIDVGVDYING
                ************************************************************

xylR            ILTDLEGTIVLDQYRHLESNSPEITKDILIDMIHHFITQMPQSPYGFIGIGICVPGLIDK
xylR_JCat       ILTDLEGTIVLDQYRHLESNSPEITKDILIDMIHHFITQMPQSPYGFIGIGICVPGLIDK
                ************************************************************

xylR            DQKIVFTPNSNWRDIDLKSSIQEKYNVSVFIENEANAGAYGEKLFGAAKNHDNIIYVSIS
xylR_JCat       DQKIVFTPNSNWRDIDLKSSIQEKYNVSVFIENEANAGAYGEKLFGAAKNHDNIIYVSIS
                ************************************************************

xylR            TGIGIGVIINNHLYRGVSGFSGEMGHMTIDFNGPKCSCGNRGCWELYASEKALLKSLQTK
xylR_JCat       TGIGIGVIINNHLYRGVSGFSGEMGHMTIDFNGPKCSCGNRGCWELYASEKALLKSLQTK
                ************************************************************

xylR            EKKLSYQDIINLAHLNDIGTLNALQNFGFYLGIGLTNILNTFNPQAVILRNSIIESHPMV
xylR_JCat       EKKLSYQDIINLAHLNDIGTLNALQNFGFYLGIGLTNILNTFNPQAVILRNSIIESHPMV
                ************************************************************

xylR            LNSMRSEVSSRVYSQLGNSYELLPSSLGQNAPALGMSSIVIDHFLDMITM**
xylR_JCat       LNSMRSEVSSRVYSQLGNSYELLPSSLGQNAPALGMSSIVIDHFLDMITM**
                **************************************************
```

APPENDIX 3

MARLG1

```
LOCUS       MARLG1                  4231 bp ds-DNA     linear       03-DEC-2014
DEFINITION  .
FEATURES             Location/Qualifiers
     misc_feature    1..150
                     /label="USHR-LG1"
     misc_feature    complement(259..261)
                     /label="Inverted Repeat 3"
     misc_feature    complement(174..190)
                     /label="Inverted Repeat"
     misc_feature    236..239
                     /label="Inverted Repeat 1"
     misc_feature    241..250
                     /label="Inverted Repeat 2"
     misc_feature    complement(207..295)
                     /label="ilvGEDA"
     misc_feature    151..295
                     /label="MARterm"
     misc_feature    151..191
                     /label="BBa_B1006 U10"
     misc_feature    252..254
                     /label="Inverted Repeat 3"
     misc_feature    complement(275..278)
                     /label="Inverted Repeat 1"
     misc_feature    151..167
                     /label="Inverted Repeat"
     misc_feature    215..218
                     /label="Inverted Repeat 4"
     misc_feature    complement(231..234)
                     /label="Inverted Repeat 4"
     misc_feature    complement(263..272)
                     /label="Inverted Repeat 2"
     -35_signal      320..325
                     /label="-35"
     protein_bind    325..343
                     /label="tetO"
     modified_base   347..347
                     /label="a52t"
     promoter        296..414
                     /label="P_revtetR-r2"
     -10_signal      343..348
                     /label="-10"
     RBS             415..422
                     /label="RBS"
     misc_feature    423..425
                     /label="Buffer 1"
     misc_feature    415..430
```

```
                     /label="Bs_Cons_RBS"
misc_feature         426..430
                     /label="Buffer 2"
misc_feature         431..736
                     /label="ccdB_Ec"
misc_feature         complement(845..847)
                     /label="Inverted Repeat 3"
misc_feature         complement(760..776)
                     /label="Inverted Repeat"
misc_feature         822..825
                     /label="Inverted Repeat 1"
misc_feature         827..836
                     /label="Inverted Repeat 2"
misc_feature         complement(793..881)
                     /label="ilvGEDA"
misc_feature         737..881
                     /label="MARterm"
misc_feature         737..777
                     /label="BBa_B1006 U10"
misc_feature         838..840
                     /label="Inverted Repeat 3"
misc_feature         complement(861..864)
                     /label="Inverted Repeat 1"
misc_feature         737..753
                     /label="Inverted Repeat"
misc_feature         801..804
                     /label="Inverted Repeat 4"
misc_feature         complement(817..820)
                     /label="Inverted Repeat 4"
misc_feature         complement(849..858)
                     /label="Inverted Repeat 2"
misc_feature         882..915
                     /label="loxP"
misc_feature         complement(903..915)
                     /label="loxP Inverted Repeat"
misc_feature         882..894
                     /label="loxP Inverted Repeat"
promoter             978..983
                     /label="Sigma A -35"
misc_feature         916..1012
                     /label="P_veg"
promoter             1001..1006
                     /label="Sigma A -10"
misc_feature         1028..1029
                     /label="Buffer 1"
misc_feature         1030..1034
                     /label="Buffer 2"
misc_feature         1019..1027
                     /label="RBS"
CDS                  1035..1850
                     /label="kanR"
misc_feature         complement(1959..1961)
                     /label="Inverted Repeat 3"
misc_feature         complement(1874..1890)
                     /label="Inverted Repeat"
misc_feature         1936..1939
                     /label="Inverted Repeat 1"
misc_feature         1941..1950
                     /label="Inverted Repeat 2"
misc_feature         complement(1907..1995)
```

```
                     /label="ilvGEDA"
misc_feature         1851..1995
                     /label="MARterm"
misc_feature         1851..1891
                     /label="BBa_B1006 U10"
misc_feature         1952..1954
                     /label="Inverted Repeat 3"
misc_feature         complement(1975..1978)
                     /label="Inverted Repeat 1"
misc_feature         1851..1867
                     /label="Inverted Repeat"
misc_feature         1915..1918
                     /label="Inverted Repeat 4"
misc_feature         complement(1931..1934)
                     /label="Inverted Repeat 4"
misc_feature         complement(1963..1972)
                     /label="Inverted Repeat 2"
misc_feature         1996..2029
                     /label="loxP"
misc_feature         complement(2017..2029)
                     /label="loxP Inverted Repeat"
misc_feature         1996..2008
                     /label="loxP Inverted Repeat"
-35_signal           2115..2120
                     /label="-35"
promoter             2066..2174
                     /label="P_glnRA"
-10_signal           2138..2143
                     /label="-10"
RBS                  2160..2166
                     /label="RBS (glnRA)"
RBS                  2175..2182
                     /label="RBS"
misc_feature         2183..2185
                     /label="Buffer 1"
misc_feature         2175..2190
                     /label="Bs_Cons_RBS"
misc_feature         2186..2190
                     /label="Buffer 2"
misc_feature         2191..2937
                     /label="spoIISA"
misc_feature         complement(3046..3048)
                     /label="Inverted Repeat 3"
misc_feature         complement(2961..2977)
                     /label="Inverted Repeat"
misc_feature         3023..3026
                     /label="Inverted Repeat 1"
misc_feature         3028..3037
                     /label="Inverted Repeat 2"
misc_feature         complement(2994..3082)
                     /label="ilvGEDA"
misc_feature         2938..3082
                     /label="MARterm"
misc_feature         2938..2978
                     /label="BBa_B1006 U10"
misc_feature         3039..3041
                     /label="Inverted Repeat 3"
misc_feature         complement(3062..3065)
                     /label="Inverted Repeat 1"
misc_feature         2938..2954
```

```
                     /label="Inverted Repeat"
     misc_feature    3002..3005
                     /label="Inverted Repeat 4"
     misc_feature    complement(3018..3021)
                     /label="Inverted Repeat 4"
     misc_feature    complement(3050..3059)
                     /label="Inverted Repeat 2"
     misc_feature    3083..3871
                     /label="lysB4"
     RBS             3872..3879
                     /label="RBS"
     misc_feature    3880..3882
                     /label="Buffer 1"
     misc_feature    3872..3887
                     /label="Bs_Cons_RBS"
     misc_feature    3883..3887
                     /label="Buffer 2"
     misc_feature    3912..3928
                     /label="OR1"
     misc_feature    3888..3936
                     /label="BBa_R0051"
     misc_feature    3888..3904
                     /label="OR2"
     promoter        3902..3907
                     /label="-35"
     promoter        3925..3930
                     /label="-10"
     misc_feature    complement(4045..4047)
                     /label="Inverted Repeat 3"
     misc_feature    complement(3960..3976)
                     /label="Inverted Repeat"
     misc_feature    4022..4025
                     /label="Inverted Repeat 1"
     misc_feature    4027..4036
                     /label="Inverted Repeat 2"
     misc_feature    complement(3993..4081)
                     /label="ilvGEDA"
     misc_feature    3937..4081
                     /label="MARterm"
     misc_feature    3937..3977
                     /label="BBa_B1006 U10"
     misc_feature    4038..4040
                     /label="Inverted Repeat 3"
     misc_feature    complement(4061..4064)
                     /label="Inverted Repeat 1"
     misc_feature    3937..3953
                     /label="Inverted Repeat"
     misc_feature    4001..4004
                     /label="Inverted Repeat 4"
     misc_feature    complement(4017..4020)
                     /label="Inverted Repeat 4"
     misc_feature    complement(4049..4058)
                     /label="Inverted Repeat 2"
     misc_feature    4082..4231
                     /label="DSHR-LG1"
ORIGIN
        1 taaagggata ttaaccctta tacatcaatg ttttaaccgt cttaaaaaac tagacaaagc
       61 gtgaataaaa aaagagaagg tctttcatca gtttactaaa ctgttgggag acctttttctc
      121 catattagcg gtcatatgag cataaatgtc aaaaaaaaac cccgcccctg acagggcggg
      181 gttttttttt ttttttttt ttttttgtc tgctcctcgg ttatgttttt aaggtcaaaa
```

```
 241 aaaaccccccg gacctttcgg tgcgggggtc ttagttcgtt aaggcttgat ctctagaatt
 301 ccaaaaacta aaaaaaatat tgacactcta tcattgatag agtatattta acgggatccc
 361 gccaagcttg ggatccccag cttgttgata cactaatgct tttatatagg gaaaaaagga
 421 ggtgtttttt atgcaattca aagtttacac atacaaacgt gaatctcgtt accgtctttt
 481 cgttgatgtt caatctgata tcatcgatac acctggccgt cgtatggtta tccctcttgc
 541 ttctgctcgt cttctttctg ataaagtttc tcgtgaactt taccctgttg ttcatatcgg
 601 cgatgaatct tggcgtatga tgacaacaga tatggcttct gttcctgttt ctgttatcgg
 661 cgaagaagtt gctgatcttt ctcatcgtga aaacgatatc aaaaacgcta tcaaccttat
 721 gttctggggc atctaaaaaa aaaaaccccg cccctgacag ggcggggttt ttttttttt
 781 ttttttttt tttgtctgct cctcggttat gtttttaagg tcaaaaaaaa cccccggacc
 841 tttcggtgcg ggggtcttag ttcgttaagg cttgatctct aataacttcg tatagcatac
 901 attatacgaa gttataattt tgtcaaaata attttattga caacgtctta ttaacgttga
 961 tataatttaa attttatttg acaaaaatgg gctcgtgttg tacaataaat gtatattaag
1021 aggaggagtt ttttatgtct catatccaac gtgaaacatc ttgctctcgt cctcgtctta
1081 actctaacat ggatgctgat ctttacggct acaaatgggc tcgtgataac gttggccaat
1141 ctggcgctac aatctaccgt ctttacggca aacctgatgc tcctgaactt ttccttaaac
1201 atggcaaagg ctctgttgct aacgatgtta cagatgaaat ggttcgtctt aactggctta
1261 cagaattcat gcctcttcct acaatcaaac atttcatccg tacacctgat gatgcttggc
1321 ttcttacaac agctatccct ggcaaaacag ctttccaagt tcttgaagaa taccctgatt
1381 ctggcgaaaa catcgttgat gctcttgctg ttttccttcg tcgtcttcat tctatccctg
1441 tttgcaactg ccctttcaac tctgatcgtg ttttccgtct tgctcaagct caatctcgta
1501 tgaacaacgg ccttgttgat gcttctgatt tcgatgatga acgtaacggc tggcctgttg
1561 aacaagtttg gaaagaaatg cataaacttc ttccttttctc tcctgattct gttgttacac
1621 atggcgattt ctctcttgat aaccttatct tcgatgaagg caaacttatc ggctgcatcg
1681 atgttggccg tgttggcatc gctgatcgtt accaagatct tgctatcctt tggaactgcc
1741 ttggcgaatt ctctccttct cttcaaaaac gtcttttcca aaaatacggc atcgataacc
1801 ctgatatgaa caaacttcaa ttccatctta tgcttgatga attcttctaa aaaaaaaaac
1861 cccgcccctg acagggcggg gttttttttt ttttttttt ttttttgtc tgctcctcgg
1921 ttatgttttt aaggtcaaaa aaaacccccg gacctttcgg tgcgggggtc ttagttcgtt
1981 aaggcttgat ctctaataac ttcgtatagc atacattata cgaagttata tggcaagatg
2041 ctaagcaaga atataaatcg caagcatttt ttaaaaattt ctctggattt gatgttaaga
2101 atccttacat cgtattgaca cataatataa catcacctat aatgaaacta agttaagaaa
2161 aggaggaaat tgagaaagga ggtgtttttt atggttcttt tcttccaaat catggtttgg
2221 tgcatcgttg ctggccttgg cctttacgtt tacgctacat ggcgtttcga agctaaagtt
2281 aaagaaaaaa tgtctgctat ccgtaaaaca tggtaccttc ttttcgttct tggcgctatg
2341 gtttactgga catacgaacc tacatctctt ttcacacatt gggaacgtta ccttatcgtt
2401 gctgtttctt tcgctcttat cgatgctttc atcttccttt ctgcttacgt taaaaaactt
2461 gctggctctg aacttgaaac agatacacgt gaaatccttg aagaaaacaa cgaaatgctt
2521 catatgtacc ttaaccgtct taaaacatac caataccttc ttaaaaacga acctatccat
2581 gtttactacg gctctatcga tgcttacgct gaaggcatcg ataaacttct taaaacatac
2641 gctgataaaa tgaaccttac agcttctctt tgccattact ctacacaagc tgataaagat
2701 cgtcttacag aacatatgga tgatcctgct gatgttcaaa cacgtcttga tcgtaaagat
2761 gtttactacg atcaatacgg caaagttgtt cttatccctt tcacaatcga aacacaaaac
2821 tacgttatca aacttacatc tgattctatc gttacagaat tcgattacct tcttttcaca
2881 tctcttacat ctatctacga tcttgttctt cctatcgaag aagaaggcga aggctaaaaa
2941 aaaaaacccc gcccctgaca gggcggggtt ttttttttt ttttttttt ttttgtctgc
3001 tcctcggtta tgtttttaag gtcaaaaaaa acccccggac ctttcggtgc gggggtctta
3061 gttcgttaag gcttgatctc tattatttga atgtgcccca gtagttttga cgtttgccgc
3121 ctttagattc gcctgtagca aggtagccgt agccgttaga acgaggttga cggatccaaa
3181 cgtagccatc gtattcgatg ccgaagccgt tgtagttaac aggagagcca gcaggaagtg
3241 tagcgataac atcagcagat gtgaaaggag ctgtacgaag tttgatagat gtgtttgtaa
3301 cgaatgtgcc tgtttctttt gtgaaccaag aagaatcgta gccgccgcct gtagagccgc
3361 cgcctgtaga gccgccgcca gagccgcctg tagagccaga gccgccgccg ttatcaacag
3421 cgccgccgtt atcagcagga ggtttttggc cgccaacaac atcgtaaagt tcgaaatgag
3481 ggtaatcttt gaaagaaacc caatcgccgc cccatttgaa gccttgagct ttcatagcag
3541 cgataacttt acggaagttg ccttcaactg tccagataac atcagagcca tcttgtgtgt
3601 aaaggcaaag atcaacagca acgccgtagt tatggttaga ttggccgcca cgagcgtttg
3661 taacgataga gccaggtttt gtacggcctt gagcgtaaag agcgttttgt tcagcgaaag
3721 aacggaagcc ttgagcaacg cagatgtaga tgccttgagc atgcatttgt gtgataacag
3781 cacgtgtacg atcagcaaca tctttacgca tgccagaaac gttaagttta cggttagctt
```

```
     3841 tatcgataag tgtttgaaga gccatagcca taaaaaacac ctcctttgca accattatca
     3901 ccgccagagg taaaatagtc aacacgcacg gtgttaaaaa aaaaaccccg cccctgacag
     3961 ggcggggttt ttttttttt ttttttttt tttgtctgct cctcggttat gtttttaagg
     4021 tcaaaaaaaa ccccccggacc tttcggtgcg ggggtcttag ttcgttaagg cttgatctct
     4081 acgagcctaa ttttccatct atttgattgg ggaacaaatg gctttttaac aagaaagaag
     4141 tccaagaata cattgattgg tggtcaattg aggttaaaag gaaaaagagg gcctgatata
     4201 tctttcactt tcgcctaact agcaaaattt a
//
```

MAREPR1

```
LOCUS       MAREPR1                 1530 bp ds-DNA     linear       03-DEC-2014
DEFINITION  .
FEATURES             Location/Qualifiers
     misc_feature    1..150
                     /label="spoIVA (final 150 bp)"
     misc_feature    1..150
                     /label="USHR-EPR1"
     misc_feature    complement(172..184)
                     /label="loxP Inverted Repeat"
     misc_feature    151..184
                     /label="loxP"
     misc_feature    151..163
                     /label="loxP Inverted Repeat"
     misc_feature    185..281
                     /label="P_veg"
     promoter        247..252
                     /label="Sigma A -35"
     promoter        270..275
                     /label="Sigma A -10"
     misc_feature    297..298
                     /label="Buffer 1"
     misc_feature    288..296
                     /label="RBS"
     misc_feature    299..303
                     /label="Buffer 2"
     CDS             304..1119
                     /label="kanR"
     misc_feature    complement(1141..1153)
                     /label="loxP Inverted Repeat"
     misc_feature    1120..1153
                     /label="loxP"
     misc_feature    1120..1132
                     /label="loxP Inverted Repeat"
     misc_feature    1255..1257
                     /label="Inverted Repeat 3"
     misc_feature    1154..1194
                     /label="BBa_B1006 U10"
     misc_feature    complement(1262..1264)
                     /label="Inverted Repeat 3"
     misc_feature    complement(1278..1281)
                     /label="Inverted Repeat 1"
     misc_feature    complement(1177..1193)
                     /label="Inverted Repeat"
     misc_feature    complement(1266..1275)
                     /label="Inverted Repeat 2"
     misc_feature    1154..1298
                     /label="MARterm"
     misc_feature    1244..1253
                     /label="Inverted Repeat 2"
     misc_feature    1218..1221
                     /label="Inverted Repeat 4"
     misc_feature    1239..1242
                     /label="Inverted Repeat 1"
     misc_feature    complement(1234..1237)
                     /label="Inverted Repeat 4"
     misc_feature    1154..1170
                     /label="Inverted Repeat"
```

```
     misc_feature    complement(1210..1298)
                     /label="ilvGEDA"
     misc_feature    1335..1380
                     /label="P_T7"
     misc_feature    1384..1390
                     /label="hbs RBS"
     misc_feature    1381..1530
                     /label="DSHR-EPR1"
     misc_feature    1398..1530
                     /label="hbs"
ORIGIN
        1 atcttcggaa ggtcgctgag ctcaattgtg agagaaggga ttcaggcaaa gctgtcattg
       61 atgcctgaaa acgcacggta taaattaaaa gaaacattag aaagaatcat aaacgaaggc
      121 tctggcggct taatcgccat catcctgtaa ataacttcgt atagcataca ttatacgaag
      181 ttataatttt gtcaaaataa ttttattgac aacgtcttat taacgttgat ataatttaaa
      241 ttttatttga caaaaatggg ctcgtgttgt acaataaatg tatattaaga ggaggagttt
      301 tttatgtctc atatccaacg tgaaacatct tgctctcgtc ctcgtcttaa ctctaacatg
      361 gatgctgatc tttacggcta caaatgggct cgtgataacg ttggccaatc tggcgctaca
      421 atctaccgtc tttacggcaa acctgatgct cctgaacttt tccttaaaca tggcaaaggc
      481 tctgttgcta acgatgttac agatgaaatg gttcgtctta actggcttac agaattcatg
      541 cctcttccta caatcaaaca tttcatccgt acacctgatg atgcttggct tcttacaaca
      601 gctatccctg gcaaaacagc tttccaagtt cttgaagaat accctgattc tggcgaaaac
      661 atcgttgatg ctcttgctgt tttccttcgt cgtcttcatt ctatccctgt ttgcaactgc
      721 cctttcaact ctgatcgtgt tttccgtctt gctcaagctc aatctcgtat gaacaacggc
      781 cttgttgatg cttctgattt cgatgatgaa cgtaacggct ggcctgttga acaagtttgg
      841 aaagaaatgc ataaacttct tcctttctct cctgattctg ttgttacaca tggcgatttc
      901 tctcttgata accttatctt cgatgaaggc aaacttatcg gctgcatcga tgttggccgt
      961 gttggcatcg ctgatcgtta ccaagatctt gctatccttt ggaactgcct tggcgaattc
     1021 tctccttctc ttcaaaaacg tcttttccaa aaatacggca tcgataaccc tgatatgaac
     1081 aaacttcaat tccatcttat gcttgatgaa ttcttctaaa taacttcgta tagcatacat
     1141 tatacgaagt tataaaaaaa aaccccgccc ctgacagggc ggggtttttt ttttttttt
     1201 ttttttttt gtctgctcct cggttatgtt tttaaggtca aaaaaaaccc ccggacctt
     1261 cggtgcgggg gtcttagttc gttaaggctt gatctctaat ggcaagatgc taagcaagaa
     1321 tataaatcgc aagctaatac gactcactat agggaataca agctacttgt tctttttgca
     1381 gggaggaggt gaaaggcatg aacaaaacag aacttatcaa tgcggttgca gaagcaagcg
     1441 aattgtctaa aaaagacgct acaaaagcag ttgactctgt ttttgatacg atcttagatg
     1501 cacttaaaaa cggtgataaa atccaactga
//
```

MAREPR2

```
LOCUS       MAREPR2                 1566 bp ds-DNA     linear       03-DEC-2014
DEFINITION  .
FEATURES             Location/Qualifiers
     misc_feature    1..150
                     /label="USHR-EPR2"
     CDS             1..150
                     /label="xsa CDS"
     misc_feature    151..184
                     /label="loxP"
     misc_feature    151..163
                     /label="loxP Inverted Repeat"
     misc_feature    complement(172..184)
                     /label="loxP Inverted Repeat"
     misc_feature    185..281
                     /label="P_veg"
     promoter        247..252
                     /label="Sigma A -35"
     promoter        270..275
                     /label="Sigma A -10"
     misc_feature    299..303
                     /label="Buffer 2"
     misc_feature    297..298
                     /label="Buffer 1"
     misc_feature    288..296
                     /label="RBS"
     CDS             304..1119
                     /label="kanR"
     misc_feature    1120..1153
                     /label="loxP"
     misc_feature    1120..1132
                     /label="loxP Inverted Repeat"
     misc_feature    complement(1141..1153)
                     /label="loxP Inverted Repeat"
     misc_feature    complement(1234..1237)
                     /label="Inverted Repeat 4"
     misc_feature    complement(1177..1193)
                     /label="Inverted Repeat"
     misc_feature    complement(1210..1298)
                     /label="ilvGEDA"
     misc_feature    complement(1278..1281)
                     /label="Inverted Repeat 1"
     misc_feature    1154..1194
                     /label="BBa_B1006 U10"
     misc_feature    complement(1262..1264)
                     /label="Inverted Repeat 3"
     misc_feature    1218..1221
                     /label="Inverted Repeat 4"
     misc_feature    1239..1242
                     /label="Inverted Repeat 1"
     misc_feature    1255..1257
                     /label="Inverted Repeat 3"
     misc_feature    1244..1253
                     /label="Inverted Repeat 2"
     misc_feature    complement(1266..1275)
                     /label="Inverted Repeat 2"
     misc_feature    1154..1298
                     /label="MARterm"
```

```
     misc_feature    1154..1170
                     /label="Inverted Repeat"
     promoter        1335..1416
                     /label="P_xylA"
     misc_feature    1385..1395
                     /label="XylR Operator"
     promoter        1347..1352
                     /label="Sigma A -35"
     promoter        1370..1375
                     /label="Sigma A -10"
     misc_feature    1399..1409
                     /label="XylR Operator"
     misc_feature    1425..1432
                     /label="Putative PA RBS"
     CDS             1442..1566
                     /label="trxA CDS"
     misc_feature    1417..1566
                     /label="DSHR-EPR2"
ORIGIN
        1 gcagaaaaaa tgaatgcgca taacacgttt gacgatcctc atcatgtcaa accggaatcc
       61 ttcagacaat acacgctcag caaaaacaaa ctgaaagtaa aactcccgcc aatgtcagtc
      121 gtcttactta cgctgcgtgc tgattcttaa ataacttcgt atagcataca ttatacgaag
      181 ttataatttt gtcaaaataa ttttattgac aacgtcttat taacgttgat ataatttaaa
      241 ttttatttga caaaaatggg ctcgtgttgt acaataaatg tatattaaga ggaggagttt
      301 tttatgtctc atatccaacg tgaaacatct tgctctcgtc ctcgtcttaa ctctaacatg
      361 gatgctgatc tttacggcta caaatgggct cgtgataacg ttggccaatc tggcgctaca
      421 atctaccgtc tttacggcaa acctgatgct cctgaacttt tccttaaaca tggcaaaggc
      481 tctgttgcta acgatgttac agatgaaatg gttcgtctta actggcttac agaattcatg
      541 cctcttccta caatcaaaca tttcatccgt acacctgatg atgcttggct tcttacaaca
      601 gctatccctg gcaaaacagc tttccaagtt cttgaagaat accctgattc tggcgaaaac
      661 atcgttgatg ctcttgctgt tttccttcgt cgtcttcatt ctatccctgt ttgcaactgc
      721 cctttcaact ctgatcgtgt tttccgtctt gctcaagctc aatctcgtat gaacaacggc
      781 cttgttgatg cttctgattt cgatgatgaa cgtaacggct ggcctgttga acaagtttgg
      841 aaagaaatgc ataaacttct tcctttctct cctgattctg ttgttacaca tggcgatttc
      901 tctcttgata accttatctt cgatgaaggc aaacttatcg gctgcatcga tgttggccgt
      961 gttggcatcg ctgatcgtta ccaagatctt gctatccttt ggaactgcct tggcgaattc
     1021 tctccttctc ttcaaaaacg tcttttccaa aaatacggca tcgataaccc tgatatgaac
     1081 aaacttcaat tccatcttat gcttgatgaa ttcttctaaa taacttcgta tagcatacat
     1141 tatacgaagt tataaaaaaa aaccccgccc ctgacagggc ggggtttttt ttttttttt
     1201 ttttttttt gtctgctcct cggttatgtt tttaaggtca aaaaaaaccc ccggacctt
     1261 cggtgcgggg gtcttagttc gttaaggctt gatctctaat ggcaagatgc taagcaagaa
     1321 tataaatcgc aagcctaaaa aaaatattga aaatactgac gaggttatat aagatgaaaa
     1381 taagttagtt tgtttaaaca acaaactaat aggtgacatt tcacattgga ggaattcaat
     1441 aatggctatc gtaaaagcaa ctgatcaatc tttctcagct gaaacaagcg aaggcgtcgt
     1501 actggcagac ttctgggctc cttggtgcgg accttgtaaa atgattgcac ctgttcttga
     1561 agaatt
//
```

MARAcc

```
LOCUS       MARAcc                  4957 bp ds-DNA     linear       03-DEC-2014
DEFINITION  .
FEATURES             Location/Qualifiers
     misc_feature    1..150
                     /label="USHR-Acc"
     misc_feature    151..295
                     /label="MARterm"
     misc_feature    241..250
                     /label="Inverted Repeat 2"
     misc_feature    complement(263..272)
                     /label="Inverted Repeat 2"
     misc_feature    236..239
                     /label="Inverted Repeat 1"
     misc_feature    215..218
                     /label="Inverted Repeat 4"
     misc_feature    252..254
                     /label="Inverted Repeat 3"
     misc_feature    complement(231..234)
                     /label="Inverted Repeat 4"
     misc_feature    151..167
                     /label="Inverted Repeat"
     misc_feature    complement(174..190)
                     /label="Inverted Repeat"
     misc_feature    complement(259..261)
                     /label="Inverted Repeat 3"
     misc_feature    151..191
                     /label="BBa_B1006 U10"
     misc_feature    complement(275..278)
                     /label="Inverted Repeat 1"
     misc_feature    complement(207..295)
                     /label="ilvGEDA"
     misc_feature    296..392
                     /label="P_veg"
     promoter        358..363
                     /label="Sigma A -35"
     promoter        381..386
                     /label="Sigma A -10"
     misc_feature    408..409
                     /label="Buffer 1"
     misc_feature    410..414
                     /label="Buffer 2"
     misc_feature    399..407
                     /label="RBS"
     misc_feature    415..1068
                     /label="revTetR"
     misc_feature    1069..1213
                     /label="MARterm"
     misc_feature    1159..1168
                     /label="Inverted Repeat 2"
     misc_feature    complement(1181..1190)
                     /label="Inverted Repeat 2"
     misc_feature    1154..1157
                     /label="Inverted Repeat 1"
     misc_feature    1133..1136
                     /label="Inverted Repeat 4"
     misc_feature    1170..1172
                     /label="Inverted Repeat 3"
```

```
misc_feature    complement(1149..1152)
                /label="Inverted Repeat 4"
misc_feature    1069..1085
                /label="Inverted Repeat"
misc_feature    complement(1092..1108)
                /label="Inverted Repeat"
misc_feature    complement(1177..1179)
                /label="Inverted Repeat 3"
misc_feature    1069..1109
                /label="BBa_B1006 U10"
misc_feature    complement(1193..1196)
                /label="Inverted Repeat 1"
misc_feature    complement(1125..1213)
                /label="ilvGEDA"
misc_feature    1214..1247
                /label="loxP"
misc_feature    complement(1235..1247)
                /label="loxP Inverted Repeat"
misc_feature    1214..1226
                /label="loxP Inverted Repeat"
misc_feature    1248..1344
                /label="P_veg"
promoter        1310..1315
                /label="Sigma A -35"
promoter        1333..1338
                /label="Sigma A -10"
misc_feature    1360..1361
                /label="Buffer 1"
misc_feature    1362..1366
                /label="Buffer 2"
misc_feature    1351..1359
                /label="RBS"
CDS             1367..2182
                /label="kanR"
misc_feature    2183..2327
                /label="MARterm"
misc_feature    2273..2282
                /label="Inverted Repeat 2"
misc_feature    complement(2295..2304)
                /label="Inverted Repeat 2"
misc_feature    2268..2271
                /label="Inverted Repeat 1"
misc_feature    2247..2250
                /label="Inverted Repeat 4"
misc_feature    2284..2286
                /label="Inverted Repeat 3"
misc_feature    complement(2263..2266)
                /label="Inverted Repeat 4"
misc_feature    2183..2199
                /label="Inverted Repeat"
misc_feature    complement(2206..2222)
                /label="Inverted Repeat"
misc_feature    complement(2291..2293)
                /label="Inverted Repeat 3"
misc_feature    2183..2223
                /label="BBa_B1006 U10"
misc_feature    complement(2307..2310)
                /label="Inverted Repeat 1"
misc_feature    complement(2239..2327)
                /label="ilvGEDA"
```

```
misc_feature    2328..2361
                /label="loxP"
misc_feature    complement(2349..2361)
                /label="loxP Inverted Repeat"
misc_feature    2328..2340
                /label="loxP Inverted Repeat"
misc_feature    2398..2494
                /label="P_veg"
promoter        2460..2465
                /label="Sigma A -35"
promoter        2483..2488
                /label="Sigma A -10"
misc_feature    2510..2511
                /label="Buffer 1"
misc_feature    2512..2516
                /label="Buffer 2"
misc_feature    2501..2509
                /label="RBS"
misc_feature    2517..3572
                /label="xylR"
misc_feature    3573..3717
                /label="MARterm"
misc_feature    3663..3672
                /label="Inverted Repeat 2"
misc_feature    complement(3685..3694)
                /label="Inverted Repeat 2"
misc_feature    3658..3661
                /label="Inverted Repeat 1"
misc_feature    3637..3640
                /label="Inverted Repeat 4"
misc_feature    3674..3676
                /label="Inverted Repeat 3"
misc_feature    complement(3653..3656)
                /label="Inverted Repeat 4"
misc_feature    3573..3589
                /label="Inverted Repeat"
misc_feature    complement(3596..3612)
                /label="Inverted Repeat"
misc_feature    complement(3681..3683)
                /label="Inverted Repeat 3"
misc_feature    3573..3613
                /label="BBa_B1006 U10"
misc_feature    complement(3697..3700)
                /label="Inverted Repeat 1"
misc_feature    complement(3629..3717)
                /label="ilvGEDA"
misc_feature    3842..3876
                /label="liaR-binding"
misc_feature    3914..3914
                /label="+1"
misc_feature    3877..3882
                /label="-35"
promoter        3718..3926
                /label="PliaI"
misc_feature    3902..3907
                /label="-10"
misc_feature    3942..3943
                /label="Buffer 1"
misc_feature    3944..3948
                /label="Buffer 2"
```

```
     misc_feature    3933..3941
                     /label="RBS"
     misc_feature    3949..4662
                     /label="cI"
     misc_feature    4663..4807
                     /label="MARterm"
     misc_feature    4753..4762
                     /label="Inverted Repeat 2"
     misc_feature    complement(4775..4784)
                     /label="Inverted Repeat 2"
     misc_feature    4748..4751
                     /label="Inverted Repeat 1"
     misc_feature    4727..4730
                     /label="Inverted Repeat 4"
     misc_feature    4764..4766
                     /label="Inverted Repeat 3"
     misc_feature    complement(4743..4746)
                     /label="Inverted Repeat 4"
     misc_feature    4663..4679
                     /label="Inverted Repeat"
     misc_feature    complement(4686..4702)
                     /label="Inverted Repeat"
     misc_feature    complement(4771..4773)
                     /label="Inverted Repeat 3"
     misc_feature    4663..4703
                     /label="BBa_B1006 U10"
     misc_feature    complement(4787..4790)
                     /label="Inverted Repeat 1"
     misc_feature    complement(4719..4807)
                     /label="ilvGEDA"
     misc_feature    4808..4957
                     /label="DSHR-Acc"
     misc_feature    complement(4808..4957)
                     /label="pit"
ORIGIN
        1 ccaaaccttc cgtaatcaac aaacttcaca cgcctcacca atttttcac tgtatcacct
       61 gacattatcc tctgtttgta tttattatat gtcacccttt aagaaaagga ataaggacaa
      121 gagctgtttt cccttgtcct tttagtgtga aaaaaaaaac cccgcccctg acagggcggg
      181 gttttttttt ttttttttt ttttttgtc tgctcctcgg ttatgttttt aaggtcaaaa
      241 aaaacccccg gacctttcgg tgcgggggtc ttagttcgtt aaggcttgat ctctaaattt
      301 tgtcaaaata attttattga caacgtctta ttaacgttga tataatttaa attttatttg
      361 acaaaaatgg gctcgtgttg tacaataaat gtatattaag aggaggagtt ttttatgtct
      421 cgtcttgata aatctaaagt tatcaactct gctcttgctc ttggcaacga agttggcatc
      481 gaaggcgtta caacacgtaa acttgctcaa aaacttggcg ttgaacaacc tacactttac
      541 tggcatgtta aaaacaaacg tgctcttctt gatgctcttg ctgttgaaat ccttgctcgt
      601 catcatgatt actctcttcc tgctgctggc gaatcttggc aatctttcct tcgtaacaac
      661 gctatgtctt tccgtcgtgc tcttcttcgt taccgtgatg gcgctaaagt tcatcttggc
      721 acacgtcctg atgaaaaaca atacgataca gttgaaacac aacttcgttt catgacagaa
      781 aacggcttct ctcttcgtga tggcctttac gctatctctg ctgtttctca tttcacactt
      841 ggcgctgttc ttgaacaaca agaacataca gctgctctta cagatcgtcc tgctgctcct
      901 gatgaaaacc ttcctcctct tcttcgtgaa gctcttcaaa tcatggattc tgatgatggc
      961 gaacaagctt tccttcatgg ccttgaatct cttatccgtg gcttcgaagt tcaacttaca
     1021 gctcttcttc aaatcgttgg cggcgataaa cttatcatcc ctttctgcaa aaaaaaaccc
     1081 cgcccctgac agggcggggt ttttttttt ttttttttt tttttgtctg ctcctcggtt
     1141 atgtttttaa ggtcaaaaaa aacccccgga cctttcggtg cgggggtctt agttcgttaa
     1201 ggcttgatct ctaataactt cgtatagcat acattatacg aagttataat tttgtcaaaa
     1261 taattttatt gacaacgtct tattaacgtt gatataattt aaattttatt tgacaaaaat
     1321 gggctcgtgt tgtacaataa atgtatatta agaggaggag ttttttatgt ctcatatcca
     1381 acgtgaaaca tcttgctctc gtcctcgtct taactctaac atggatgctg atctttacgg
     1441 ctacaaatgg gctcgtgata acgttggcca atctggcgct acaatctacc gtctttacgg
```

```
1501 caaacctgat gctcctgaac ttttccttaa acatggcaaa ggctctgttg ctaacgatgt
1561 tacagatgaa atggttcgtc ttaactggct tacagaattc atgcctcttc ctacaatcaa
1621 acatttcatc cgtacacctg atgatgcttg gcttcttaca acagctatcc ctggcaaaac
1681 agctttccaa gttcttgaag aataccctga ttctggcgaa aacatcgttg atgctcttgc
1741 tgttttcctt cgtcgtcttc attctatccc tgtttgcaac tgccctttca actctgatcg
1801 tgttttccgt cttgctcaag ctcaatctcg tatgaacaac ggccttgttg atgcttctga
1861 tttcgatgat gaacgtaacg gctggcctgt tgaacaagtt tggaaagaaa tgcataaact
1921 tcttcctttc tctcctgatt ctgttgttac acatggcgat ttctctcttg ataaccttat
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2101 acgtcttttc caaaaatacg gcatcgataa ccctgatatg aacaaacttc aattccatct
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2221 tttttttttt ttttttttgg tctgctcctc ggttatgttt ttaaggtcaa aaaaaacccc
2281 cggacctttc ggtgcggggg tcttagttcg ttaaggcttg atctctaata acttcgtata
2341 gcatacatta tacgaagtta tatggcaaga tgctaagcaa gaatataaat cgcaagcaat
2401 tttgtcaaaa taatttttatt gacaacgtct tattaacgtt gatataattt aaattttatt
2461 tgacaaaaat gggctcgtgt tgtacaataa atgtatatta agaggaggag ttttttatga
2521 caggccttaa caaatctaca gtttcttctc aagttaacac acttatgaaa gaatctatgg
2581 ttttcgaaat cggccaaggc caatcttctg gcggccgtcg tcctgttatg cttgttttca
2641 acaaaaaagc tggctactct gttggcatcg atgttggcgt tgattacatc aacggcatcc
2701 ttacagatct tgaaggcaca atcgttcttg atcaataccg tcatcttgaa tctaactctc
2761 ctgaaatcac aaaagatatc cttatcgata tgatccatca tttcatcaca caaatgcctc
2821 aatctcctta cggcttcatc ggcatcggca tctgcgttcc tggccttatc gataaagatc
2881 aaaaaatcgt tttcacacct aactctaact ggcgtgatat cgatcttaaa tcttctatcc
2941 aagaaaaata caacgtttct gttttcatcg aaaacgaagc taacgctggc gcttacggcg
3001 aaaaactttt cggcgctgct aaaaaccatg ataacatcat ctacgtttct atctctacag
3061 gcatcggcat cggcgttatc atcaacaacc atctttaccg tggcgtttct ggcttctctg
3121 gcgaaatggg ccatatgaca atcgatttca acggccctaa atgctcttgc ggcaaccgtg
3181 gctgctggga actttacgct tctgaaaaag ctcttcttaa atctcttcaa acaaaagaaa
3241 aaaaactttc ttaccaagat atcatcaacc ttgctcatct taacgatatc ggcacactta
3301 acgctcttca aaacttcggc ttctaccttg gcatcggcct tacaaacatc cttaacacat
3361 tcaaccctca agctgttatc cttcgtaact ctatcatcga atctcatcct atggttctta
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3481 ttcttccttc ttctcttggc caaaacgctc ctgctcttgg catgtcttct atcgttatcg
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3601 gggttttttt ttttttttt ttttttttg tctgctcctc ggttatgttt ttaaggtcaa
3661 aaaaaacccc cggacctttc ggtgcggggg tcttagttcg ttaaggcttg atctctaatt
3721 ggccaaagca gaaaggtccg acctaattaa agaaagggaa gcaagtgttc atctgtaaag
3781 ggttttaaaa cgccatgcct cgtgcatggc gttttttttgt gccaatgggt ccggtgcgag
3841 atacgactcc ggtcttatat aaaaatcaat ctctgattcg ttttgcatat cttccaactt
3901 gtataagatg aagacaagga aaacgaatat taagaggagg agtttttat gtctacaaaa
3961 aaaaaacctc ttacacaaga acaacttgaa gatgctcgtc gtcttaaagc tatctacgaa
4021 aaaaaaaaaa acgaacttgg cctttctcaa gaatctgttg ctgataaaat gggcatgggc
4081 caatctggcg ttggcgctct tttcaacggc atcaacgctc ttaacgctta caacgctgct
4141 cttcttgcta aaatccttaa agtttctgtt gaagaattct ctccttctat cgctcgtgaa
4201 atctacgaaa tgtacgaagc tgtttctatg caaccttctc ttcgttctga atacgaatac
4261 cctgtttttct ctcatgttca agctggcatg ttctctcctg aacttcgtac attcacaaaa
4321 ggcgatgctg aacgttgggt ttctacaaca aaaaaagctt ctgattctgc tttctggctt
4381 gaagttgaag gcaactctat gacagctcct acaggctcta aaccttcttt ccctgatggc
4441 atgcttatcc ttgttgatcc tgaacaagct gttgaacctg gcgatttctg catcgctcgt
4501 cttggcggcg atgaattcac attcaaaaaa cttatccgtg attctggcca agttttcctt
4561 caacctctta accctcaata ccctatgatc ccttgcaacg aatcttgctc tgttgttggc
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MARAND

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