ENGINEERING THE SKIN MICROBIOME FOR DRUG DELIVERY

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ENGINEERING THE SKIN MICROBIOME FOR DRUG DELIVERY

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To my parents, Sharon and Ron Montgomery

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2-PE	2-phenylethanol
$aroG^{fbr}$	Feedback resistant mutant of 3-deoxy-D-arabinoheptulosonate-7phosphate synthase
bp	Base pairs
BS	Bacillus subtilis
DegQ	Regulation protein DegQ
DNA	Deoxyribonucleic acid
GFP	Green fluorescent protein
HPLC	High performance liquid chromatography
kbp	Kilo-base pairs
kDa	Kilodalton
KDC	2-keto acid decarboxylase
kivd	Keto-acid decarboxylase
LB	Lysogeny broth
LD_{50}	Dose corresponding to 50% cell death
PCR	Polymerase chain reaction
$pheA^{fbr}$	Feedback resistant mutant of chorismite mutase/ prephenate dehydratase
RBS	Ribosome binding site
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
WT	Wildtype
yjgb	Aldehyde reductase

SUMMARY

Topical drug delivery is convenient for patients because the skin is a highly accessible tissue that allows simple self-application of therapies. However, topical drug delivery formulations are limited in their ability to deliver drugs for long periods of time, which constrains their suitability for chronic conditions. The skin microbiome, which consists of many commensal species of bacteria that can colonize the skin for days to months or years at a time, could be used to address these limitations if commensal bacteria were engineered to act as drug delivery platforms for the skin. The objective of this thesis was to evaluate a bacteria-based drug delivery platform for topical therapy using *Bacillus subtilis*, a genetically tractable organism that is found in the skin microbiome. The feasibility of using *B. subtilis* as a topical drug delivery platform was first assessed using a combination of computational and experimental skin models, with green fluorescent protein (GFP) serving as a model heterologous protein drug. We found that *B. subtilis* appeared safe for use on skin and was able to survive while producing GFP for at least 1 day across multiple skin models. We next explored two therapeutically relevant applications for engineered *B. subtilis* on the skin as an antifungal treatment and mosquito repellant. We identified challenges in the specific use of *B. subtilis* as a drug delivery platform as well as more broad challenges for the field of using engineered bacteria for skin treatments.

CHAPTER 1. INTRODUCTION

Non-adherence to medication refers to a situation in which patients do not follow their treatment regimen as prescribed. Non-adherence is a global health problem that can result in worse treatment outcomes and preventable hospital admissions, adding strain to the healthcare system and worsening patients' quality of life [1-3]. There are several factors contributing to non-adherence, including forgetfulness, inconvenience of the treatment, and fear of unwanted side effects [4-6]. Each of these concerns can be addressed with improved drug delivery platforms that limit side effects by targeting treatments to the site of action, make treatments more comfortable with noninvasive delivery routes, and reduce the number of required administrations by delivering drugs over an extended period of time. For skin conditions, topical drug delivery is a natural choice as the therapeutic can be delivered directly to the skin in a noninvasive way. However, gels, creams, ointments, and sprays often require frequent administration, which can be inconvenient to patients and can result in improper adherence as a result [7].

For this project, we sought to develop a longer-acting topical delivery platform using bacteria from the skin microbiome. The skin microbiome includes many species of harmless and even beneficial bacteria which are believed to colonize the skin and persist there stably for a long time. The central hypothesis of this project was that bacteria from the skin microbiome can be engineered to produce useful therapeutic molecules and then be applied to the skin to deliver those molecules continuously over time. *Bacillus subtilis* is a member of the skin microbiome and is frequently used to produce industrial products such as vitamins and enzymes [8-10]. Due to its safety and genetic tractability, we chose

B. subtilis to study the feasibility of using bacteria from the skin microbiome for drug delivery.

For one application of this technology, we sought to engineer *B. subtilis* to overproduce the antifungal molecule iturin A for the treatment of fungal skin infections. An estimated 20 to 25 percent of the world's population is affected by fungal skin infections, and the treatment for these conditions usually involves daily or twice daily topical application of an antifungal agent for one to six weeks [11-13]. The requirement for frequent application, however, can reduce patient adherence, making a more convenient delivery method ideal. *B. subtilis* naturally produces multiple antifungal molecules from the iturin, surfactin, and fengycin families [14-16]. In particular, iturin A is a broad antifungal agent that is effective against relevant fungi, including the main causes of athlete's foot [17-19]. Thus, our strategy was to engineer *B. subtilis* to overproduce iturin A in order to develop a more convenient antifungal treatment.

For the second application, we sought to engineer *B. subtilis* to produce the mosquito-repelling molecule 2-phenyethanol (2-PE) as a longer lasting mosquito repellant. Approximately half of the world's population lives in areas with risk of malaria transmission, with over 400,000 mortalities per year [20]. Other mosquito borne diseases such as dengue, chikungunya virus, and Zika virus similarly represent widespread global health threats. A critical approach to fighting these diseases is preventing mosquitoes from biting the skin in order to stop transmission of the disease. 2-PE has been identified as a volatile compound produced by human skin bacteria that repels mosquitoes, though its activity is limited to 1 to 2 hours due to its volatility [21-24]. We expected that continuous production of 2-PE on the skin by *B. subtilis* would overcome the volatility limitation and provide a safe, long-lasting protection against mosquitoes.

CHAPTER 2. BACKGROUND

2.1 Long term drug delivery to the skin

2.1.1 Medication adherence

Medication adherence refers to the degree to which patients follow through with their prescribed treatment regimen and represents a surprisingly difficult issue in the medical field. In fact, a review by Haynes et al. predicted that more global health benefits could be achieved by improving patient adherence to medications than any improvement to specific medications themselves [25]. Non-adherence to medication can result in sub-optimal treatment outcomes or severe complications later in life and is a particular concern for child, elderly, chronically ill, and poor patient populations [3, 26]. Non-adherence has been estimated to account for 33-69 percent of preventable drug-related hospital admissions, and proper adherence to medication is less than 50 percent in developed countries and is expected to be much lower in other parts of the world [1-3]. Forgetfulness, inconvenience, and fear of side effects are among the primary reasons for nonadherence [4-6]. To address these issues, the field of drug delivery works towards improving adherence by designing safer treatments, such as targeted delivery systems to minimize unwanted side effects, non-invasive delivery to make administration more comfortable, and extended-release platforms to decrease the number of required administrations [27].

2.1.2 Topical drug delivery

In the case of dermatology, topical drug delivery platforms are ideal because the therapeutic can be delivered directly to the site of action (the skin) in a minimally invasive way, typically by the patients themselves. This can help reduce the number of hospital visits, needles, unwanted side effects, and other barriers to obtaining treatment. However, despite the advantages associated with topical drug delivery, the ability to deliver drugs topically for an extended time is limited, and this can negatively impact patient adherence, especially in the case of chronic diseases such as psoriasis and atopic dermatitis [7]. Numerous studies have found high rates of non-adherence for topical drug delivery, and in some studies the rate of non-adherence was higher for topical drug formulations than for oral drug formulations [7]. There are many potential reasons for non-adherence to topical drugs, including delayed efficacy, aesthetic concerns, side effects, confusing application instructions, and time-consuming applications [7]. In a study of patients with psoriasis, once-daily treatment had significantly higher adherence than twice-daily treatment [28].

2.1.3 Current topical delivery platforms

Delivery platforms for the skin could either target drugs to the skin itself (topical delivery) or across the skin for systemic delivery (transdermal delivery). While most skin medications are intended for transdermal delivery, the applications described in this work are intended for topical delivery.

For both topical and transdermal delivery, medications are commonly applied to the skin via gels, ointments, lotions, creams, and sprays. An advantage of these types of topical formulations is their ease of application over the affected area of skin [29]. However, these formulations are also easily removed from the skin in a short amount of time and often require daily or more frequent application to be effective [7].

Patches have been developed to deliver therapeutics into the skin for a longer time, but these are limited in surface area, making them non-optimal for many potential applications [30].

2.2 The skin microenvironment

The skin serves as a barrier to maintain moisture levels inside the body and protect from pathogens on the outside [31, 32]. The main barrier function of the skin is provided by the stratum corneum, which is the outermost layer and about 10 μm thick; stratum corneum is composed of stacked, terminally differentiated keratinocytes (corneocytes) and intercellular lipids [31]. Through a process called desquamation, the stratum corneum is also continuously shed, with a total turnover time of 2-3 weeks [31]. Beneath the stratum corneum, the viable epidermis is an avascular tissue about 50 μm thick that is composed of keratinocytes, melanocytes (melanin production), Langerhans cells (immune system), and Merkel cells (sense of touch) [31, 32]. Below the epidermis, the dermis contains blood vessels, nerve fibers, and lymphatic vessels which serve many functions including immune responses, pressure regulation, temperature regulation, and nutrition for the epidermis [31]. Sweat glands are found on most of the skin and secrete sweat, which contains a mixture of water, salt, and nutrients such as vitamins and amino acids [33, 34]. Finally, hair follicles are found almost everywhere on the skin and contain sebaceous glands, which provide a relatively anoxic environment and secrete the fatty substance sebum that helps protect and maintain the pH of skin while also serving as a source of nutrition for certain bacteria [31, 33]. Hair follicles are considered reservoirs for bacteria in the skin, and hair follicle maps have been used to estimate that about a quarter of the bacteria found on skin originates from hair follicles [35].

The skin surface is a relatively inhospitable environment for microbes due to its low pH (around 5), low moisture, salty sweat, antimicrobial molecules, and continuous shedding of the stratum corneum [31, 33, 36]. Nevertheless, many different types of bacteria are able to survive on the skin, often in a mutually beneficial relationship with their hosts [36]. The skin microenvironment varies greatly across the body with differences in skin thickness, hair follicle density, gland activity, and level of occlusion [33, 36]. For the purposes of microbiome analyses, the skin is generally divided into sebaceous (e.g., back), moist (e.g., armpit), and dry (e.g., forearm) sites [33, 37].

2.3 The skin microbiome

The skin microbiome consists of a diverse community of microorganisms (viruses, fungi, and bacteria) colonizing many different areas of the body [33]. It is estimated that there are about 10^6 bacteria per square centimeter of skin based on analyses of skin biopsies, although the density varies by skin site [38, 39]. *Corynebacteria*, *Propionibacteria*, and *Staphylococci* are the most common genera found on the skin [36, 37].

2.3.1 Distribution and composition

Bacteria live on the skin surface as well as the inner layers of the stratum corneum, hair follicles, and sebaceous glands [33, 35, 40]. Some evidence suggests that the bacteria of the skin also extend below the epidermis into the dermis [41, 42]. In general, the dry and salty skin environment tends to promote the survival of gram-positive bacteria, such as *S. epidermidis*, over gram-negative bacteria, such as *E. coli* [43]. The density and diversity of bacteria on the skin varies according to skin site, and characteristic populations of bacteria inhabit each type of skin site, with sebaceous sites dominated by *Propionibacteria* and *Staphylococci*, moist sites dominated by *Corynebacteria* and *Staphylococci*, and dry sites exhibiting the most diverse bacterial population [33, 37]. Bacterial transplant studies between different body sites suggest that the characteristics of sebaceous sites exert more influence in shaping the resident bacterial community than those of dry sites [44]. Other factors influencing the composition of the skin microbiota include age, gender, and environmental factors such as cosmetic usage and geographical location [33].

2.3.2 Stability over time

An individual's microbiota is relatively stable over time [44]. The temporal stability of the skin microbiota is correlated with skin site [37]. In general, the differences in skin bacterial communities are smaller between days for a given individual than between individuals on a given day [44]. Certain strains of bacteria have been found to stably survive on human skin for a time scale of months to years [37, 45]. Analyses at the strain and single nucleotide variant level suggest that these bacteria are retained on the skin rather than reacquired over time from the environment [45]. Microbes that do not colonize the skin are considered transient and persist on the skin for hours to days [46].

2.3.3 Host-microbiome interactions

Commensal skin bacteria help protect the skin from pathogenic bacteria through competition over space and resources, release of antimicrobials, and interactions with the host immune system [36, 47]. The skin's immune system is able to discriminate between commensal bacteria and pathogens, and some commensal bacteria have been found to modulate the skin's innate immune response [33, 36].

2.3.4 *Bacillus subtilis*

Bacillus subtilis is a member of the skin microbiome that has been identified on forehead and plantar skin [8, 9, 43]. It is nonpathogenic and has natural antimicrobial properties against pathogenic staphylococci and fungi [48-50]. A *B. subtilis* strain as well as several enzymes produced by *B. subtilis* have generally regarded as safe (GRAS) status from the FDA, further supporting its potential for safe use in long term treatments [51]. It is also commonly used in biotechnology for the production of proteins, vitamins, and antibiotics because of its efficient protein secretion system, ease of cultivation, high genetic tractability, and low codon bias [10, 52, 53]. As a result, many tools and libraries have been developed for the genetic manipulation of *B. subtilis* [10, 54-57].

While *B. subtilis* can live and is metabolically active on human skin, it is not commonly found in the skin microbiome and is considered a more transient species acquired from the environment [8, 9, 34]. A study in which *B. subtilis* was applied topically to mouse ears found that *B. subtilis* in skin swabs returned to baseline levels within four days of the last application [58].

2.3.5 *Introducing a new strain into the skin microbiome*

Whether a new strain of bacteria can colonize a particular body site is an ongoing question that appears to depend on characteristics of both the host environment and the strain of bacteria [59-61]. A few studies have sought to address this topic using probiotics delivered to the gut microbiome. One study found that persistence of the probiotic *Bifidobacterium longum* in the human gut was correlated with low abundance of *B. longum* and underrepresentation of certain genes in the host's gut microbiome before treatment

[60]. This is consistent with the classical biological hypothesis of phylogenetic limiting similarity, in which competition between closely related species is greater than competition between distant species due to their shared niches, which suggests that the optimal choice for a successful engrafting strain may not be the most common species found in that niche [62]. Conversely, another study in mice found that high baseline levels of closely related species in the host gut microbiome was correlated with successful colonization by a new species, suggesting that the presence of a closely related species may indicate a favorable environment that supports growth of the new species as well [59]. Thus, the best choice for a successfully engrafting strain is complicated and not entirely predictable. In addition to the inherent challenge of introducing a new strain to an established community, producing a heterologous molecule such as a drug adds an additional metabolic burden on a bacterial cell which could hurt its ability to survive in a microbial community like the skin microbiome [63].

2.4 Genetic engineering

Modern biotechnology began about 40 years ago when *Escherichia coli* was first engineered to express a foreign antibiotic resistance gene, leading to a period of innovation in which microbes and even plant and mammalian cells were engineered to produce various biological molecules. Medicinal and industrial proteins are now commonly produced in industry by the genetic engineering of microbes, and many tools and techniques have been developed for the genetic manipulation of living organisms [10]. The most extensively studied organism for genetic engineering is *E. coli*, but there has been considerable work conducted using other organisms such as *B. subtilis* and *Saccharomyces cerevisiae* [10, 52].

A straightforward example of genetic engineering is engineering a cell to produce a heterologous protein that is encoded by inserted DNA. Small molecules, including many drugs, are more complicated because they are not encoded directly by the DNA and might require a pathway of multiple proteins that the cell would need to produce to obtain the small molecule.

2.4.1 Plasmid DNA

Plasmids are small, circular pieces of double-stranded DNA that are separate from a cell's chromosomal DNA. Plasmids are passed between bacteria cells through both vertical transmission, where copies are passed down to daughter cells during cell division, and horizontal transmission, in which genetic material is acquired from unrelated organisms [64]. Plasmids can be manipulated in the lab and inserted into bacteria cells to express foreign genes.

There are several methods for manipulating and assembling plasmid DNA. Restriction digest is a classic technique in which restriction enzymes that cleave specific DNA sequences are used to cut DNA at a particular site. Two pieces of DNA that have been cut to have complementary ends can then be joined together by ligation [65]. The disadvantages of restriction enzyme cloning are that it leaves a small 'scar' in the assembled DNA, and it requires a compatible plasmid with restriction sites in the appropriate location [66].

Several alternative plasmid assembly techniques have been developed, such as Gibson assembly, Golden Gate Assembly, Gateway cloning, TA cloning, and Ligation independent cloning [65, 67]. We used the Gibson assembly method for plasmid assembly in these studies. In this method, the DNA pieces to be assembled are PCR-amplified to have overlapping ends. A 5' exonuclease removes the 5' ends, allowing the exposed complementary DNA fragments to anneal; DNA polymerase then extends the 3' ends, and DNA ligase seals the nicks in the DNA [68]. The Gibson assembly method has been used to assemble plasmid DNA that is hundreds of kilobases in length, though the efficiency of the reaction decreases as the size and number of fragments increases [68, 69].

2.4.2 Genome insertion

While plasmids provide a relatively easy way to express high levels of proteins, they are limited by the need for antibiotics in the media for plasmid maintenance as well as issues with structural and segregational instability. Structural instability refers to changes of the DNA sequence through insertions, deletions, or point mutations; segregational instability refers to the appearance of plasmid-free cells in the culture due to some daughter cells not receiving the plasmid [70].

Due to these limitations, inserting the foreign genes directly into the bacterial genome is sometimes the preferred approach. Genome insertion can also lower the metabolic burden of protein expression because there is only one copy of the gene insert per cell, compared to tens to hundreds of copies per cell for plasmid-based expression, depending on the plasmid copy number [70]. Methods for inserting genes into the chromosome include transposon-based methods, phages, λ-red recombinase recombination, recA homologous recombination, cre-loxP recombination, and CRISPR/Cas9 [71].

For these proof-of-concept experiments, we used plasmid DNA for all manipulations of cells due to its ease of use, but commercial use would likely require the genes to be inserted into the chromosome for heightened stability and reduced risk of horizontal transmission.

2.4.3 Public opinions of genetic engineering

Probably the most widely known application of genetic engineering is in agriculture, where genetically modified plants save farmers time and money through advantages such as higher yields and disease resistance [72]. The use of genetic engineering in agriculture has brought the concept of genetically modified organisms (GMOs) to political debates, where it has been met with varying degrees of skepticism and regulation, especially in Europe [72]. However, studies on the source of this skepticism have found that negative opinions about genetic engineering are largely the result of a lack of perceived benefits rather than an excess of perceived risks [72-74]. In fact, benefit perception is thought to be a dominant factor in the acceptance of genetic engineering [73-75]. The use of GMOs in agriculture offers many advantages for farmers, who have widely adopted their use, but the main benefit for consumers, price reduction, is not as obviously connected to the use of GMOs [72]. For medical applications, where the end benefits are more obvious, the use of genetic engineering is viewed more positively [74, 75]. While the medical applications in the referenced studies are different from the applications proposed in this research, benefit perception being a primary driver of public opinion towards genetic engineering indicates that there could be a path to adoption for novel uses of GMOs provided the benefits are clear.

2.5 Bacteria as drug delivery vehicles

The natural symbiotic relationship between bacteria and the humans they colonize combined with existing technology to engineer bacteria to produce heterologous molecules creates an opportunity to engineer bacteria as a form of *in situ* drug delivery. Engineered live biotherapeutics are living microorganisms that have been engineered to treat, prevent, or cure human diseases.

2.5.1 Bacteria in medicine

The use of various forms of bacteria in medicine can be traced back thousands of years in human history. Reports from fourth-century China described the use of feces to treat multiple diseases, and fecal microbiota transplants (FMT), in which the feces of a healthy donor is given to a patient, became a part of mainstream medicine in the 1950s [76]. FMT is now a recommended treatment for patients with recurrent *C. difficile* infection, with a reported cure rate of up to 90% [76]. While the exact mechanism behind the success of FMT is not known, the general understanding is that the 'good bacteria' that are transplanted into the patient's colon out-compete pathogenic bacteria, such as *C. difficile*, allowing balance in the gut microbiome to be restored [76].

Probiotics have also gained attention as the importance of bacteria to human health has become increasingly apparent. Probiotics are live microorganisms that have beneficial effects, such as supporting a balanced gut microbiota and stimulating the host immune system [77]. The most evidence for the positive effects of probiotics has come from treatment of gastrointestinal disorders such as gastroenteritis and traveler's diarrhea, but probiotics have also been studied for many other conditions including allergies, asthma, and neurological diseases [77]. However, although probiotic strains have received GRAS status from the FDA, they have not been approved as a medical treatment due to a lack of sufficient data [77].

Non-virulent or attenuated strains of pathogenic bacteria are used as vaccines against some bacterial infections because they can induce immunity by mimicking a natural infection [78]. Live bacterial vaccines currently in use include the BCG vaccine, a live attenuated strain of *Mycobacterium bovis* for the prevention of tuberculosis; Vivotif, a live attenuated strain of *Salmonella typhi* for the prevention of typhoid fever; and Vaxchora, a live attenuated strain of *Vibrio cholerae* for the prevention of cholera [78-81].

2.5.2 Engineered live biotherapeutics in academic studies

There are several academic studies in which bacteria have been engineered to produce therapeutic molecules in multiple different body sites *in vivo.* For example, *Lactobacillus zeae* was engineered to express single chain fragment variable antibodies (scFv) in the mouth to reduce development of caries in rats [82]. *E. coli* was engineered to express N-acylphosphatidylethanolamines in the gut to reduce obesity in mice [83]. *Streptococcus gordonii* expressing scFv was used to treat vaginitis in rats [84]. An engineered strain of *Lactococcus lactis* producing interleukin-10 has even been tested in a phase 1 human clinical trial to determine the safety and feasibility of using this platform as a treatment for Crohn’s disease [85]. Less work has been done using bacteria to deliver drugs to the skin, except for *Lactobacillus reuteri* engineered to produce the molecule CXCL12 in the wound environment to accelerate wound healing [86].

2.5.3 Engineered live biotherapeutics in industry

There is commercial interest in this field from two companies working to develop engineered bacteria as topical drug delivery agents [87]. Azitra has three preclinical products using engineered *Staphylococcus epidermidis* to treat skin barrier disorders with proteins such as fillagrin [87, 88]. Likewise, Xycrobe Therapeutics' lead product is a strain of *Propionibacterium acnes* engineered to deliver IL-10 for inflammatory skin disorders [87, 89]. Other companies working in the space of engineered live biotherapeutics for areas other than the skin include Osel Inc, which has a preclinical stage engineered strain of *Lactobacillus* expressing an HIV inhibitor intended for topical vaginal application [90]; Oragenics, which conducted phase 2 clinical trials with an engineered strain of *Lactococcus Lactis* expressing human Trefoil Factor 1 for the treatment of oral mucositis [91]; Novome, which has an engineered strain of gut bacteria in phase 1 clinical trials for the treatment of enteric hyperoxaluria [61]; and Synlogic, which has multiple preclinical and clinical stage strains of engineered bacteria intended to treat metabolic disorders in the gastrointestinal tract. Zbiotics has a commercially available strain of *B. subtilis* marketed as a genetically modified food which produces acetaldehyde dehydrogenase in the gut for hangover prevention [92].

2.6 Skin fungal infections

Dermatophytoses are fungal infections of the skin. Common dermatophytoses include tinea pedis (Athletes' foot), tinea corporis (ringworm), tinea capitis (scalp infection), and onchomycosis (nail infection). An estimated 20 to 25 percent of the world's population is affected by fungal skin infections, and it is estimated that over $500 million is spent each year to treat these conditions [11, 12].

2.6.1 Athlete's foot

Athlete's foot is a fungal skin infection in the feet that can result in white and soggy skin between the toes, dry and scaly skin on the soles of the feet, or red and blistered skin all over the foot [93]. *Trichophyton rubrum, Trichophyton mentagrophytes,* and *Epidermophyton floccosum* are the most common causes of athlete's foot [11]. Higher levels of athlete's foot are associated with urbanization, communal showers, sports, and occlusive footwear [11, 12].

2.6.2 Treatments for Athlete's foot

Topical antifungal application is the primary form of treatment for athlete's foot. These treatments involve daily or twice daily application of an antifungal agent (typically azoles or allylamines), such as terbinafine (Lamisil AT), clotrimazole (Lotrimin AF), or miconazole (Monistat), for one to six weeks [13, 94]. More severe infections may require oral antifungal medication instead of topical medication [13].

B. subtilis naturally produces multiple antifungal molecules from the iturin, surfactin, and fengycin families [14-16]. In particular, iturin A is a broadly antifungal lipopeptide that is effective against relevant fungi, including the main causes of athlete's foot, but is inactive against most bacteria [17-19, 95]. Iturin A exerts its effect by disrupting the fungi's plasma membrane, resulting in the release of cellular contents, degradation of phospholipids, and cell death [18, 95].

2.7 Mosquito-borne diseases

2.7.1 Malaria

Malaria is a mosquito-borne disease caused by a parasite that causes fever, headache, and chills, leading to severe illness or death if left untreated [20]. The two predominant malaria parasites (*Plasmodium falciparum* and *Plasmodium vivax*) cause over 200 million clinical cases of malaria each year across approximately 100 countries, with over 400,000 mortalities per year, disproportionately occurring in children under five years old [20].

2.7.2 Arboviruses

Arboviruses are viruses that are transmitted to humans through infected arthropods (e.g., mosquitoes, fleas, and ticks). There are hundreds of arboviruses world-wide, and the most common viruses causing human disease include Dengue fever, chikungunya virus, and Zika virus, all of which are transmitted by mosquitoes [96].

There are 96 million clinical cases of Dengue per year across 128 countries. While half of Dengue infections are asymptomatic, symptoms can include fever, rash, muscle pain, headache, and vomiting, and severe cases can lead to death [96]. Chikungunya transmission has been identified in over 60 countries worldwide, and symptoms include severe joint stiffness and pain [96]. Zika virus is active in 86 countries; zika infections are mostly asymptomatic, but it is associated with birth defects in infants born to mothers who were infected during pregnancy and can also cause symptoms including fever, rash, pink eye, joint stiffness, muscle pain, eye pain, and weakness [96].

2.7.3 Strategies to prevent mosquito-borne diseases

While preventative vaccines have been a long-pursued area of research, there is currently no commercially available vaccine for Zika virus or chikungunya virus, and the newly approved malaria vaccine is about 30% effective, indicating that it is critical to address transmission to prevent the disease [96-98].

The clearest way to prevent transmission of mosquito-borne diseases is to prevent mosquitoes from biting the skin. Efforts to prevent mosquito bites have led to significant reductions in prevalence with tools such as larval source-reduction campaigns, toxic sugar traps, lethal ovitraps, insecticidal bed nets, indoor spraying of insecticides, and mosquito repellants [20, 96]. Insecticidal bed nets have been the most successful approach, accounting for 69% of prevented malaria cases [20]. However, the finite lifetime of bed nets and their inability to protect people outside of bed suggest the importance of complementary approaches to prevent transmission.

2.7.4 Mosquito repellents

It is important for mosquito repellants to both repel mosquitoes and remain effective for at least several hours after application to the skin [23]. Thousands of chemicals have been tested to determine their suitability as a mosquito repellant [23].

N,N-diethyl-m-toluamide (DEET) has been considered the gold standard for mosquito repellants for decades. Formulations containing DEET concentrations at 20% or higher have a complete protection time of 4 to 6 hours [99]. While there are some lingering safety concerns about DEET in the public opinion, the EPA concluded that insect repellants containing DEET were safe [100]. However, there is some uncertainty about the maximum safe concentration of DEET products [101].

2-phenylethanol (2-PE) is a naturally occurring molecule with a rose-like odor that has been isolated from the headspace of skin microbiome samples from individuals that are less attractive to mosquitoes [21, 102]. It is a volatile molecule that repels mosquitoes at least in part through activation of the odorant receptor CquiOR4 [24]. Though it activates a different receptor, 2-PE has been found to have repellant activity comparable to DEET, but it has a lower duration of protection due to its volatility [23, 24].

CHAPTER 3. OVERVIEW OF AIMS

3.1 Aim 1 Assess feasibility and safety of *Bacillus subtilis* as a skin drug delivery platform

Non-adherence to medication is a major challenge in healthcare that results in worsened treatment outcomes for patients. Reducing the frequency of required administrations could improve adherence but is challenging for topical drug delivery due to the generally short residence time of topical formulations on the skin. In this study, we sought to determine the feasibility of developing a microbiome-based, long-acting, topical delivery platform using *Bacillus subtilis* for drug production and delivery on the skin, which was assessed using green fluorescent protein as a model heterologous protein for delivery. We developed a computational model of bacteria population dynamics on the skin and used its qualitative predictions to guide experimental design choices. Using an *ex vivo* pig skin model and a human skin tissue culture model, we saw persistence of delivered bacteria for multiple days and observed little evidence of cytotoxicity to human keratinocyte cells *in vitro*. Finally, using an *in vivo* mouse model, we found that the delivered bacteria persisted on the skin for at least one day during every-other-day application and did not appear to present safety concerns. Taken together, our results support the feasibility of using engineered *B. subtilis* for topical drug delivery.

3.2 Aim 2 Determine efficacy of engineered *Bacillus subtilis* for topical delivery of antifungal agent

Fungal skin infections are a common condition affecting 20-25 percent of the world population. While these conditions are treatable with regular application of an antifungal medication, we sought to develop a more convenient, longer-lasting topical antifungal platform that could increase patient adherence to treatment regimens by using *Bacillus subtilis*, a naturally antifungal bacteria found on the skin, for drug production and delivery. In this aim, we engineered *B. subtilis* for increased production of the antifungal lipopeptide iturin A by overexpression of the pleiotropic regulator DegQ. The engineered strain had a more-than 200% increase in iturin A production accompanied by slower growth but the same terminal cell density. In an *in vitro* antifungal assay, we found that despite its higher iturin A production, the engineered strain was less effective at reducing the growth of the pathogenic fungus *T. mentagrophytes* compared to the parent strain. The reduced efficacy of the engineered strain may be explained by its reduced growth rate, which highlights the need to address trade-offs between titers and other figures of merit during metabolic engineering.

3.3 Aim 3 Determine efficacy of engineered *Bacillus subtilis* producing mosquito repellent

Mosquito-borne diseases such as malaria, dengue, chikungunya virus, and Zika virus represent a widespread health burden resulting in hundreds of thousands of deaths each year. In this aim, we sought to develop a long-acting mosquito repellant based on *B. subtilis* engineered to continuously produce the repellant 2-PE on the skin. Initial progress was made towards expressing an expanded shikimate pathway for 2-PE production, but we encountered challenges related to plasmid stability in *B. subtilis*. Challenges related to developing genetically engineered bacteria-based therapies as well as specific challenges of working with *B. subtilis* are discussed.

CHAPTER 4. ASSESS FEASIBILITY AND SAFETY OF BACILLUS SUBTILIS AS SKIN DRUG DELIVERY PLATFORM

4.1 Introduction

Improving patient adherence to existing medications is a major challenge in healthcare that, due to its wide-reaching nature, could yield greater health benefits than improvements to any specific medication [25]. Non-adherence can result in sub-optimal treatment outcomes or significant complications, which are particular concerns for pediatric, geriatric, chronically ill, and poor patient populations [3, 26]. In fact, nonadherence accounts for an estimated 10% of drug-related hospital admissions, and proper adherence to medication is less than 50% in countries with advanced economies; it is expected to be much lower in other parts of the world [3].

Improved drug delivery platforms have the potential to make medication adherence easier for patients by minimizing side effects, allowing more comfortable administration routes, and reducing the number of required administrations [103]. In the case of dermatology, topical drug delivery platforms are attractive because the therapeutic can be delivered directly to the site of action (i.e., the skin) in a non-invasive way, typically by patients themselves. This approach avoids the use of needles and other barriers to obtaining treatment, which can help minimize hospital visits and unwanted side effects.

Despite the advantages of topical drug delivery, it is difficult to deliver drugs topically for an extended time. Gels, ointments, and lotions often require daily or more frequent application because of their limited residence time on the skin due to removal by clothing, sweating, rubbing and other effects [104]. Patches have been developed to deliver therapeutics to the skin for a longer time, but patches are limited in surface area, making them non-optimal for indications requiring treatment over large areas of skin [30]. The need for frequent administration of topical delivery formulations can negatively impact patient adherence, especially in the case of chronic diseases such as psoriasis and atopic dermatitis [7].

An exciting area emerging in the field of drug delivery is the use of bacteria as drug delivery vehicles [91, 105]. The symbiotic relationship between bacteria and the humans they colonize combined with existing technology to engineer bacteria to produce heterologous molecules creates an opportunity to used engineered bacteria as a form of *in situ* drug production and delivery. A growing body of work has reported the design of engineered bacteria to produce therapeutic molecules in the gut, vaginal tract, skin, and mouth [91, 105, 106].

In the context of dermatology, there are preclinical and clinical-stage companies currently working to develop engineered bacteria as topical drug delivery agents. Notable examples include Azitra, which has multiple preclinical products using engineered *Staphylococcus epidermidis* to treat skin disorders [87]; Ilya Pharma, which has developed a clinical-stage strain of *Limosilactobacillus reuteri* expressing the chemokine CXCL12 to promote wound healing [86, 106]; and Xycrobe Therapeutics, which is developing a preclinical strain of *Cutibacterium acnes* engineered to deliver IL-10 for acne vulgaris [106].

In this study, we investigate the potential use of engineered *Bacillus subtilis* as a topical drug delivery platform. *B. subtilis* may have advantages over other candidate bacteria as a platform for drug delivery to the skin because of its safety profile and genetic tractability. It is found in the skin microflora and is metabolically active on the skin [8, 9]. It is nonpathogenic and has natural antimicrobial properties against pathogenic staphylococci and fungi [48, 49, 107]. *B. subtilis* has Generally Regarded As Safe (GRAS) status from the FDA, and multiple *B. subtilis* probiotic products as well as a genetically modified strain of *B. subtilis* are currently commercially available [51, 105, 108].

An important characteristic of *B. subtilis* is that it is commonly used in biotechnology for the production of proteins, vitamins, and antibiotics because of its efficient protein secretion system, ease of cultivation, and high genetic tractability [10]. As a result, many tools and libraries have been developed for its genetic manipulation, making the design and implementation of engineered *B. subtilis*-based biotherapeutics more straightforward than the similar use of other, non-model organisms [10, 57].

Motivated by these characteristics of *B. subtilis*, we sought to assess the feasibility of using *B. subtilis* as a topical drug delivery platform in terms of the duration of drug production on skin and safety. To facilitate characterization, we used an engineered strain of *B. subtilis* producing green fluorescent protein (GFP) as an easily detectable, model heterologous protein. Through a combination of experimental studies supported by computational modeling, we identified that microbiome-based drug delivery via *B. subtilis* is feasible, with certain constraints.

4.2 Methods

4.2.1 Computational model

We used an agent-based model to predict the survival of bacteria on the skin because of the flexibility of this modeling approach in situations where detailed mathematical knowledge of the governing equations is incomplete [109, 110]. In agent-based modeling, individual agents are defined with a set of rules that determine their behavior, and the model simulates interactions between different agents [109].

We adapted the code for Gutlogo, an agent-based model made in NetLogo to simulate population dynamics of the gut microbiota, to represent the skin environment [110]. Code for our model, Skinlogo, is publicly available at https://github.com/gtStyLab/skinlogo.git. The basic rules of the Gutlogo model were preserved in our skin model. Each agent represents a bacterium that can 'eat' a set of defined carbon sources. Each agent has an energy level that starts at 100 and decreases at each time step down to 0 within a simulated day if it does not eat. Agents with an energy level of 0 die, and agents with an energy level above 50 divide based on their age and a defined doubling time at which their energy level is halved and a new agent of the same species with half the energy level is created. Agents with an energy level below 80 are considered 'hungry'; on each time step (representing one minute), the model runs through a list of the hungry bacteria and the available nutrients. If a nutrient that is present is a carbon source for a hungry bacterium, the bacterium will consume that nutrient, the number of available units of that nutrient will decrease by 1, and the energy level of the bacterium increases by the energy number assigned to that nutrient. The model incorporates stochasticity through random placement of bacteria in the modeling space, random assignment of each bacterium's age when the simulation is initiated, and a random selection process through which hungry bacteria are matched with available nutrients.

Beyond these basic principles, other aspects of the model were adjusted to better reflect the skin microbiome. The original Gutlogo model includes a flow component to simulate movement through the gut, which we removed for modeling the skin. We modified the species represented in the model to be reflective of the skin microbiome, and we changed the types and amounts of carbon sources to reflect those found on human forearm skin in previously reported literature [111, 112]. We also included the effects of antibiotics in the model. The endogenous populations were defined as having a minimum inhibitory concentration (MIC) of 10 $\mu g/cm^2$ antibiotic. If there was greater than 10 $\mu g/cm^2$ antibiotic around a bacteria agent, it died, and the amount of antibiotic in the region decreased by 10 $\mu g/cm^2$. The added, engineered *B. subtilis* population was modeled as having no sensitivity to the antibiotic.

The agents in our model comprised three genera of bacteria that are commonly found in the skin microbiome at different abundances, consisting of endogenous *Corynebacteria*, S*taphylococci*, and *Acinetobacter*, as well as our introduced *B. subtilis* [113]. The model was initiated with equal starting numbers of the endogenous populations, and *B. subtilis* was introduced after they appeared to reach steady state (3.125 days). Cell doubling times were based on reported doubling times of representative species in nutrient-rich media (Table 4.1). Carbon sources for each type of bacteria were approximated according to reported carbon source utilization for representative species (Table 4.1). For the purpose of hypothesis generation in the model, malate was considered a carbon source for only the *B. subtilis* agents because malate is a preferred carbon source for *B. subtilis* that has been shown to selectively recruit *B. subtilis* compared to other soil bacteria when secreted from plant roots [114, 115].

Table 4.1. Bacterial agents in model

Bacteria Agent Name	**Doubling Time**	**Carbon Sources**	**References**
Acinetobacter	40	Alanine, histidine, proline, phenylalanine, tyrosine	[116, 117]
Corynebacteria	90	Glucose, serine, alanine, histidine, proline, fatty acids	[118-120]
Staphylococci	50	Glucose, serine, threonine, glycine, alanine, histidine, proline, valine, leucine, isoleucine	[121-123]
B. subtilis	24	Glucose, alanine, histidine, proline, malate	[114, 124-127]

Amounts of glucose, amino acids, and fatty acids initially on the skin and their replenishing rates (Table 4.2) were calculated from reports of values extracted from the forearm of human volunteers [111, 112]. For simplicity, fatty acids were assumed to be saturated with a chain length of 16 carbons [128]. Energy numbers in the model refer to the increase in energy gained from eating a given nutrient and were determined based on the net yield of ATP generated from complete catabolism through the TCA cycle, assuming ratios of 2.5 NADH per ATP and 1.5 $FADH_2$ per ATP [129]. Using values obtained from literature to provide initial estimates, carbon source starting amounts and replenishing rates were then adjusted by increasing their values 10-fold and energy numbers were reduced 10-fold to prevent complete death or overgrowth of populations in the model (Table 4.2), following an approach similar to that taken in the Gutlogo model [110]. For each simulation, the model was run three times, and the average and standard deviation across three simulations was reported.

Table 4.2. Carbon sources in model

Carbon source	**Starting amount** ($nmol/cm^2$)	**Replenishing rate** ($nmol/cm^2$)	**Energy** (ATP)	**References**
Glucose	12	0.01	3.2	[111, 129]
Serine	3700	4.46	1.25	[111, 130]
Threonine	840	1.00	1.9	[111, 130]
Glycine	2500	3.42	1.25	[111, 130]

Table 4.2 continued				
Alanine	1840	2.08	1.25	[111, 130]
Histidine	1010	1.04	2.25	[111, 130]
Proline	420	0.46	2.75	[111, 130]
Leucine/Isoleucine	330	0.31	3.3	[111, 130]
Valine	300	0.33	2.75	[111, 130]
Fatty acids	1170	4.46	10.6	[112, 129]
Phenylalanine/ Tyrosine	295	0.31	3.0	[111, 130]
Malate	n/a	n/a	1.0	[129]

4.2.2 Bacterial culture

Strains and plasmids used in this study are listed in Table 4.3. *B. subtilis* 168 and *Escherichia coli* DH5α were cultured in lysogeny broth (LB), consisting of 0.5% w/v yeast extract (Life Technologies, Carlsbad, CA), 1% w/v NaCl, and 1% w/v tryptone (Life Technologies), at 37 °C with shaking at 200 rpm. Growth was measured by absorbance at 600 nm. Fluorescence and absorbance were measured with a Biotek Synergy H4 Hybrid Microplate Reader (Agilent, Santa Clara, CA). For fitness comparisons, each strain containing a different ribosome binding site (RBS) was inoculated into a co-culture with equal volume of the parent strain *B. subtilis* 168 in LB media without antibiotics, and fluorescence was compared with that of the engineered strain in monoculture. Fluorescence was normalized by absorbance at 600 nm, and the normalized fluorescence values after 24 h in culture were reported.

Table 4.3. DNA plasmids and bacterial strains

Name	**Description**	**Reference/Source**
DNA plasmids		
pRB374	*E. coli* - *B. subtilis* shuttle plasmid; amp^r, kan^r	ATCC[1], [55]
pGFP-RBSX	pRB374; vegII: *GFP*; containing ribosome binding sites 0-5 (X = 0-5)	This study
Bacterial strains		
DH5α	*E. coli* high efficiency competent cells	NEB[2]
EC-GFP	DH5α carrying pGFP-RBS5	This study
168	*B. subtilis* parent strain	ATCC
15841	Antifungal lipopeptide-producing strain of *B. subtilis*	ATCC, [131]
BS-GFP	168 carrying pGFP-RBS5	This study

[1] ATCC, American Type Culture Collection (Manassas, VA). [2] NEB, New England Biolabs (Ipswich, MA)

4.2.3 Plasmid assembly

DNA primers used in this study are listed in Supplementary Table 4.4. All plasmids were assembled in *E. coli* DH5α and then transformed into *B. subtilis* 168. *E. coli* cells were transformed by heat shock following the NEB high efficiency transformation protocol (New England Biolabs, Ipswich, MA). *B. subtilis* cells were transformed by electroporation using an ECM 600 electroporator (BTX, Holliston, MA) set to 2.1 kV, 129 Ω and 50 µF with 1 mm gap cuvettes. Superfolder green fluorescent protein (GFP) was synthesized by Eurofins (Louisville, KY). GFP was inserted into pRB374 by Gibson assembly, resulting in plasmid pGFP-RBS0 [68]. For RBS analysis, each RBS was incorporated into the forward primer for amplification of GFP, which was inserted into pRB374 by Gibson assembly, resulting in the plasmids pGFP-RBSX (X = 1-5).

The plasmid pGFP-RBS5 was used to express GFP in *E. coli* and *B. subtilis* for *ex vivo* and *in vivo* experiments (*EC-GFP* and *BS-GFP*, respectively). During strain and plasmid construction, *E. coli* and *B. subtilis* were grown in LB media with 100 µg/ml carbenicillin and 10 µg/ml kanamycin, respectively, at 37°C.

4.2.4 Mammalian cell culture

HaCaT immortalized keratinocyte cells (AddexBio, San Diego, CA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM, 4.5 g/L glucose, 1 mM sodium pyruvate, 4 mM L-glutamine, ThermoFisher, Waltham, MA) containing 10% v/v fetal bovine serum (FBS, ThermoFisher) and 1% v/v penicillin-streptomycin (ThermoFisher). Cell cultures were grown in a humidified incubator set to 37 °C and 5% CO_2.

4.2.5 LD_{50} Cytotoxicity assays

HaCaT cells were seeded in 96-well plates at a density of $1x10^3$ cells per well and incubated for 3 days at 37 °C and 5% CO_2 in a humidified incubator. After 3 days, the medium in the wells was replaced with keratinocyte media containing either LB medium or sterile-filtered supernatant from overnight *B. subtilis* 168 or 15841 cultures at concentrations ranging from 0.00019% to 50% v/v. Bacterial supernatants were adjusted to pH 7 with 1 M NaOH before being added to mammalian cells. Plates were incubated another 3 days, after which the medium was replaced with sterile phosphate-buffered saline (PBS), 10 µL alamarBlue (ThermoFisher) was added to the wells, and plates were incubated for 1-4 h at 37 °C.

Wells were read using a Biotek Synergy H4 Hybrid Microplate Reader according to the assay protocol, either with absorbance at 570 nm and 600 nm or with fluorescence using a 560 nm excitation wavelength and 590 nm emission wavelength. For the positive toxicity control, 2% Tween 20 was added to the cell culture media to kill the cells. For the negative toxicity control, no supernatant was added to the cell culture media.

For absorbance measurements, the percent reduction of alamarBlue reagent was calculated according to the assay protocol using the equation $100*(\varepsilon_2 A_1 - \varepsilon_1 A_2)/(\varepsilon_2 A'_1 - \varepsilon_1 A'_2)$, where ε_1 and ε_2 are the molar extinction coefficients of alamarBlue at 570 nm and 600 nm, respectively, A_1 and A_2 are the absorbances of the treated wells at 570 nm and 600 nm, respectively, and A'_1 and A'_2 are the absorbances of the negative toxicity control at 570 nm and 600 nm, respectively. The average value for the positive toxicity control (2% Tween 20, expected to correspond to complete cell death) was subtracted from all measurements, and the resulting values were normalized by the negative toxicity (no treatment) control. The calculated values were plotted and fitted to a line using a simple linear regression, and LD_{50} was calculated as the concentration that corresponded to 50% cell survival.

4.2.6 Preparation of bacteria for skin model experiments

Overnight cultures were diluted in LB and grown with shaking at 200 rpm and 37 °C with appropriate antibiotics (100 µg/ml carbenicillin for *E. coli* and 10 µg/ml kanamycin for *B. subtilis*) until they reached an absorbance at 600 nm wavelength of approximately 0.6. Cells were then diluted 1:10 in fresh LB with appropriate antibiotics to be inoculated onto skin models.

4.2.7 Pig skin model

Six mm biopsy punches were taken from *ex-vivo* pig ear inner pinna skin (Pel-freez, Rogers, AR). The skin pieces were rinsed in 0.1% v/v peracetic acid (Pfaltz & Bauer, Waterbury, CT) diluted in sterile PBS, adjusted to pH 7.0-7.4 with 1 M NaOH, for 3 h to clean microorganisms from the skin surface [132]. The skin was then rinsed in two washes of sterile PBS for a total of 30 min to remove residual peracetic acid. Skin pieces were loaded into an HTS Transwell 96-well Permeable Support plate with a black receiver plate (8 µm pore size, Corning), with sterile PBS in the bottom wells refilled daily to maintain moisture in the skin.

Bacteria were inoculated onto the surface of the skin pieces as 10 µL of *BS-GFP* or *EC-GFP* in LB. For the malate and kanamycin experiments, the added bacteria were supplemented with L-malic acid (10 µL of either 0.1 M or 1 M in sterile PBS, Eastman, Kingsport, TN), which was neutralized to pH 7.0 with 5 M NaOH before application to skin, or kanamycin (10 µL of 50 µg/ml or 100 µg/ml in sterile PBS). *B. subtilis* 168 in LB and sterile PBS were used as negative controls, and 100 µg/mL rGFP (VWR, Radnor, PA) in sterile PBS was used as a positive control. Plates were stored at 32 °C, with lids raised slightly up from the plate to prevent condensation on the skin pieces.

Fluorescence was measured at 475 nm excitation and 510 nm emission wavelengths using a fluorescence area scan with matrix size of 5 x 5, set to gain 70, with a Biotek Synergy H4 Hybrid Microplate Reader. The average fluorescence value of the sterile PBS group was subtracted as background at each time point. The derivatives of the resulting fluorescence curves were estimated using a central difference approximation. GFP production was considered to have stopped when the derivative became negative.

Rate constants were determined by plotting GFP fluorescence values over time for individual samples on a log-log scale and taking the average slope of the trendlines. We interpret a greater rate constant to indicate a greater GFP production rate.

4.2.8 Human skin tissue culture model

Full thickness human skin tissue cultures were obtained from MatTek (Epiderm FT model, Ashland, MA). Upon arrival, skin tissues were equilibrated at 37°C and 5% CO_2 with antibiotic- and antifungal-free media provided by MatTek. Skin tissue culture media was changed daily, per the manufacturer protocol.

After equilibration, 20 µL of human skin swab solution was applied to the surface of the skin tissue. For human skin swabs, a sterile HydraFlock swab (Puritan, Guilford, ME) was soaked in sterile PBS with 0.1% v/v Tween 20 and gently rubbed on forearm skin for about 30 s. The flocked tip of the swab was then broken off into the same tube of sterile PBS with 0.1% v/v Tween 20 and stored for use. The same volume of swab solution (20 µL) was plated on LB agar to monitor microbial load (Supplementary Figure 4.12).

After inoculation of human skin swab solution, skin tissue cultures were incubated at 37°C and 5% CO_2 for 24 h, at which time 20 µL of either LB media or BS-GFP solution was applied to the tissue surface. For fluorescence measurements, cassettes holding the skin tissue culture samples were transferred to a 6-well plate with black tape lining the walls of the individual wells. Fluorescence was measured with 475 nm excitation and 510 nm emission wavelengths using a fluorescence area scan with matrix size of 5 x 5, set to gain 70, with a Biotek Synergy H4 Hybrid Microplate Reader. The average fluorescence value of the LB media group was subtracted at each time point. The derivative of the resulting fluorescence curve for BS-GFP was estimated using a central difference approximation. GFP production was considered to have stopped when the derivative became negative.

4.2.9 Mouse model

Experiments with mice were approved by the Georgia Tech Institutional Animal Care and Use Committee. Six- to eight-week-old SKH1 hairless mice from Charles River (Wilmington, MA) were used for *in vivo* mouse experiments. A Covance Infusion Harness (Instech, Plymouth Meeting, PA) was used to minimize grooming at the application site once bacteria were applied to the skin. Mouse toenails were clipped at the start of the study and once per week thereafter to prevent tampering with the harnesses. Mice were housed individually to prevent contamination and tampering with harnesses.

Two days before the start of the experiment, the back of each mouse was cleaned with an isopropyl alcohol wipe and fitted with a harness. Test solutions of either *BS-GFP*, *B. subtilis* 168, or LB alone (20 µL) were applied to the protected area of skin under the harness every 2 days, with daily skin swabs under the protected area of the harness to determine survival of bacteria. For swabbing, a sterile HydraFlock swab was soaked in sterile PBS with 0.1% v/v Tween 20 and gently rubbed on the skin for about 30 s. The flocked tip of the swab was then broken off into the same tube of sterile PBS with 0.1% v/v Tween 20 and stored for plating.

Swab samples were plated on LB agar with 10 µg/mL kanamycin as well as LB agar with no antibiotic to monitor the bacterial load of swabs. Presence of *BS-GFP* in swabs was determined by counting green colony forming units (c.f.u.) on kanamycin plates. Daily swabbing continued until c.f.u. counts were negligible. Mice were then sacrificed, and a 5 mm biopsy skin sample was taken from the area covered by the harness for histological analysis.

4.2.10 Histology

Mouse skin was stored in 10 % neutral buffered formalin for at least 24 h after harvesting. Samples were then transferred to Optimal Cutting Temperature Compound (ThermoFisher) and frozen on dry ice. Skin sections measuring 10 µm thick were taken using a Cryostar NX70 cryostat (ThermoFisher) with blade and block temperature set to -

20 °C. Sections were hematoxylin and eosin stained with an Autostainer XL (Leica, Wetzlar, Germany).

4.2.11 Statistical analysis

Results are presented as mean +/- standard deviation. Two-way ANOVA followed by Dunnett's multiple comparisons test was used to fluorescence in pig skin model. One-way ANOVA followed by Dunnett's multiple comparisons test was used to compare rate constants, LD_{50} values, fluorescence in liquid culture, and doubling times. Doubling times were calculated by fitting the mid-exponential phase of the growth curves to an exponential growth equation. Statistical analyses and linear regressions were performed using GraphPad Prism version 9.4.1 for Windows (San Diego, California, www.graphpad.com). P-values less than 0.05 were considered significant.

4.3 Results

4.3.1 Survival and production of heterologous protein by B. subtilis on skin

Model simulation. In our overall approach to assess the viability of *B. subtilis*-based skin drug delivery, we relied on a hybrid computational and experimental approach where initial model predictions helped to guide experiments. We first developed an agent-based model for bacterial population dynamics on skin based on the Gutlogo model of population dynamics in the gut [110]. This model predicted that the populations representing *Corynebacteria*, *Staphylococci*, and *Acinetobacter* would reach a steady state comprising about 84% *Corynebacteria*, 15% *Staphylococci*, and 1% *Acinetobacter*. When *B. subtilis* was introduced at timestep 4500 (3.125 days), it survived for about half a day before quickly dying off, after which the other species largely returned to their previous steady-state levels (Figure 4.1).

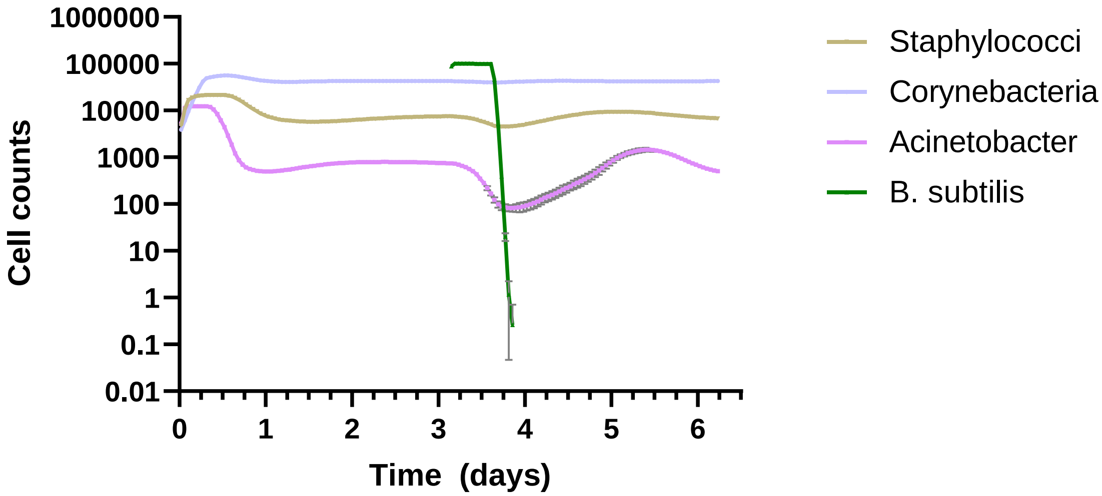

Figure 4.1. Simulation of skin bacteria population dynamics using an agent-based model. Population representing B. subtilis was added on day 3.125. Lines represent mean values and error bars represent standard deviation of three simulations. In most cases, the error bars are smaller than the line thickness.

Experimental findings. Before assessing survival time of *B. subtilis* on skin experimentally, we first wanted to account for an additional effect not considered by the computational model, which could further decrease *B. subtilis* survival. *In situ* production of a therapeutic for delivery could have a substantial growth burden on the cells, providing a negative selection pressure, so we sought to identify the impact on cell growth of expressing a heterologous reporter protein from a plasmid through cell culture experiments. We used GFP as a simple model protein, which could represent either a protein directly serving as a therapeutic or an enzyme used to synthesize a small-molecule therapeutic.

On the reporter plasmid, we tested six different RBS candidates expected to yield a variety of expression levels to determine if there were expression-dependent effects on cell fitness. Using doubling time, fluorescence in culture, and fluorescence in co-culture with the parent (non-fluorescent) strain to monitor cell fitness, we identified that GFP expression had small (albeit significant) effects on cell fitness (Supplemental Figure 4.11). To make later measurements of *B. subtilis* survival and protein expression as sensitive as possible, we used RBS5 in strain *BS-GFP* for experiments moving forward, as it yielded almost an order of magnitude more GFP expression than any other RBS with minimal additional impact on cell growth.

We used the resulting strain as the basis for experiments in an *ex vivo* pig skin model. On pig skin that had microorganisms cleaned from the surface (i.e., cleansed, but not sterile), the *BS-GFP* strain apparently survived on the skin and produced GFP for about 4 days, after which fluorescence reached a plateau (Figure 4.2). This duration of survival was different from that seen in the model simulation, probably because there was only limited competition with other bacteria on the cleansed pig skin and because there was no source of additional nutrients *ex vivo*. Comparing *B. subtilis* with *E. coli*, a gram-negative species with limited survival on human skin [43], we found that *BS-GFP* produced more GFP than *EC-GFP* in the pig skin model (two-way ANOVA, $p < 0.0001$). Taken together, these results support the potential viability of *B. subtilis* as a skin drug delivery platform.

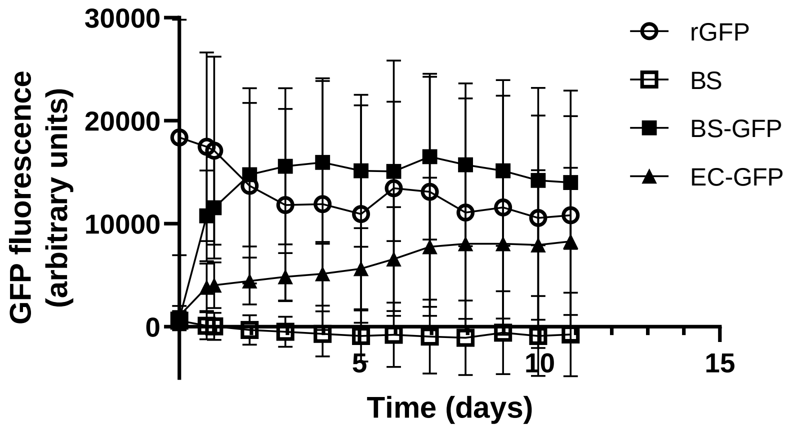

Figure 4.2. Fluorescence of GFP expressed by B. subtilis over time in an ex vivo pig skin model. rGFP: recombinant GFP added to skin surface at time 0; BS: B. subtilis strain 168; BS-GFP: B. subtilis strain 168 harboring pGFP-RBS5 plasmid; EC-GFP: E. coli strain DH5α harboring pGFP-RBS5 plasmid. Data show mean +/- standard deviation of 8 replicates.

4.3.2 Effect of added antibiotics

A way to extend the lifetime of added *B. subtilis* in a skin microbiome may be to add a selective pressure that provides an advantage for *B. subtilis* compared to the native microbiome. We investigated one way of forcing a selective advantage: adding an antibiotic to the environment to which *B. subtilis* is resistant but the rest of the microbiome is sensitive.

Model simulation. We first used the computational model to simulate the potential impact of adding such an antibiotic. According to the model, antibiotic concentrations less than 1000 times the defined MIC for the native microbiome species had almost no effect on the survival of *B. subtilis*, with the other populations recovering shortly after the *B. subtilis* population died. At the highest concentration tested (1000 times the defined MIC for the native microbiome species), the *B. subtilis* population appeared to achieve long-term survival, at the expense of the *Acinetobacter* population dying completely (Figure 4.3).

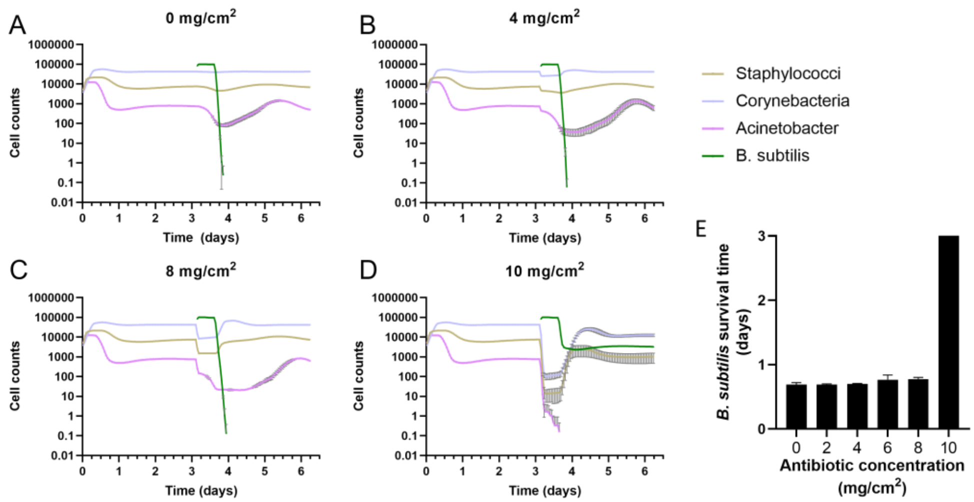

Figure 4.3. Simulation of skin bacteria population dynamics when adding antibiotics concurrently with B. subtilis. (A) 0 mg/cm^2; (B) 4 mg/cm^2; (C) 8 mg/cm^2; (D) 10 mg/cm^2 of antibiotic. (E) Survival time of B. subtilis with different concentrations of antibiotic. At 10 mg/cm^2, B. subtilis appeared to survive long-term, but is shown at 3 days because the simulation was ended at that point. Lines and bars show mean +/- standard deviation of 3 replicate simulations.

Experimental findings. We next tested the effects of antibiotics in the *ex vivo* pig skin model using the antibiotic kanamycin, to which the *BS-GFP* strain has resistance due to the selection marker on the plasmid. We found that there was a dose-dependent effect of antibiotic on total GFP production, as well as an increase in the length of time over which GFP was produced from 4 days to 5 days when antibiotic was added (Figure 4.4). Consistent with the model simulation, the dose-dependent antibiotic effect may be due to decreased competition with residual microorganisms on the cleansed skin. It is also possible that effect of the antibiotic was due to decreased competition with *B. subtilis* cells that spontaneously lose plasmid and would otherwise have a growth advantage by virtue of not producing heterologous protein.

We characterized the GFP production curves (Figure 4.4A) with a rate constant (Figure 4.4B). There was a significant increase in the rate constant of the fluorescence curve for the lower antibiotic concentration and a trend towards an increased rate constant for the higher antibiotic concentration that was not significant (Figure 4.4B). Taken together, these results suggest that using an antibiotic adjuvant for bacterial-based drug delivery to skin could have positive impacts on total dosage and persistence of delivery vehicle viability.

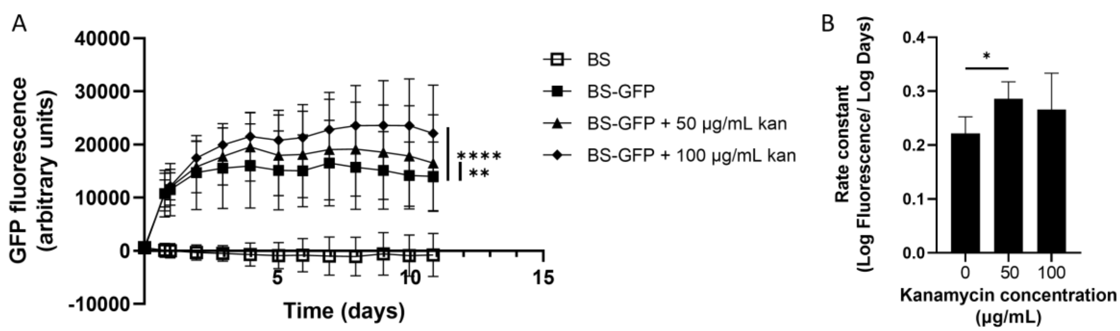

Figure 4.4. GFP production in ex vivo pig skin model with antibiotic addition. (A) Fluorescence measurements over time. BS: B. subtilis strain 168; BS-GFP: B. subtilis strain 168 harboring pGFP-RBS5 plasmid; BS-GFP + 50 ug/mL kan: BS-GFP with 50 ug/mL kanamycin added immediately before bacteria; BS-GFP + 100 ug/mL kan: BS-GFP with 100 ug/mL kanamycin added immediately before bacteria. ** $p < 0.01$, **** $p < 0.0001$, two-way ANOVA compared to BS-GFP. (B) Rate constant of fluorescence curves shown in part (A). * $p < 0.05$, one-way ANOVA compared to BS-GFP. Data show mean +/- standard deviation of 8 replicates.

4.3.3 Effect of added carbon source

Another way to extend the lifetime of added *B. subtilis* in a skin microbiome may be to supplement a nutrient source that *B. subtilis* would have a distinct advantage in using compared to the native microbiome. It has previously been shown that creation of an exclusive metabolic niche in the gut can lead to long term colonization of a probiotic in mice [61]. We hypothesized that creating a similar niche on the skin may yield similar results.

Model simulation. We thus used the computational model to evaluate the potential effect of adding a carbon source to specifically benefit *B. subtilis*. We chose to use malate as the additional carbon source; while malate can be consumed by other species, it is a preferred carbon source for *B. subtilis* (in addition to glucose), so we would expect malate addition to most benefit *B. subtilis*. Since the model does not differentiate between preferred and non-preferred carbon sources, we modeled this supplementation as the creation of an exclusive niche where no other species could use malate as a carbon source. Addition of increasing concentrations of malate led to an increase in predicted *B. subtilis* survival time from about half a day to about one day (Figure 4.5).

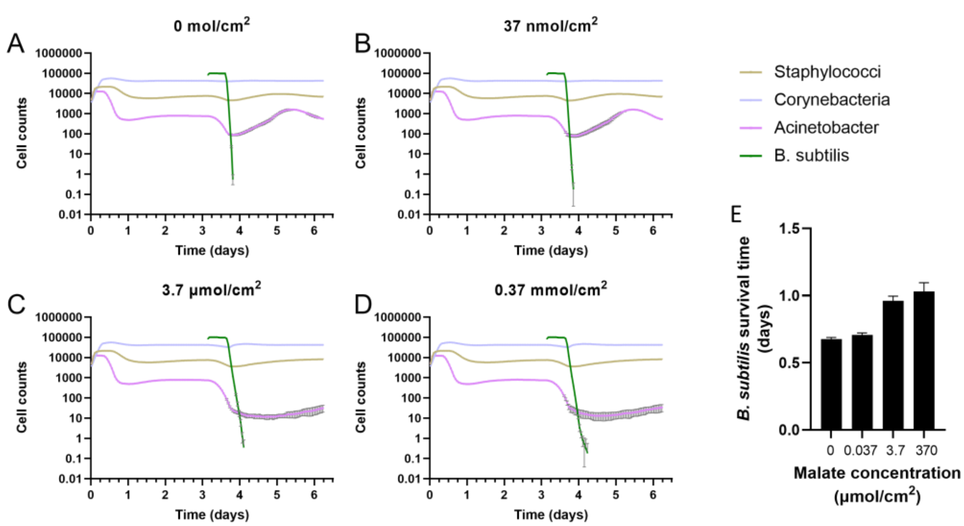

Figure 4.5. Simulation of skin bacteria population dynamics when adding supplemental carbon source concurrently with B. subtilis. (A) 0 mol/cm^2 malate added; (B) 37 nmol/cm^2

malate; (C) 3.7 umol/cm^2 malate; (D) 0.37 mmol/cm^2 malate. Lines represent mean values and error bars show standard deviation across 3 simulations. In most cases, the error bars are smaller than the line thickness. (E) survival time of *B. subtilis* with different concentrations of carbon source. Data show mean +/- standard deviations of 3 replicate simulations.

Experimental findings. Malate was added to the *ex vivo* pig skin model at two doses (0.1 M and 1 M, which correspond to approximately 3.6 μmol/cm^2 and 36 μmol/cm^2, respectively). We observed a dose-dependent effect of the added malate on total GFP production. We also observed an increase in the length of time over which GFP was produced to about 5 days for the 1 M malate group, as well as significant increases in the rate constants for both malate concentrations (Figure 4.6). Thus, malate could serve as a useful added nutrient source for *B. subtilis* on skin, enabling increased protein production and a longer duration of protein production.

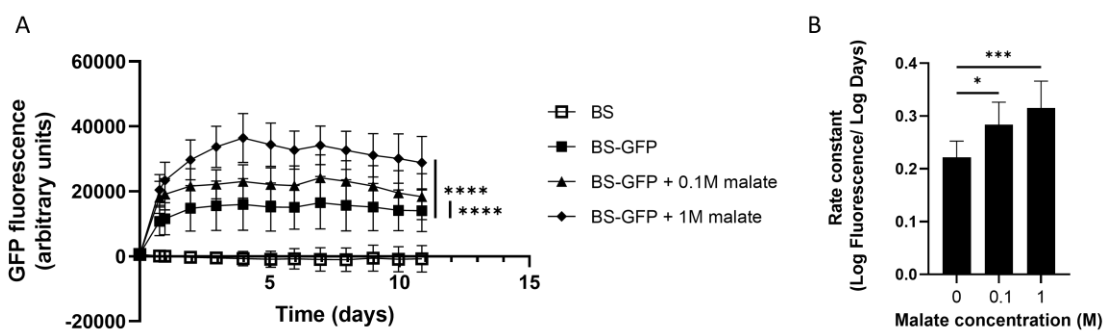

Figure 4.6. GFP production in ex vivo pig skin model with malate addition. (A) Fluorescence measurements over time. BS: B. subtilis strain 168; BS-GFP: B. subtilis strain 168 harboring pGFP-RBS5 plasmid; BS-GFP + 0.1 M malate: BS-GFP with 0.1 M malate added immediately before bacteria; BS-GFP + 1 M malate: BS-GFP with 1 M malate added immediately before bacteria. **** $p < 0.0001$, one-way ANOVA compared to BS-GFP. (B) Rate constant of fluorescence curves shown in part (A). * $p < 0.05$, *** $p < 0.001$, one- way ANOVA compared to 0 M. Data show mean +/- standard deviation of 8 replicates.

4.3.4 Survival of B. subtilis on human skin culture in vitro

Experimental findings. Guided by evidence of *B. subtilis* survival on pig skin, we next used a full thickness human skin tissue culture model (Epiderm FT, from MatTek) to assess the ability of *B. subtilis* to survive and produce GFP on human skin that had been pre-colonized with microorganisms from a swab of human forearm skin (Supplemental Figure 4.12). Using fluorescence measurements to track GFP production, we found that *BS-GFP* survived and produced GFP for 2.1 ± 0.7 days. (Figure 4.7), which is generally consistent with the findings from our pig skin model.

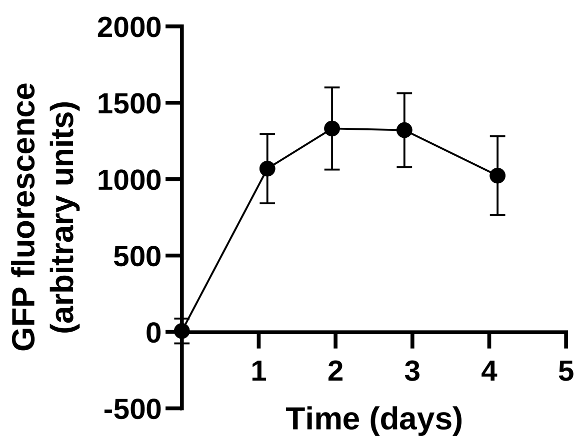

Figure 4.7. GFP production in in vitro human skin tissue culture model by BS-GFP. Data show mean +/- standard deviation of 6 replicates.

4.3.5 Safety of B. subtilis on human skin cells in vitro

Experimental findings. For *B. subtilis* to be a viable vehicle for therapeutic delivery to the skin, it must not be toxic to the human cells that it will encounter. The potential toxicity of *B. subtilis* was thus tested against HaCaT human keratinocyte cells by exposing the keratinocytes to *B. subtilis* culture supernatants as part of the keratinocyte growth medium. We tested two strains of *B. subtilis*, the common laboratory strain 168 and the antifungal lipopeptide-producing strain 15841, using exposure to LB media alone as a negative control. We expressed the LD_{50} in terms of the percentage of the supplemented keratinocyte medium composed of LB or supernatant.

The LD_{50} of LB and supernatants from 168 and 15841 cells were 64.0% (95% CI: 49.8 – 90.50), 33.5% (95% CI: 31.6 – 35.5) and 28.6% (95% CI: 26.2 – 31.4), respectively, after 3 days of exposure (Figure 4.8). The LD_{50} values for both strains 168 and 15841 were significantly different from that of LB media ($p < 0.0001$), which means that the supernatants of both strains had greater toxicity than the media alone but were of the same order of magnitude.

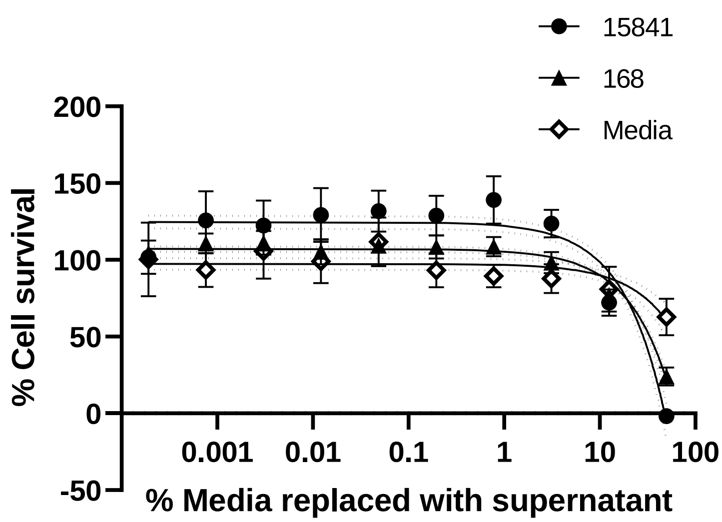

Figure 4.8. Cytotoxicity of bacterial supernatants against HaCaT human keratinocyte cells in vitro. Dotted lines show 95% confidence intervals for linear regression analysis. Data points show mean +/- standard deviation of 8 replicates.

4.3.6 B. subtilis safety and survival in vivo

Experimental findings. Given that the *in vitro*, *ex vivo,* and computational model results suggest the viability and safety of *B. subtilis* on skin, we used a mouse model *in vivo* for further validation. *BS-GFP*, the parent strain *B. subtilis* 168, and LB medium were inoculated onto the backs of hairless mice every other day, with daily swabbing to track whether *BS-GFP* was still present on mouse skin by counting green colonies after plating on selective medium. We found that *BS-GFP* was consistently detected on the day following application and sometimes on the following day (Figure 4.9).

After the last application of bacteria, *BS-GFP* was detectable by swab for 3 days and then returned to negligible levels. Mice were then sacrificed, and biopsy samples of skin exposed to bacteria or media were taken for histological analysis. No evidence of tissue damage or infiltrating immune cells were observed for the mice receiving *B. subtilis* treatment (*BS* and *BS-GFP*) compared to mice treated with just medium (Figure 4.10).

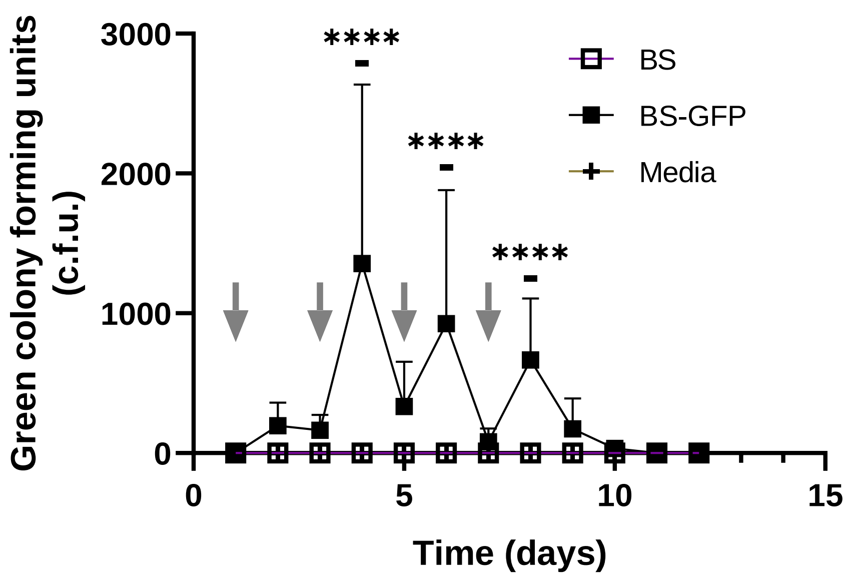

Figure 4.9. Persistence of BS-GFP on mouse skin in vivo. Bacterial or medium solutions were added every other day (indicated by arrows), and mice were swabbed daily. BS: B.

subtilis strain 168; BS-GFP: B. subtilis strain 168 harboring pGFP-RBS5 plasmid; Media: LB medium. **** $p < 0.0001$, two-way ANOVA comparing BS-GFP to media. Data show mean +/- standard deviation of 3 mice per treatment and 3 technical replicates of swab plating for each mouse.

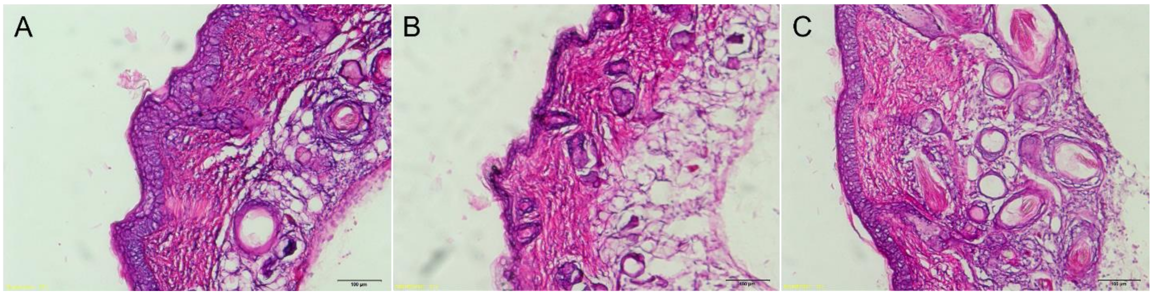

Figure 4.10. Representative images of mouse skin stained with hematoxylin and eosin. (A) treated with B. subtilis strain 168; (B) treated with B. subtilis strain BS-GFP; (C) treated with LB medium. Mouse skin in vivo treated with *B. subtilis* or media as shown in Figure 4.9 was biopsied on day 13, sectioned, stained, and imaged by brightfield microscopy.

4.4 Discussion

For topical drug delivery, medications are commonly applied to the skin via gels, ointments, lotions, creams, and sprays, which are advantageous for their ease of application over the affected area of skin [29]. However, topical formulations are also easily removed from the skin after a short amount of time and often require daily or more-frequent application to be effective [104]. Continuous production of a drug or other molecule of interest on skin throughout the day could support enhanced adherence and better outcomes with these forms of treatment. Skin-resident bacteria like *B. subtilis* could be engineered to continuously produce drugs on the skin for lasting therapy.

Across our various models, we found that *B. subtilis* survived on the skin while producing GFP for at least one day and appeared to be safe. Although *B. subtilis* is found on healthy skin, we wanted to confirm that doses of interest for therapeutic applications would not be harmful to the skin, even if at levels much higher than typically found on human skin [9]. While we found that the supernatants of the two *B. subtilis* strains tested were more toxic to human keratinocyte cells than just LB medium alone, the LD_{50} values were similar, leading us to conclude that the strains would likely be safe for use on skin, but more work is needed to fully answer this question. Furthermore, no signs of negative skin reaction were observed on mouse skin *in vivo* after one week of every-other-day application of *B. subtilis*. Altogether, our data suggest that *B. subtilis* may be well tolerated by skin, at least at the levels used in this study.

A safety component which was not experimentally investigated in this study was the effect of *B. subtilis* treatment on the existing microbiome. The microbial community present on the skin is associated with skin health, so it would be important to also ensure that any changes to the existing community are not harmful [133]. It is worth noting that the computational model predicted that, without antibiotic addition, after the delivered species dies off the existing community would return to its previous levels. This suggests that perturbations to the skin microbiome due to the use of *B. subtilis* as a delivery vehicle may just be transient. In agreement with this observation, other work analyzing the shift in mouse ear microbiota composition during and after *B. subtilis* administration found that there were shifts in the microbiota composition during administration of the bacteria, but the microbiota composition was similar to that of the untreated control group within a week after stopping administration of bacteria [58].

Starting with equal numbers of the representative skin microbiome species in this model, the relative steady state percentages predicted by the computational model qualitatively matched relative percentages of their genera identified from swabs of human forearm skin [113]. The computational model also predicted that a species with doubling time and carbon source utilization characteristics representative of *B. subtilis* could survive within an established community representing the skin microbiome for a little over half a day. It predicted that adding a carbon source to specifically benefit the added species could lead to a modest increase in survival time and that application of an antibiotic to which the added species has resistance could lead to long-term survival, although with long-term consequences to the existing community. These predictions generally aligned with experimental results using an *ex vivo* pig skin model, where we found that the engineered strain of *B. subtilis* survived on skin while producing GFP for multiple days, and the addition of either kanamycin, to which the engineered strain had resistance, or malate, a preferred carbon source for *B. subtilis*, led to significantly increased GFP production. While addition of antibiotics increased *B. subtilis* survival in both models, the FDA has recommended against the use of antibiotic selection markers in clinical stage products [134].

We used a full thickness human skin tissue culture model to evaluate the survival of *B. subtilis* on human skin in the presence of microorganisms obtained from human forearm skin and found that BS-GFP survived while producing GFP for about 3 days. While this model provided further evidence that *B. subtilis* can survive on human skin, we applied a low density of human skin microorganisms to the tissue surface, so further studies would be required to further determine the ability of *B. subtilis* to survive in the context of the skin microbiome.

Finally, when testing survival of *B. subtilis* on mouse skin *in vivo*, we found that *BS-GFP* was repeatedly detectable by swab 1 day after application, which was generally consistent with the computational model predictions. After applications ceased, *BS-GFP* was still detectable by swab for 2 days but returned to baseline within 3 days of the last application. A study in which *B. subtilis* was applied to mouse ears for 7 consecutive days found that *B. subtilis* levels returned to baseline levels within 4 days after the last application, which is in general agreement with our results [58]. Taken together, these findings led us to conclude that *B. subtilis* could be suitable as a drug delivery platform for a daily or every-other-day application but may not be suitable for longer-term delivery.

Wild-type *B. subtilis* may be a more transient member of the skin microbiome compared to other skin-resident bacteria [34], such that longer-resident species, such as *S. epidermidis*, may be more suited to multi-day delivery scenarios [33]. For example, the company, Azitra, has multiple clinical and preclinical stage products incorporating engineered *S. epidermidis* to treat various skin disorders [87]. However, variable colonization efficacy has remained a challenge in the field of topical probiotics utilizing resident skin commensal species including *S. epidermidis*, possibly due to competition with the existing microbiota or variability in the skin sites within and between individuals [135]. Furthermore, due to biocontainment concerns, a common strategy to prevent transfer of the engineered strain to undesired areas is to render the strain an auxotroph requiring regular application of an additional substance, such as D-alanine, to maintain viability [136]. With these considerations in mind, the genetic tractability of *B. subtilis* makes it an attractive option for situations in which daily or every-other-day application is appropriate.

A limitation of this study is that GFP is only a model for protein drugs that do not interfere with cell metabolism. GFP expression had little effect on the fitness of our cells, but small molecule drugs that require more complex pathways for production would likely have a greater effect on cell fitness and ability to survive on skin. A protein drug that directly interfered with the cell metabolism would also likely have a greater effect on the cell's fitness and survival. Also, logistical considerations of human routines such as hand and body washing, and transfer of bacteria from skin to other surfaces could limit the potential applications of using engineered bacteria as a topical drug delivery platform.

4.5 Conclusion

In this study, we assessed the feasibility of using *B. subtilis* as a topical drug delivery platform. Using GFP as a model heterologous protein, we found that engineered *B. subtilis* could survive on multiple skin models for at least one day and appeared to be safe for skin. Due to its wide commercial use and relative ease of genetic modification, we believe that *B. subtilis* could be an attractive candidate to make the development of bacteria-based topical delivery platforms more straightforward and accessible.

4.6 Supplementary Materials

Table 4.4. DNA primers Aim 1

Name	Sequence (5'-3')[1]
RBS0_GFP_5p.F	aatttgcaagcttaaggaggacaaacatgagcaaaggtgaagaactg
RBS1_GFP_5p.F	cttagaggtggtgtacaaacatgagcaaaggtgaagaactg
RBS2_GFP_5p.F	cttaagaggagataacaaacatgagcaaaggtgaagaactg
RBS3_GFP_5p.F	cttaaggagagataacaaacatgagcaaaggtgaagaactg
RBS4_GFP_5p.F	atattaagaggaggagacaaacatgagcaaaggtgaagaactg

Table 4.4 continued	
RBS5_GFP_5p.F	cttaaaggaggtgtacaaacatgagcaaaggtgaagaactg
GFP_3p.R	gagtcgacctgcaggcatgcttatttttcgaactgcggatgg
RB_5p.F	atccgcagttcgaaaaataagcatgcctgcaggtcg
RBS0_RB_3p.R	agttcttcacctttgctcatgtttgtcctccttaagcttgcaaattatatcaacg
RBS1_RB_3p.R	catgtttgtacaccacctctaagcttgcaaattatatcaacgttaataagac
RBS2_RB_3p.R	catgtttgttatctcctcttaagcttgcaaattatatcaacgttaataagac
RBS3_RB_3p.R	catgtttgttatctctccttaagcttgcaaattatatcaacgttaataagac
RBS4_RB_3p.R	ttgtctcctcctcttaatataagcttgcaaattatatcaacgttaataagac
RBS5_RB_3p.R	catgtttgtacacctcctttaagcttgcaaattatatcaacgttaataagac

[1]Ribosome binding sites are underlined

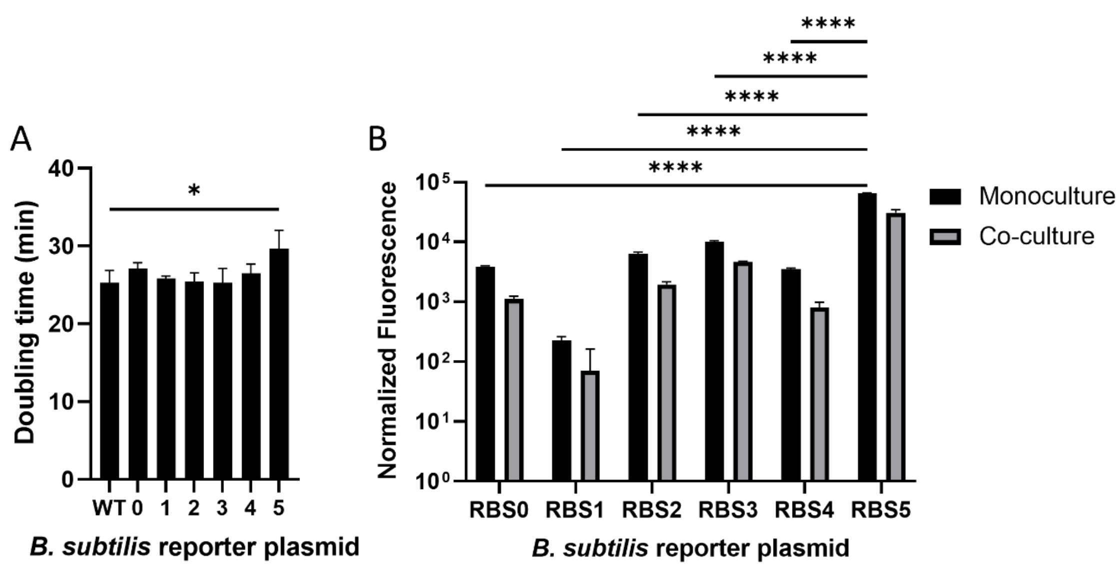

Figure 4.11. Survival and production of heterologous protein by B. subtilis in culture. (A) Doubling times for wild-type (WT) B. subtilis and strains harboring plasmids pGFP-RBS0

through pGFP-RBS5. * $p < 0.05$, one-way ANOVA comparing doubling times to wild-type. (B) Fluorescence of GFP expressed in B. subtilis strains harboring different reporter plasmids in monoculture and in co-culture with parent strain B. subtilis 168. Data show mean +/- standard deviation of three replicates. **** $p < 0.0001$, one-way ANOVA comparing fluorescence values in monoculture.

Figure 4.12. Same volume (20 µL) of arm swab sample that was used on human skin tissue culture plated on non-selecting LB agar.

CHAPTER 5. DETERMINE EFFICACY OF ENGINEERED BACILLUS SUBTILIS FOR TOPICAL DELIVERY OF ANTIFUNGAL AGENT

5.1 Introduction

Dermatophytoses are fungal infections of the skin [137]. Common dermatophytoses include tinea pedis (athletes' foot), tinea corporis (ringworm), tinea capitis (scalp infection), and onchomycosis (nail infection). An estimated 20 to 25 percent of the world's population is affected by fungal skin infections, and it is estimated that over $500 million is spent each year to treat these conditions [11, 12]. Topical application of antifungal agents is the primary form of treatment for tinea pedis and tinea corporis. These treatment regimens involve daily or twice-daily application of an antifungal agent (typically azoles or allylamines) for 1 to 6 weeks [13]. The requirement for frequent application, however, can reduce patient adherence, making a more convenient delivery method desirable.

B. subtilis naturally produces antifungal compounds in the iturin, surfactin, and fengycin families. In fact, the commercial agricultural product Serenade uses *B. subtilis* to fight plant fungal pathogens, and *B. subtilis* has been incorporated into microneedles and a topical gel for treatment of skin fungal infections in mice [49, 107, 138]. Further supporting its use as a topical antifungal treatment, *B. subtilis* is found within the skin microflora, has Generally Regarded As Safe status from the FDA, and is commercially available for human consumption [9, 51, 108]. In this study, we sought to increase the antifungal activity of *B. subtilis* by engineering cells to overproduce the antifungal lipopeptide iturin A.

Iturin A is a broad antifungal agent active against several relevant fungi, including *Trichophyton mentagrophytes* and *Trichophyton rubrum*, two of the main causes of athlete's foot [11, 17, 19]. While iturin A has not been widely used as a therapeutic due to high production costs, low yields, and hemolytic activity, we hypothesized that *in situ* production of iturin A by *B. subtilis* cells localized to the skin surface would eliminate the need for costly extraction procedures and exhibit minimal safety concerns [49, 139, 140]. We thus expected this strategy to serve as an effective means to reduce the frequency of required antifungal administrations by providing continuous drug exposure.

In this study, we engineered *B. subtilis* to have increased production of iturin A using the pleiotropic regulator DegQ, which has been used by others to increase iturin A production in strains of *Bacillus* [141, 142]. We then studied the effectiveness of this engineered strain in an *in vitro* antifungal assay.

5.2 Methods

5.2.1 Media and growth conditions

Bacterial and fungal strains used in this study are listed in Table 5.1. *Escherichia coli* and *B. subtilis* were grown in lysogeny broth (LB), consisting of 0.5% w/v yeast extract (Life Technologies, Carlsbad, CA), 1% w/v NaCl, and 1% w/v tryptone (Life Technologies) at 37 °C and shaking at 200 rpm with appropriate antibiotics (100 µg/ml carbenicillin and 10 µg/ml kanamycin for *E. coli* and *B. subtilis*, respectively). Growth of cells was monitored by reading absorbance at 600 nm using a Biotek Synergy H4 Hybrid Microplate Reader (Agilent, Santa Clara, CA).

Table 5.1. Bacteria, fungi, and DNA plasmids

Name	**Description**	**Source/Reference**
Strains		
168	*B. subtilis* common laboratory strain	ATCC[1]
15841	Antifungal lipopeptide-producing strain of *B. subtilis*	ATCC, [131]
DH5α	*E. coli* competent cells	NEB[2]
T. mentagrophytes	Fungus that causes athlete's foot	ATCC
Plasmids		
pRB374	*E. coli* - *B. subtilis* shuttle plasmid; amp^r, kan^r	ATCC, [55]
pDegQ	vegII:DegQ; amp^r, kan^r	This study

[1] ATCC, American Type Culture Collection (Manassas, VA). [2] NEB, New England Biolabs (Ipswich, MA)

[2] NEB, New England Biolabs (Ipswich, MA)

Landy medium, consisting of 20 g/L glucose, 5 g/L L-glutamic acid, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, 1 g/L KH_2PO_4, 0.15 mg/L $FeSO_4$, 5 mg/L $MnSO_4$, and 0.16 mg/L $CuSO_4$ and raised to pH 7 with 1 N NaOH, was used for iturin A production [15].

T. mentagrophytes was grown on Emmons modified sabouraud dextrose agar (BD Difco, Franklin Lakes, NJ) at 25°C. After a lawn was formed (about 15-20 days), spores were harvested by adding sterile PBS with 0.05% v/v Tween 20 to the plate surface and then gently scraping the surface with a sterile L-shaped cell spreader. After harvesting, the spore suspension was either plated on sabouraud agar for continued culture or stored in 15% glycerol at -80°C for future use.

5.2.2 Plasmid assembly

Plasmids used in this study are listed in Table 5.1. Primers used in this study are listed in Supplementary Table 5.3. All plasmids were assembled in *E. coli* DH5α and then transformed into *B. subtilis* 15841. *E. coli* cells were transformed by heat shock following the NEB High Efficiency Transformation protocol (New England Biolabs, Ipswich, MA). *B. subtilis* cells were transformed by electroporation using an ECM 600 electroporator (BTX, Holliston, MA) set to 2.1 kV, 129 Ω and 50 μF with 1 mm gap cuvettes.

Iturin A is a non-ribosomal antifungal lipopeptide that is encoded in the *B. subtilis* 15841 genome by a 37.2 kb gene cluster, encompassing genes ItuD (1203 bp), ItuA (11949 bp), ItuB (16089 bp), and ItuC (7854 bp). The genome of *B. subtilis* 15841 was extracted using the Purelink Genomic DNA Minikit (ThermoFisher, Waltham, MA) and sequenced with the Illumina MiSeq Nano kit (San Diego, CA). The resulting reads were aligned with the published sequence for the iturin A gene cluster in *B. subtilis* strain RB14 using the Illumina BWA Aligner online application [143, 144]. The annotated sequence of the iturin A gene cluster in strain 15841 is available in the Appendix. Due to the size of the gene cluster, it was amplified from the 15841 genome extract by PCR in 3 parts: ItuD/A (13152 bp, primers ItuDA 5p.F and ItuDA 3p.R), ItuB1 (12052 bp, primers ItuB1 5p.F and ItuB1 3p.R), and ItuB/C (12019 bp, primers ItuBC 5p.F and ItuBC 3p.R).

The DegQ gene (WP_003220708.1) was synthesized by Eurofins (Louisville, KY). Plasmid pDegQ for the overexpression of DegQ in *B. subtilis* 15841 was created by inserting the DegQ gene into the shuttle plasmid pRB374 by Gibson assembly [68].

5.2.3 *Iturin A extraction from liquid culture*

Overnight cultures of *B. subtilis* strain 168 or 15841 were diluted in fresh Landy medium and grown for 3 to 7 days at 37°C, shaking at 200 rpm. Cultures were then centrifuged for 15 min at 3000 rcf and 4°C, and iturin A was extracted from culture supernatant using an aqueous two-phase extraction method [145]. To create a turbid solution, 1 mL of culture supernatant was combined with 0.2 mL ethanol. The pH was adjusted to 9 using 1 N NaOH, and 0.5 g $(NH_4)_2SO_4$ was added. The mixture was vortexed until the salt dissolved completely and then centrifuged for 5 min at 6000 rcf. The upper phase was preserved for analysis, and then the lower phase was extracted again with 0.2 mL ethanol. The upper phases were then combined, diluted to 1 mL with methanol, and then filtered through a 0.2 µm filter for HPLC analysis.

5.2.4 *Analytical methods*

Iturin A was detected using an Agilent 1200 series HPLC system (Agilent, Santa Clara, CA) equipped with a reversed-phase HPLC column (Eclipse XDB-C18, 5 µm, 4.6 x 150 mm, Agilent) at a wavelength of 230 nm. The mobile phase consisted of 60% water with 0.1% trifluoroacetic acid and 40% acetonitrile at a flow rate of 0.6 mL/min and a temperature of 20°C. Commercial iturin A was used as a standard (Sigma, St. Louis, MO).

5.2.5 *Antifungal assays*

An 8-mm biopsy punch was taken from a lawn of *T. mentagrophytes* on sabouraud agar and transferred to a clean sabouraud agar plate. Overnight cultures of bacteria were diluted in fresh LB media and grown at 37°C and shaking at 200 rpm until they reached early log phase. Cultures were then adjusted to a concentration of 10^6 CFU/mL and streaked along a line 3 cm away from the fungal plug using a sterile cotton swab (Puritan Medical Products, Guilford, ME). The plates were left at room temperature (20 – 25˚C) for 15 days and then imaged using a UVP UVsolo touch stand-alone gel documentation system (Analytik Jena, Jena, Germany).

5.2.6 Statistical analysis

Results are presented as mean +/- standard deviation. One-way ANOVA followed by Dunnett's multiple comparisons test was used to compare doubling times, which were calculated by fitting the mid-exponential phase of the growth curves to an exponential growth equation. Statistical analyses were performed using GraphPad Prism version 9.4.1 for Windows (San Diego, California, www.graphpad.com). A p-value less than 0.05 was considered significant.

5.3 Results

We first confirmed that *B. subtilis* strain 15841 has the iturin A gene cluster in its genome by amplifying the entire gene cluster from the genome extract. Due to the size of the gene cluster, it was amplified in three parts (ItuD/A, 13152 bp; ItuB1, 12052 bp; ItuB/C, 12019 bp) (Figure 5.1). We attempted to combine these parts into a single plasmid for potential plasmid-borne overexpression but were not successful, likely due to an insert of such a large size being better suited for a cosmid or alternative vector [146].

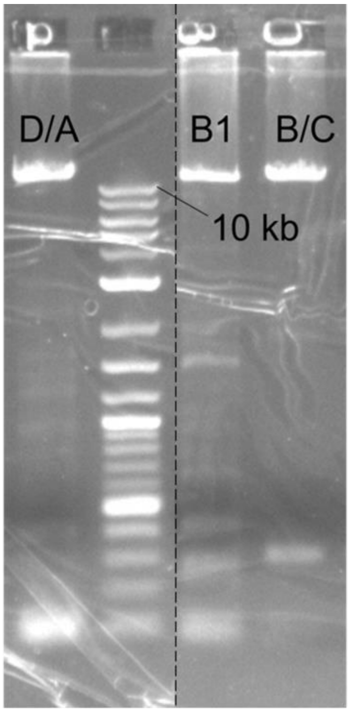

Figure 5.1. Amplification of iturin A gene cluster from B. subtilis strain 15841 genomic DNA by PCR. D/A encompasses a region including ituD and ituA. B1 encompasses part of ItuB. B/C encompasses the remaining section of ItuB and ItuC. Image has been cropped to remove duplicate lanes (dashed line). The full image is available in Supplementary Figure 5.5.

We then engineered *B. subtilis* strain 15841 for increased production of iturin A by overexpressing the pleiotropic regulator DegQ on plasmid DNA. We tested whether the engineered *B. subtilis* strain (BS-DegQ) was successfully engineered to produce higher levels of iturin A by HPLC analysis of the cell culture supernatant. Iturin A contains a cyclic heptapeptide with a β-amino fatty acid chain [95]. There are multiple iturin A homologues corresponding to different carbon chain lengths (primarily 14, 15 and 16 carbons), which can be characterized by three distinct HPLC peaks at 6.1, 8.1 and 8.6 min (Figure 5.2B) [145].

There was a peak in Landy medium (i.e., the negative control) around one of the expected peaks for iturin A (5.8 min) (Figure 5.2A), so peaks near that elution time were not considered for calculations of extracted iturin A. Landy medium spiked with 10 µg/mL of commercial iturin A was used as a positive control and exhibited the expected characteristic peaks at 8.1 and 8.6 min (Figure 5.2E).

B. subtilis strain 15841, which naturally produces iturin A, was used as another positive control. It produced detectable iturin A in the culture supernatant after 3 days, as evidenced by a peak at 8.6 min (Figure 5.2C), but no iturin A was detected after 7 days (Figure 5.2D). Iturin A was also not detectable in Landy medium spiked with 10 µg/mL iturin A after 7 days in culture conditions (Figure 5.2F), suggesting that iturin A synthesized by cells in Landy medium can only be measured reliably within a few days of its production. BS-DegQ did not show evidence of iturin A production after 3 days (Figure 5.2G), but a peak corresponding to iturin A at 8.6 min appeared after 7 days of culture (Figure 5.2H). We believe that only one of the characteristic iturin A peaks was seen in these culture supernatants because other peaks may have been below the limit of detection. Differences in the ratio of iturin A homologues have been observed between different strains of *B. subtilis* as well as by the same strain of *B. subtilis* grown in different culture conditions [141, 147].

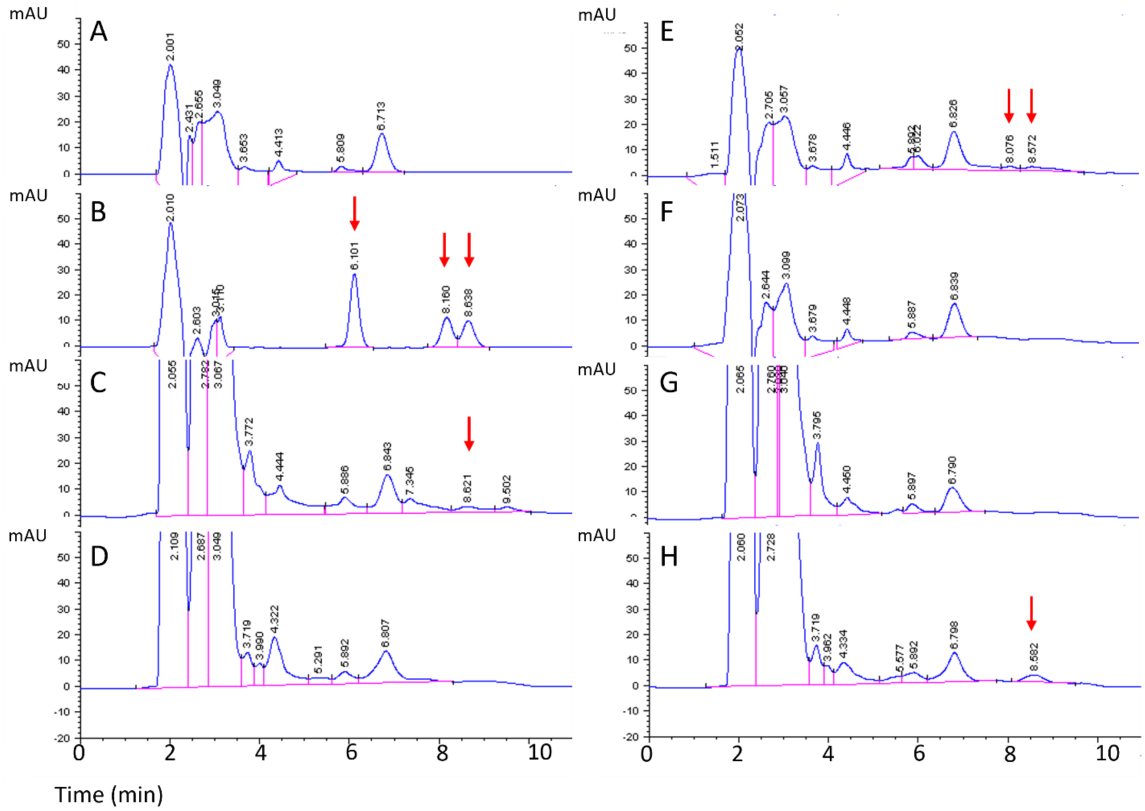

Figure 5.2. Representative HPLC chromatograms for analysis of iturin A concentration. (A) Landy medium. (B) Iturin A at a concentration of 75 µg/mL in methanol. (C) B. subtilis strain 15841 cultured for (C) 3 days or (D) 7 days. Landy medium (E) spiked with 10 µg/mL iturin A. *B. subtilis* engineered to over-express iturin A (BS-DegQ) cultured for (G) 3 days or (H) 7 days. Arrows indicate peaks that were considered characteristic of iturin A.

We next quantified iturin A concentration in the culture supernatant of the *B. subtilis* strains. The iturin A concentration in the culture supernatant of strains 15841 and BS-DegQ after 3 and 7 days of culture, respectively, were of similar magnitude, but iturin A produced by BS-DegQ achieved a concentration approximately three-fold higher than strain 15841 (Student's t-test, $p < 0.01$) (Table 5.2). Altogether, these data indicate that BS-DegQ, at the time points measured, produced a higher concentration of iturin A than the parent stain 15841.

Table 5.2. Iturin A detected from liquid culture by HPLC.

	Iturin A concentration (μg/mL)[1]	
***B. subtilis* strain**	**Day 3**	**Day 7**
15841 (parent)	2.24 +/- 0.16	0
BS-DegQ	0	7.39 +/- 0.63

[1]Values represent average of 3 replicates +/- standard deviation.

We wanted to better understand the kinetics of iturin A production by the different *B. subtilis* strains and thought it might relate to their growth kinetics. The unmodified strains 15841 (parent strain) and 168 (negative control) exhibited similar doubling times of 35.8 +/- 0.9 min ($n = 3$) and 35.0 +/- 2.1 min ($n = 3$), respectively ($p > 0.05$) (Figure 5.3). In contrast, the engineered BS-DegQ strain had a significantly longer doubling time of 75.9 +/- 17.27 min ($n = 3$) ($p < 0.01$). We hypothesize that the longer doubling time of BS-DegQ may explain the delayed appearance of iturin A in the supernatant during liquid culture compared to the parent strain 15841.

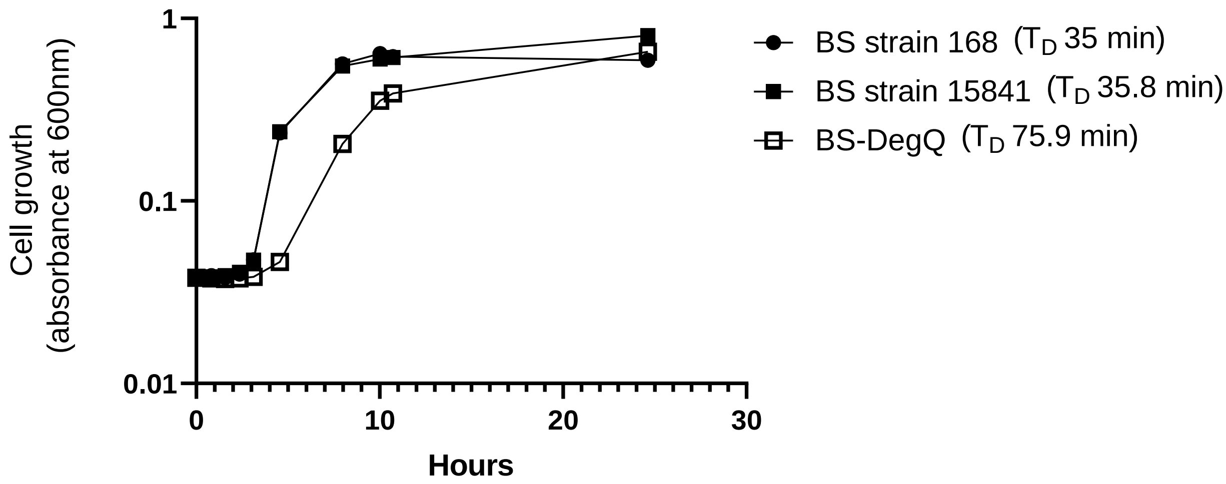

Figure 5.3. Representative growth curves of B. subtilis (BS) strains 168, 15841, and BS-DegQ. Cells were cultured for one day in LB media with no antibiotic at 37°C with shaking.

We finally tested the hypothesis that increased iturin A production by BS-DegQ would result in increased antifungal activity. In an *in vitro* antifungal assay, we compared the ability of different strains to control the growth of a plug of *T. mentagrophytes* on an agar plate (Figure 5.4). In cultures containing the negative control 168 strain (i.e., does not produce iturin A), we saw growth of the fungal cells (appearing black) as well as growth of the 168 cells (appearing gray) (Figure 5.4A).

In contrast, fungal cells in the presence of the parent strain 15841 exhibited very little growth (Figure 5.4B), presumably due to the endogenous antifungal activity of these bacterial cells [131]. The 15841 cells appear to be diffuse on the agar plate, possibly because of the production of other biosurfactants such as surfactin. While we did not analyze surfactin production in this study, strain 15841 has also been reported to produce surfactin, and surfactin production facilitates cell sliding on agar plates [148, 149].

Finally, we found that the BS-DegQ strain did not appear to inhibit fungal cell growth (Figure 5.4C). The increased growth of fungus was accompanied by less growth of bacteria for the *BS-DegQ* strain, which is consistent with the slower growth kinetics seen in Figure 5.3.

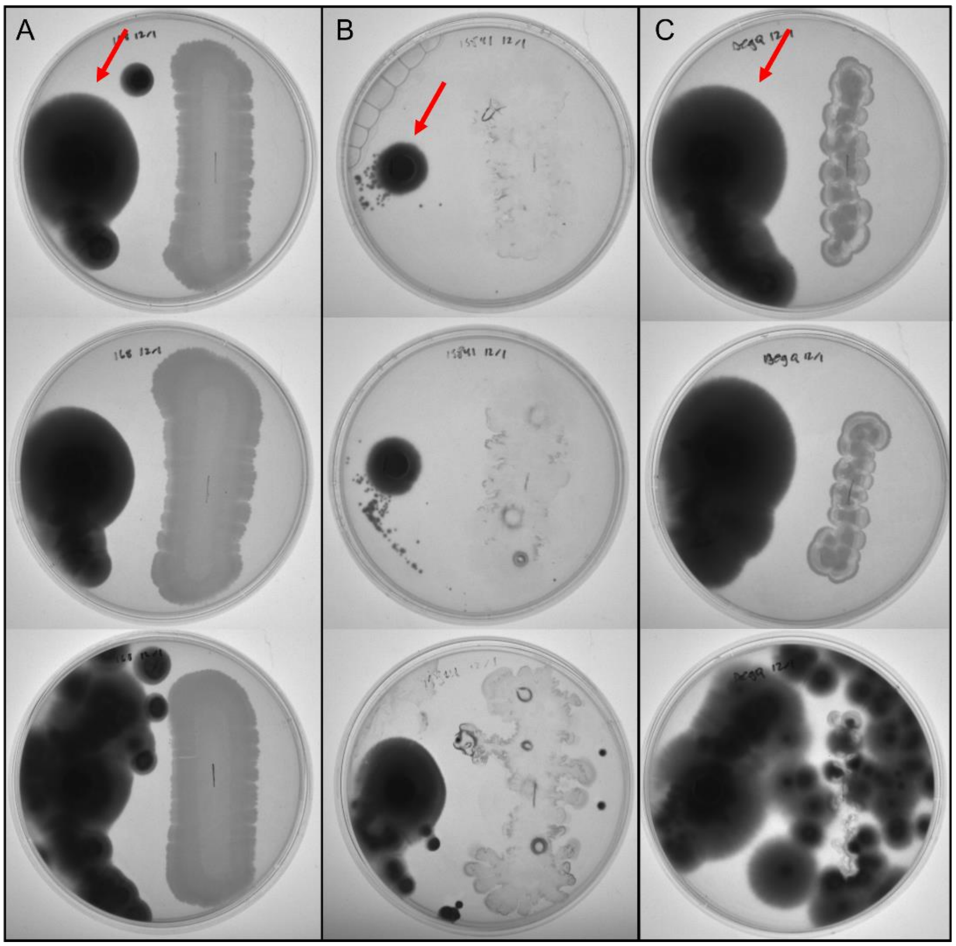

Figure 5.4. In vitro antifungal assay. T. mentagrophytes were cultured with B. subtilis (A) strain 168, (B) strain 15841 or (C) strain BS-DegQ for 15 days at room temperature. Each column shows three replicates of the same B. subtilis strain. Arrows in the top row indicate the fungus T. mentagrophytes.

5.4 Discussion

While fungal skin infections are generally treatable with topical application of an antifungal medication, the requirement for frequent administrations (1 to 2 times per day) is an inconvenience that can lead to poor adherence by patients. Adherence could potentially be improved by the use of longer-lasting topical delivery platforms that require less frequent application [13].

In this study we explored the possibility of using *B. subtilis* as an extended-release topical antifungal treatment. *B. subtilis* naturally produces multiple antifungal molecules, including iturin A, an antifungal lipopeptide that is active against key fungi involved in athlete's foot infections [11, 17, 19]. We sought to increase the antifungal activity of *B. subtilis* by engineering it to have increased production of iturin A. To accomplish this, we chose overexpression of the regulator DegQ, as this strategy offered the most straightforward genetic engineering approach and has been used in literature to successfully increase iturin A production in species of *Bacillus* [141, 142].

While we found evidence of a more-than 200% increase in iturin A production in our engineered strain in its liquid culture supernatant, this increased production was accompanied by strong growth defects manifesting in a much slower doubling time for the engineered strain. Growth defects have previously been observed when using regulators to increase iturin A production in *Bacillus amyloliquefaciens* [142]. Furthermore, heterologous DegQ expression has also been found to decrease production of another antifungal lipopeptide, surfactin [150].

Despite the engineered strain producing a higher titer of iturin A, it showed significantly reduced efficacy in an *in vitro* antifungal assay. This reduced efficacy may be due to slower growth, potentially decreased levels of other antifungals produced by the cells, or a combination of these and other factors. This result highlights how it is essential to consider more than just total production titer when engineering cells for *in situ* drug production, as growth defects in particular will affect a vehicle's ability to both effectively produce drug *in situ* and survive in the competitive skin environment.

Since several *B. subtilis* strains already have the gene cluster for iturin A production, one potential future strategy to increase iturin A production might be direct manipulation of regulatory elements in the genome, which has been done successfully to increase iturin A expression in *B. amyloliquefaciens* [142]. Also, the performance of unmodified *B. subtilis* strain 15841 in controlling growth of *T. mentagrophytes* in an *in vitro* antifungal assay suggests another avenue for successful bacterial-based topical delivery. For example, other studies have also found that unmodified strains of *B. subtilis* performed similarly to the antifungal drug ketoconazole in an *in vivo* mouse infection model [49, 107]. Thus, choosing a naturally highly antifungal strain of *B. subtilis* may limit the need for engineering increased antifungal activity.

5.5 Conclusion

In this study we engineered *B. subtilis* strain 15841 for increased production of the antifungal lipopeptide iturin A by overexpression of the pleiotropic regulator DegQ. We saw a more-than 200% increase in iturin A in liquid culture for the engineered strain, but also slower growth. In an *in vitro* antifungal assay, we found that the engineered strain reduced the growth of the pathogenic fungus *T. mentagrophytes* less effectively than the parent strain. Future studies should seek to balance the side effects of engineering overproduction strains when designing strains for drug delivery applications, as growth defects can be particularly problematic for the desired clinical results.

5.6 Supplementary Materials

Table 5.3. DNA primers Aim 2

Name	Sequence (5'-3')
ItuDA 5p.F	atgaacaatcttgccttttt
ItuDA 3p.R	ctaaaagttcaattgaatagaatc
ItuB1 5p.F	atgtcggtatttagaaatcaagaaacgtactgg
ItuB1 3p.R	ttccgtcagaaataatgtgatgcatatcaaacatcagaat
ItuBC 5p.F	attctgatgtttgatatgcatcacattatttctgacggaa
ItuBC 3p.R	ttacattttgtcaaactttgtgtctcccgcaaac
pDegQ_RB 5p.F	atgcaatgaaaatttcgtgagcatgcctgcaggtcga
pDegQ_RB 3p.R	tcttcaagtttcttttccatgtttgtacacctcctttaagcttgc
pDegQ_DegQ 5p.F	cttaaaggaggtgtacaaacatggaaaagaaacttgaaga
pDegQ_DegQ 3p.R	gagtcgacctgcaggcatgctcacgaaattttcattgcat

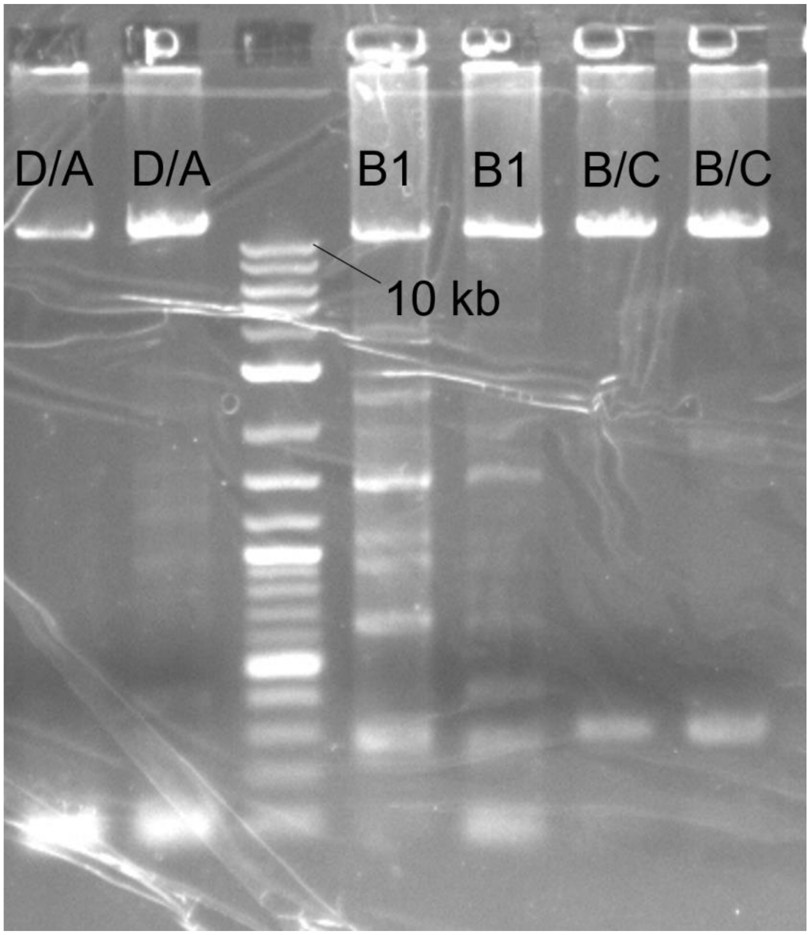

Figure 5.5 Full gel image for amplification of iturin A gene cluster from B. subtilis strain 15841 genomic DNA by PCR. D/A encompasses a region including ituD and ituA. B1 encompasses part of ItuB. B/C encompasses the remaining section of ItuB and ItuC. Duplicate lanes show replicates of the same PCR reaction.

CHAPTER 6. DETERMINE EFFICACY OF ENGINEERED BACILLUS SUBTILIS PRODUCING MOSQUITO REPELLENT

6.1 Introduction

Approximately half of the world's population lives in areas with risk of malaria transmission [151]. The two predominant malaria parasites (*Plasmodium falciparum* and *Plasmodium vivax*) cause over 200 million clinical cases of malaria each year across approximately 100 countries, with over 400,000 mortalities per year, disproportionately occurring in children under five years old [20]. Other mosquito-borne diseases such as dengue, chikungunya virus, and Zika virus similarly represent widespread global health threats [96]. While preventive vaccines have been a long-pursued area of research, there is currently no commercially available vaccine for Zika virus or chikungunya virus, and the newly approved malaria vaccine is only about 30% effective, which means that it is critical to address transmission to prevent the disease [96-98].

The clearest way to prevent transmission of malaria and other mosquito-borne diseases is to prevent mosquitoes from biting the skin. Efforts to prevent mosquito bites have led to significant reductions in malaria prevalence, with the most successful intervention being insecticidal bed nets [20]. However, the finite lifetime of bed nets and their inability to protect people outside of bed suggest the importance of developing complementary approaches to prevent transmission.

The host-seeking behavior of mosquitoes is primarily mediated by odor, which provides an opportunity for intervention to decrease transmission. Bacteria on the skin play an essential role in the production of human body odor and are believed to influence the differential attractiveness of certain people to mosquitoes via the production of volatile organic compounds [152, 153]. Notably, 2-phenylethanol (2-PE) has been identified as a volatile compound produced by human skin bacteria and is associated with individuals that are less attractive to *Anopheles gambiae* mosquitoes [21]. Laboratory studies using *An. gambiae* mosquitoes obtained from Liberia and a semi-field setting study in Kenya have shown that the addition of 2-PE to a blend of known mosquito attractants produced by the skin microbiome (i.e., ammonia, lactic acid, tetradecanoic acid, and CO_2) dramatically reduced attraction of *An. gambiae* mosquitoes [22]. Furthermore, 2-PE has previously been found to have repellent activity against *Aedes aegypti* mosquitoes for 1-2 h, and 2-PE was reported to repel *Culex quinquefasciatus* mosquitoes with comparable efficacy to DEET [23, 24]. However, 2-PE is not used commercially as a mosquito repellent, likely due to its volatility [24].

In this study, we sought to develop a long-acting 2-PE-based mosquito repellent by engineering *Bacillus subtilis* to continuously produce 2-PE on the skin. *B. subtilis* is widely used in the biotechnology industry for the production of proteins, vitamins, and antibiotics, and it has been identified as part of the skin microflora [8-10]. Furthermore, 2-PE is commonly used as an agent for flavoring and fragrances, and significant literature exists documenting the ability to produce this compound in bacteria such as *E. coli*, via pathways that are common to *B. subtilis* [102, 154-156].

We expected the continuous production of 2-PE on the skin by *B. subtilis* to be safe for users because 2-PE is an inactive ingredient in many FDA-approved products administered parenterally or topically at concentrations up to 0.5%, is a common component of soaps, and has not shown irritation or sensitization reactions after extended dermal contact [157, 158]. Likewise, *B. subtilis* has generally regarded as safe status from the FDA and is commercially available for human consumption [51, 108].

In this study, we made initial attempts to engineer *B. subtilis* for 2-PE production via phenylpyruvate and have outlined a strategy for future work.

6.2 Methods

6.2.1 Bacterial culture

Bacterial strains used in this study are listed in Table 6.1. *B. subtilis* 168 and *Escherichia coli* DH5α were cultured in lysogeny broth (LB), consisting of 0.5% w/v yeast extract (Life Technologies, Carlsbad, CA), 1% w/v NaCl, and 1% w/v tryptone (Life Technologies), at 37 °C with shaking at 200 rpm. Growth was measured by absorbance at 600 nm using a Biotek Synergy H4 Hybrid Microplate Reader (Agilent, Santa Clara, CA).

Table 6.1. DNA plasmids and bacterial strains

Name	Description	Reference/Source
DNA plasmids		
pRB374	*E. coli – B. subtilis* shuttle plasmid; amp^r, kan^r	ATCC[1], [55]
pHP13	*E. coli – B. subtilis* shuttle plasmid; Cm^r , Em^r	BGSC[2], [159]
pRB-2PE	pRB374; SpoVG: $AroG^{fbr}$, $PheA^{fbr}$, *KDC, yjgb;* amp^r, kan^r	Blue Heron[3]

Table 6.1 continued		
pRB-yjgb	pRB374; SpoVG: yjgb; amp^r, kan^r	This study
pRB-KDC-yjgb	pRB374; vegII: *KDC, yjgb;* amp^r, kan^r	This study
pRB-2KDC-yjgb	pRB-KDC-yjgb with weaker RBS in front of KDC	This study
pRB-kivd-yjgb	pRB374; vegII: *kivd, yjgb*; amp^r, kan^r	This study
pHP-kivd-yjgb	pHP13; vegII: *kivd, yjgb*; Cm^r, Em^r	This study
Bacterial strains		
DH5α	*E. coli* high efficiency competent cells	NEB[4]
EC-GFP	*DH5α* expressing GFP	CHAPTER 4
EC-KDC-yjgb	DH5α carrying pRB-KDC-yjgb	This study
168	*B. subtilis* parent strain	ATCC

[1] ATCC, American Type Culture Collection (Manassas, VA).

[2] BGSC, Bacillus Genetic Stock Center (Columbus, OH)

[3] Blue Heron Biotech (Bothell, WA)

[4] NEB, New England Biolabs (Ipswich, MA)

6.2.2 Plasmid assembly

DNA plasmids used in this study are listed in Table 6.1, and DNA primers are listed in Table 6.2. All plasmids were assembled in *E. coli* DH5α and then transformed into *B. subtilis* 168. *E. coli* cells were transformed by heat shock following the NEB high efficiency transformation protocol (New England Biolabs, Ipswich, MA). *B. subtilis* cells were transformed by electroporation using an ECM 600 electroporator (BTX, Holliston, MA) set to 2.1 kV, 129 Ω and 50 µF with 1 mm gap cuvettes. Plasmids were sequenced by Sanger sequencing (Eurofins Genomics, Louisville, KY).

Table 6.2. DNA primers Aim 3

Name	Sequence (5'-3')
yjgb_5p.F	GGAATTGATACACTAagcttaaaggaggtgtACAAACatgagcatgatta
yjgb_3p.R	gagtcgacctgcaggcatgctcaaaaatccgctttcagcaccac
KDC_5p.F	GGAATTGATACACTAagcttTCTGCTTTCCTCTACGGCGA
KDC_3p.R	gagtcgacctgcaggcatg
RBS2 _5p.F	aagaggagataACAAACATGGCTCCGGTGAC
RBS2_ 3p.R	aagcttgcaaattatatcaacgttaataagacg
kivd_yjgb_5p.F	gctatgaccatgattacgccttgacaacgtcttattaacgttgatataatttgc
kivd_yjgb_3p.R	ggggatccgtcgacctgcagcggcggatttgtcctactca
RB_yjgb_5p.F	tgctgaaagcggatttttgagcatgcctgcaggtcga
RB_yjgb_3p.R	catGTTTGTacacctcctttaagctTAGTGTATCAATTCCACGATTTTT
RB_KDC_y_5p.F	tgctgaaagcggatttttgagcatgcctgcaggtcga

Table 6.2 continued	
RB_KDC_y_3p.R	catGTTTGTacacctccttttcattttttgttgcgtttcagcgc
RB_kivd_5p.F	CTGAACAAAATAAATCATAAaaaggaggtgtACAAACatgagcatg
RB_kivd_3p.R	TAATCTCCTACTGTATACATGTTTGTtatctcctcttaagcttgcaaatt

Plasmid pRB-2PE, including a feedback resistant mutant of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (AroG^{fbr}, D146N, GenBank WP_001109196.1), a feedback resistant mutant of chorismate mutase / prephenate dehydratase (PheA^{fbr}, GenBank NP_311489.1, AA 286-386 truncated to remove R domain responsible for feedback inhibition), 2-keto acid decarboxylase (KDC, GenBank NP_010668.3), and aldehyde reductase (yjgb, GenBank CAQ34616.1), was synthesized by Blue Heron Biotech (Bothell, WA) (Guo 2018, Zhang 1998, Guo 2017). For construction of pRB-KDC-yjgb, the yjgb gene was amplified from pRB-2PE by PCR and inserted into pRB374 by Gibson assembly, resulting in plasmid pRB-yjgb (Gibson 2009). KDC was then amplified from pRB-2PE by PCR and inserted into pRB-yjgb by Gibson assembly, resulting in plasmid pRB-KDC-yjgb.

pRB-2KDC-yjgb was generated by inverse PCR, in which the plasmid pRB-KDC-yjgb was amplified by PCR with RBS2 incorporated into the 5' end of the forward primer and then ligated by T4 DNA Ligase (NEB).

The gene for kivd (GenBank 51870501) was synthesized by Eurofins (Louisville, KY) and inserted into pRB-2KDC-yjgb in place of KDC by Gibson assembly, resulting in plasmid pRB-kivd-yjgb. Plasmid pHP-kivd-yjgb was constructed by amplifying the kivd-yjgb construct from pRB-kivd-yjgb and inserting it into pHP13 by Gibson assembly.

Unless otherwise specified, during strain and plasmid construction, *E. coli* and *B. subtilis* were grown in LB media at 37°C. For pRB374-based vectors, *E. coli* and *B. subtilis* transformants were selected with 100 µg/ml carbenicillin and 10 µg/ml kanamycin, respectively. For pHP13-based vectors, *E. coli* and *B. subtilis* transformants were selected with 33 µg/mL chloramphenicol and 1 µg/mL erythromycin, respectively.

6.2.3 Lysate preparations

Lysis buffer consisted of 50 mM Na_2HPO_4, 500 mM NaCl, 10 mM imidazole at pH 8. Bacterial cultures were centrifuged at 2700 rcf and 4°C for 15 min to collect a cell pellet. The cells were then washed in phosphate buffered saline (PBS) and centrifuged at 2700 rcf and 4°C for 10 min to collect a cell pellet. Cells were resuspended in 2 mL lysis buffer per 1 g of wet cell mass and sonicated using a Qsonica Q125 sonicator (Newton, CT). A sample of the resulting lysate was saved for analysis of insoluble protein fraction. The remaining lysate was then centrifuged at 12000 rcf and 4°C for 15 min to obtain the soluble protein fraction.

6.2.4 Protein detection via SDS-PAGE

SDS loading buffer was prepared in a 5x solution, consisting of 5 mL 0.5 M Tris HCL, pH 6.8, 5 mL of 80% glycerol, 8 mL of 10% sodium dodecyl sulfate (SDS), and 0.05% Bromophenol Blue (Alfa Aesar, Haverhill, MA). SDS running buffer was prepared in a 5x solution, consisting of 7.5 g Tris base, 36 g Glycine, and 25 mL of 10% SDS.

Coomassie staining solution consisted of 0.1% Coomassie Brilliant Blue G-250 (Amresco, Solon, OH), 10% acetic acid, and 40% methanol. De-staining solution consisted of 10% glacial acetic acid and 20% methanol.

Samples were prepared by combining 50 μg protein with 100 mM dithiothreitol (VWR, Radnor, PA) and 1x SDS loading buffer. In order to visualize the insoluble protein fraction, the same volume corresponding to 50 μg soluble protein was used from the insoluble samples collected after sonication. Samples were heated for 5 min at 90°C and then loaded into 12% Mini-PROTEAN precast polyacrylamide protein gels (Bio-Rad Laboratories, Hercules, CA). Gels were run at 200 V for 45 min using a Bio-Rad Mini-PROTEAN Tetra System attached to an EC 300 XL Power Supply (Thermo Scientific, Waltham, MA) with 1x SDS running buffer diluted in deionized water. Gels were stained in staining solution on a rocker for 10 min and de-stained in de-staining solution on a rocker for about 1 h. Gels were imaged using a UVP UVsolo touch stand-alone gel documentation system (Analytik Jena, Jena, Germany).

6.2.5 2-PE extraction

To extract 2-PE from liquid culture, 1 mL samples from liquid culture were combined with 0.05 mL 2 M hydrochloric acid and 0.2 mL methanol, centrifuged for 5 min at 10,000 rcf, and then filtered through a 0.2 μm filter for HPLC analysis.

6.2.6 Analytical methods

2-PE was detected using an Agilent 1200 series HPLC system (Agilent, Santa Clara, CA) equipped with a reversed-phase HPLC column (Eclipse XDB-C18, 5 μm, 4.6 x 150 mm, Agilent) at a wavelength of 210 nm. The mobile phase consisted of 60% water with 0.1% trifluoroacetic acid and 40% methanol at a flow rate of 1 mL/min. Commercial 2-PE was used as a standard (TCI Chemicals, Portland, OR).

6.3 Results

The initial plasmid design was based on the strategy utilized by Guo et al. for 2-PE production in *E. coli*, which expanded the endogenous shikimate pathway to convert phenylpyruvic acid to 2-PE (Figure 6.1**)** [154]. This strategy expanded the endogenous shikimate pathway by introducing feedback resistant mutants of the genes 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (AroG^{fbr}) and chorismate mutase / prephenate dehydratase (PheA^{fbr}), which are normally inhibited by L-phenylalanine, to produce high levels of the precursor phenylpyruvate [160, 161]. The strategy then utilized heterologous expression of 2-keto acid decarboxylase (KDC) and aldehyde reductase (yjgb) to convert phenylpyruvate to 2-PE (Figure 6.1). When this initial strategy did not yield 2-PE production detectable by HPLC or protein production detectable by SDS-PAGE, we simplified the genetic engineering strategy to express the two essential proteins, KDC and yjgb, to assist with troubleshooting. While the additional genes (AroG^{fbr} and PheA^{fbr}) may be useful to increase yield of 2-PE, 2-PE has been detected in *B. subtilis* with the introduction of just a keto acid dehydrogenase and an alcohol reductase, so we expected the simplified strategy to still produce detectable levels of 2-PE [162].

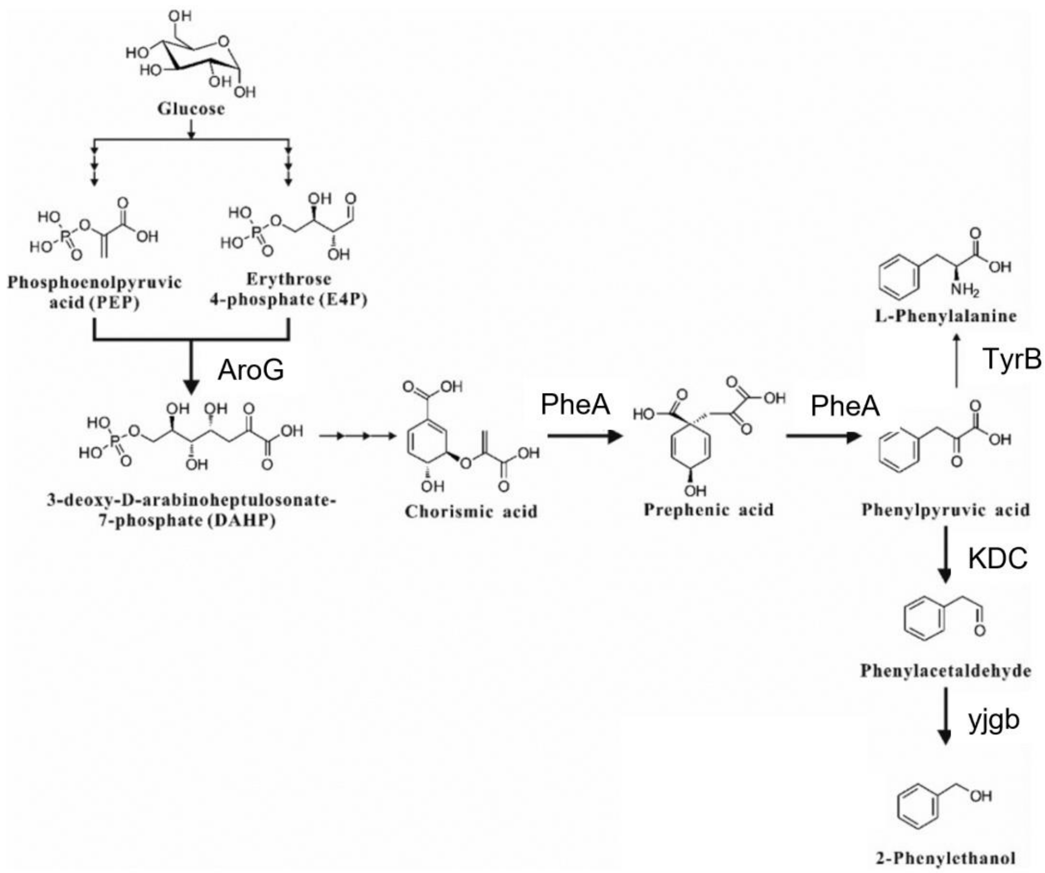

Figure 6.1. Expanded shikimate pathway for production of 2-PE. The shikimate pathway is an endogenous pathway used by bacteria to produce aromatic amino acids such as L-phenylalanine from glucose. Heterologous expression of the proteins KDC and yjgb is used to build upon this pathway for 2-PE production. Figure adapted from Guo et al. 2018 [154]

The modified expression strategy used plasmid pRB-KDC-yjgb. After transforming this plasmid into *E. coli*, we found that we only obtained colony PCR hits when cells were recovered at 25°C instead of 37°C (data not shown), indicating potential toxicity of the plasmid. When we analyzed protein expression in successful *E. coli* transformants via SDS-PAGE, we found that yjgb was detectable in both the soluble and insoluble protein fraction, but KDC was only detectable in the insoluble fraction (Figure 6.2). Having confirmed protein expression in *E. coli*, albeit not in the soluble fraction for both proteins, we next moved on to transform the plasmid into *B. subtilis* since expression in *E. coli* was not the end goal.

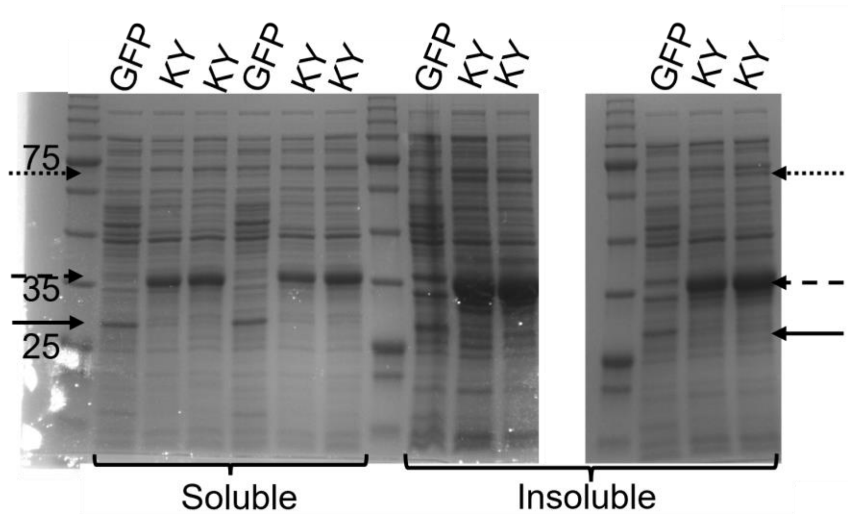

Figure 6.2. SDS-PAGE gel of lysates from E. coli strains EC-GFP (GFP) and EC-KDC-yjgb (KY). Dotted arrow shows expected band for KDC protein (around 70 kDa). Dashed arrow shows expected band for yjgb protein (around 35 kDa). Solid arrow shows expected band for GFP (around 26 kDa). Lanes show 4 separate replicates of EC-KDC-yjgb cultures and 2 replicates of EC-GFP cultures, which were used as a positive lysate control. Two separate gels were run and processed in parallel.

After transforming the plasmid pRB-KDC-yjgb into *B. subtilis* strain 168, we similarly found that successful transformants were only obtained after recovering the cells at room temperature, further indicating potential toxicity of the plasmid (data not shown). Moreover, we found that this plasmid was highly unstable in *B. subtilis*, which resulted in apparently successful transformants mutating during liquid culture (Figure 6.3).

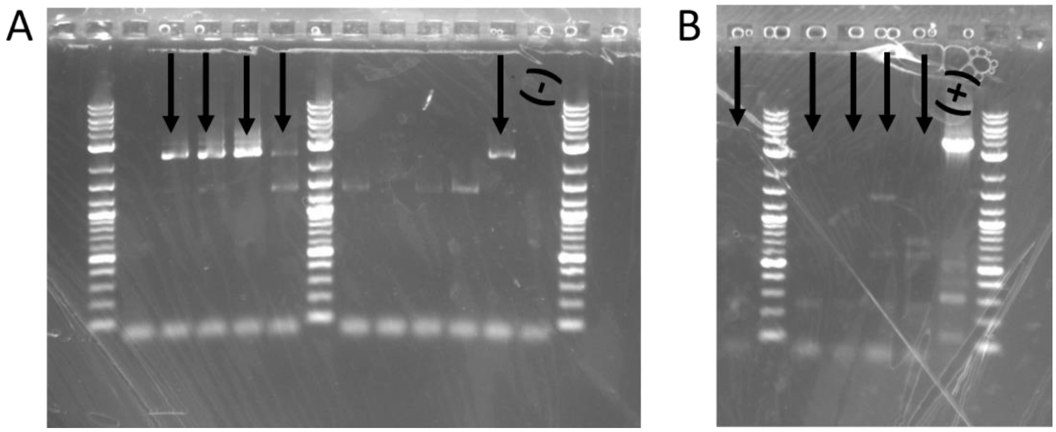

Figure 6.3. Example of PCR results indicating plasmid instability in *B. subtilis*. Arrows show (A) colony PCR amplification of region containing part of KDC and yjgb gene of colonies that were considered hits and (B) PCR of plasmid DNA isolated from the same colonies grown in liquid culture. (-): negative PCR control in which no template DNA was added. (+) positive PCR control in which plasmid isolated from *E. coli* was added.

Having observed that KDC seemed to be a potential source of toxicity in *E. coli* due to its exclusive presence in the insoluble protein fraction and because its source organism is yeast, we presumed that this gene may also be the cause of toxicity and plasmid instability in *B. subtilis*. We attempted two strategies to ease the burden caused by KDC: putting KDC under a weaker ribosome binding site (RBS2 from CHAPTER 4, **Error! Reference source not found.**) and replacing KDC with an alternative decarboxylase, kivd from *Lactococcus lactis*. These strategies did not appear to improve plasmid stability in *B.*

subtilis, as indicated by a lack of PCR hits after liquid culture for putative colony PCR hits.

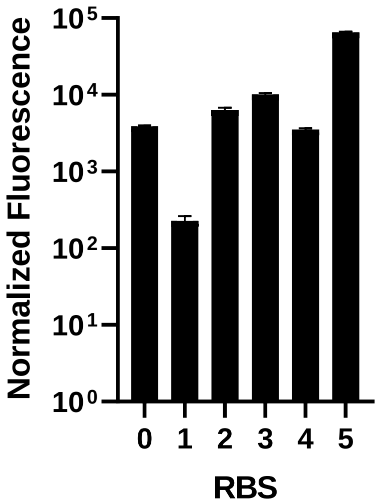

Figure 6.4 Copy of ribosome binding site fluorescence in monoculture from CHAPTER 4

Finally, we attempted to improve plasmid stability in *B. subtilis* by changing the plasmid vector to an endogenous *B. subtilis* low copy number vector (pHP13) that has been presumed to have better stability in *B. subtilis* compared to pUB110-derived vectors, such as pRB374 [159]. The results of this effort were inconclusive due to a lack of colonies when the plasmid was transformed into *E. coli*.

6.4 Discussion

Mosquito-borne diseases such as malaria, dengue, chikungunya virus, and Zika virus represent a widespread health burden resulting in hundreds of thousands of deaths each year. These diseases are spread between humans via mosquito bites, so the most obvious way to prevent transmission of disease is to prevent mosquitoes from biting the skin. The host-seeking behavior of mosquitoes is mediated by odor, and the skin microbiome has been found to affect people's attractiveness to mosquitoes by the production of volatile organic compounds. 2-PE is one such compound associated with people who are less attractive to mosquitoes and has known repellant activity against mosquitoes. In this study, we sought to engineer the skin resident bacteria *B. subtilis* to produce 2-PE as a form of long-acting mosquito repellant.

We utilized an expression strategy based on an expanded shikimate pathway and uncovered several challenges related to toxicity of the pathway and plasmid instability in *B. subtilis*. Further improvements to express an expanded shikimate pathway in *B. subtilis* for 2-PE production are left to future work. While we encountered challenges with plasmid instability when expressing this pathway on the pRB374 plasmid, we expect this to be feasible using the php13 plasmid because this is a low copy number plasmid that is endogenous to *B. subtilis* and was successfully used to express kivd and an alcohol reductase in *B. subtilis* [159, 162]. While we sought to implement plasmid-borne expression of these enzymes as an initial proof-of-concept, directly inserting genes into the *B. subtilis* genome may instead be a preferred approach. Strains engineered for commercial use generally require genome integration to ensure genetic stability and to reduce horizontal gene transfer, so this step would eventually be necessary regardless of initial expression strategy [134, 163].

Challenges expressing these genes even in the model organism *E. coli* further demonstrate the importance of minimizing metabolic burden on cells for the application of using engineered bacteria to deliver a therapeutic to the skin. While toxicity issues may be possible to overcome by optimization of media and growth conditions in a lab environment, these options are not feasible when continuous production on skin is required.

6.5 Conclusion

In this study, we sought to develop a long-acting mosquito repellant based on *B. subtilis* engineered to continuously produce the repellant 2-PE on the skin. Initial progress was made towards expressing an expanded shikimate pathway for 2-PE production, and challenges related to developing genetically engineered bacteria-based therapies as well as specific challenges of working with *B. subtilis* were highlighted.

CHAPTER 7. DISCUSSION

Improper adherence to medication is a health concern that limits the effectiveness of treatments and can hurt patient outcomes. Efforts to address this issue include strategies to make medication adherence easier for patients, for example by making administrations more comfortable and reducing the number of required administrations. Topical delivery is attractive because skin is a highly accessible tissue that can facilitate simple self-application of treatments, but existing topical delivery platforms are limited in their ability to deliver drugs over a long period of time and often require re-administration throughout the day to achieve the desired effect.

The central hypothesis of this thesis was that these limitations to existing topical delivery platforms can be overcome by taking advantage of the skin microbiome. The skin microbiome includes many species of commensal bacteria which colonize the skin and live there for days to years at a time, depending on the species. We hypothesized that we could employ synthetic biology techniques to engineer skin-resident species of bacteria to produce therapeutic molecules continuously on the skin.

Genetic engineering is not a simple task, and it becomes increasingly more difficult with non-model organisms. We thus assessed the feasibility of using *B. subtilis* as an engineered bacteria-based drug delivery platform. *B. subtilis* is found on the skin, but at much lower levels than other species of bacteria, such as species of *Staphylococci* and *Corynebacteria*, which are more traditionally considered residents of the skin microbiome. While *B. subtilis* may not be the obvious choice of candidate bacteria in terms of prevalence on skin, we considered it an attractive option because of its established safety profile and extensive use in the biotechnology industry.

In the first chapter of work, we sought to determine the timeframe over which engineered *B. subtilis* could survive and produce a heterologous protein on skin and to determine the safety of treating skin with much higher doses of *B. subtilis* than are normally found on skin. Using a combination of computational, *in vitro, ex vivo*, and *in vivo* skin models, we were able to show that *B. subtilis* engineered to produce green fluorescent protein (GFP) as a model heterologous protein could survive on skin while producing GFP for at least one day. Using *in vitro* cytotoxicity measurements and *in vivo* histological analysis, we further showed that *B. subtilis* appeared to be safe for skin at the levels tested, although further studies are needed. We thus concluded that *B. subtilis* has potential for use as a topical drug delivery platform for situations in which daily or every-other-day administration is appropriate.

In the second chapter of work, we assessed the feasibility of using engineered *B. subtilis* as an antifungal treatment. Many strains of *B. subtilis* naturally produce antifungal molecules, and unmodified *B. subtilis* has been used in *in vivo* mouse models to treat fungal skin infections. We hypothesized that we could increase the antifungal activity of *B. subtilis* by engineering cells to overproduce the antifungal lipopeptide iturin A. Iturin A is a non-ribosomal lipopeptide whose production is encoded by a 37.2 kb gene cluster. We chose a simple genetic engineering strategy to increase iturin A production by overexpressing the regulator protein DegQ, which has been used in literature to increase iturin A production in species of *Bacillus*. We found that DegQ overexpression did lead to higher levels of iturin A detected in *B. subtilis* culture. However, we also found that this approach led to significant growth defects which we suspect led the engineered strain to perform worse than the parent strain in an *in vitro* antifungal assay. This study highlighted a key challenge of using engineered bacteria for *in situ* drug production, as growth defects and other unintended effects to cells resulting from genetic engineering will limit a cell's ability to effectively produce therapeutic molecules on the skin, even if the cells are able to produce the molecules in culture.

In the final chapter of work, we assessed the use of engineered *B. subtilis* as a mosquito repellant. We chose to engineer *B. subtilis* to produce the repellant molecule 2-phenylethanol (2-PE), as this molecule has an established record of use in consumer products, has been produced heterologously in bacteria, and has established mosquito repellant activity. Our strategy for 2-PE production in *B. subtilis* built off of the endogenous metabolic pathway for phenylalanine production by adding a decarboxylase to convert phenylpyruvic acid to phenylacetaldehyde and a reductase to convert phenylacetaldehyde to 2-PE. Both proteins were co-expressed from the same plasmid DNA in *E. coli*, although the decarboxylase was only detectable in the insoluble protein fraction. We also experienced challenges transforming the plasmid DNA for this pathway into *B. subtilis*. While *B. subtilis* is a highly genetically tractable organism, a challenge highlighted by this study is limited plasmid stability in *B. subtilis*. This challenge can be overcome by careful selection of the plasmid vector for enhanced stability (e.g., low copy number) or by inserting desired genes directly into the *B. subtilis* genome. Another challenge highlighted by this study is the fact that engineered bacteria must be able to produce the desired molecules in the skin environment. Thus, genetic engineering strategies requiring optimized media or other alternative growth conditions are not feasible for production of drug *in situ*.

CHAPTER 8. CONCLUSIONS

In this work we proposed that the skin microbiome could be utilized to develop a bacteria-based topical drug delivery platform to prolong the delivery time of desired therapeutics and reduce the number of required administrations, thus making proper adherence to topical treatments easier for patients. We chose *B. subtilis* as a candidate organism for this purpose because it has an established record of safe use in humans and is highly genetically tractable.

Since *B. subtilis* is found in low levels on the skin and is likely a more transient member of the skin microbiome, we first sought to determine how long *B. subtilis* could survive on skin while producing a heterologous protein and whether *B. subtilis* is safe when applied at levels much higher than are normally found on skin. Using multiple laboratory models of skin, we found that *B. subtilis* could survive on skin while producing a model heterologous protein for at least one day and that it appeared to be safe for use on skin at the levels tested.

This work also highlighted some key challenges in the use of engineered bacteria for *in situ* drug production. Therapeutic targets and genetic engineering strategies should ideally focus on protein therapeutics that add minimal metabolic burden to cells. Complicated expression strategies imposing metabolic burden on cells can result in growth defects or other unintended effects that limit the cell's ability to produce therapeutics on the skin and survive within the competitive skin microbiome. Furthermore, expression strategies that require specialized growth conditions to produce the therapeutic would not be compatible for production on skin.

In summary, this thesis contributed to better understanding challenges associated with using engineered bacteria for drug delivery to the skin and established the potential of ameliorating some genetic engineering challenges by using *B. subtilis* as the engineered organism. While the use of engineered bacteria for *in situ* drug production is an exciting area of research, many challenges still exist in terms of colonization efficacy and the genetic engineering of non-model organisms. Development of bacteria-based drug delivery platforms suitable for use in humans will require deep understanding of the various factors affecting the platform's success *in vivo* and careful selection of candidate therapeutics.

CHAPTER 9. FUTURE DIRECTIONS

9.1 Establishing feasibility of using *B. subtilis* for drug delivery to the skin

While we demonstrated preliminary feasibility of using *B. subtilis* for drug delivery to the skin in terms of expected survival time and initial safety analysis, there are more studies needed to further establish the potential of using this organism. For example, this work did not analyze the effect of regular application of *B. subtilis* to the existing microbiome. While this issue was addressed in our computational model and other literature, more explicit studies of the changes to the skin microbiome resulting from continued application of *B. subtilis* would help further establish its safety. We also found that the strains of *B. subtilis* tested were more toxic to human keratinocyte cells compared to their culture media alone. Further studies would be required to determine the clinical significance of this toxicity and the implications for appropriate doses of *B. subtilis* for use on skin. Furthermore, other safety and biocontainment concerns would need to be addressed to prevent undesired gene transfer to neighboring bacteria and undesired transfer of the engineered bacteria to other surfaces. These issues have been addressed in industry by inserting desired genes into the genome of the engineered organisms and by using auxotrophy to ensure that the organism will not be able to survive without application of an additional substance.

In terms of the expected timeframe of therapeutic delivery, more quantitative studies to determine the actual levels of *B. subtilis* on the skin over time would help further establish an appropriate administration frequency. Another factor that could affect *B. subtilis* survival on skin which we did not address in this thesis is the initial density of bacteria applied to the skin. A future study could address this by applying engineered *B. subtilis* to the skin at different densities to determine if there is an effect on *B. subtilis* survival. Finally, studies analyzing the survival of *B. subtilis* on human skin *in vivo* are necessary to truly determine whether *B. subtilis* can survive on skin while producing heterologous protein for a useful amount of time.

9.2 Determining efficacy of engineered *B. subtilis* for use as an antifungal treatment

Since many strains of *B. subtilis* are naturally antifungal, an analysis of unmodified strains with high antifungal activity in an *in vivo* infection model would be useful to determine whether genetic engineering to increase antifungal activity is necessary. A couple of studies have addressed this topic using special formulations to protect *B. subtilis* on the skin to successfully treat *C. albicans* infection in mice. A study screening multiple naturally antifungal strains of *B. subtilis* in a more relevant infection model such as *T. mentagrophytes* would be useful to determine whether *B. subtilis* can be used for this application without additional engineering. If enhanced antifungal production through genetic engineering is desired, this screen would also be useful to select a strain with high antifungal activity as a starting point.

The genetic engineering strategy should increase antifungal activity while limiting growth defects. This can potentially be accomplished in future work by inserting a stronger promoter ahead of the iturin A gene cluster in the bacterial genome. This strategy could also be used to increase production of other antifungal molecules produced by *B. subtilis*, such as fengycin or bacillomycin D.

9.3 Determining efficacy of engineered *B. subtilis* for use as a mosquito repellant

While this work made initial progress towards engineering our strain of *B. subtilis* to produce 2-PE, continued plasmid and strain development is required to achieve production of this molecule. We expect that strain development will be more feasible using the php13 vector because it is a low copy number plasmid that is endogenous to *B. subtilis*. Once a strain is developed that produces 2-PE in culture, experiments to determine the feasibility of using this strain *in vivo* will be required. First, the strain would need to be analyzed in a skin model to determine whether useful levels of 2-PE can be detected for a useful period of time. If the results are promising, the production of 2-PE would need to be further characterized *in vivo* and finally would need to be studied for actual mosquito repellant activity *in vivo* using a repellency assay.

APPENDIX. ANNOTATED ITURIN A GENE CLUSTER FOR STRAIN 15841

ItuD ItuA ItuB ItuC

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tccttgctttattattgagttgcttcactgggaagtctgcgtatgctgccagcactccaattgcaaaacatgttgggaattc

REFERENCES

1. Patrick J McDonnell, M.R.J., *Hospital Admissions Resulting from Preventable Adverse Drug Reactions.* The Annals of Pharmacotherapy 2002. **36**.

2. Bonnie L Senst, L.E.A., Robert P Genest, Laurey A Cosentino, Corey C Ford, James A Little, Sabrina J Raybon, David W Bates, *Practical approach to determining costs and frequency of adverse drug events in a health care network.* Am J Health-Syst Pharm, 2001. **58**.

3. WHO, *Adherence to Long-Term Therapies Evidence for Action* 2003.

4. Brown, K.K., W.E. Rehmus, and A.B. Kimball, *Determining the relative importance of patient motivations for nonadherence to topical corticosteroid therapy in psoriasis.* J Am Acad Dermatol, 2006. **55**(4): p. 607-13.

5. Choi, J.W., B.R. Kim, and S.W. Youn, *Adherence to Topical Therapies for the Treatment of Psoriasis: Surveys of Physicians and Patients.* Ann Dermatol, 2017. **29**(5): p. 559-564.

6. Lars Osterberg, T.B., *Adherence to Medication* New England Journal of Medicine 2005. **353**.

7. Tan, X., et al., *Topical drug delivery systems in dermatology: a review of patient adherence issues.* Expert Opin Drug Deliv, 2012. **9**(10): p. 1263-71.

8. Katsutoshi ARa, M.H., Syunichi Akiba, Kenzo Koike, Koichi Okisaka, Toyoki Hagura, Tetsuro Kamiya, Fusao Tomita, *Foot odor due to microbial metabolism and its control.* Canadian Journal of Micobiology 2006. **52**(4).

9. Dekio, I., et al., *Detection of potentially novel bacterial components of the human skin microbiota using culture-independent molecular profiling.* J Med Microbiol, 2005. **54**(Pt 12): p. 1231-1238.

10. Su, Y., et al., *Bacillus subtilis: a universal cell factory for industry, agriculture, biomaterials and medicine.* Microb Cell Fact, 2020. **19**(1): p. 173.

11. Ameen, M., *Epidemiology of superficial fungal infections.* Clin Dermatol, 2010. **28**(2): p. 197-201.

12. Achterman, R.R. and T.C. White, *A foot in the door for dermatophyte research.* PLoS Pathog, 2012. **8**(3): p. e1002564.

13. Gupta, A.K. and E.A. Cooper, *Update in antifungal therapy of dermatophytosis.* Mycopathologia, 2008. **166**(5-6): p. 353-67.

14. Kim, P.I., Jaewon Ryu, Young Hwan Kim, Youn-Tae ChI, *Production of Biosurfactant Lipopeptides Iturin A, Fengycin, and Surfactin A from Bacillus subtilis CMB32 for Control of Colletotrichum gloeosporioides* Journal of Microbiology and Biotechnology 2009. **20**(1): p. 138-145.

15. M. Landy, G.H.W., S.B. Rosenman, L.G. Colio, *Bacillomycin: An Antibiotic from Bacillus Subtilis Active against Pathogenic Fungi.* Proc Soc Exp Biol Med, 1948. **67**(4).

16. Pathak, K.V. and H. Keharia, *Identification of surfactins and iturins produced by potent fungal antagonist, Bacillus subtilis K1 isolated from aerial roots of banyan (Ficus benghalensis) tree using mass spectrometry.* 3 Biotech, 2014. **4**(3): p. 283-295.

17. Kim, Y.T., et al., *Organization and characterization of genetic regions in Bacillus subtilis subsp. krictiensis ATCC55079 associated with the biosynthesis of iturin and surfactin compounds.* PLoS One, 2017. **12**(12): p. e0188179.

18. Laurence Thimon, F.P., Jean Wallach, Georges Michel, *Effect of the lipopeptide antibiotic, iturin A, on morphology and membrane ultrastructure of yeast cells* FEMS Microbiology Letters, 1995. **128**: p. 101-106.

19. O'Connor, N.K., et al., *Novel fluorinated lipopeptides from Bacillus sp. CS93 via precursor-directed biosynthesis.* Amino Acids, 2014. **46**(12): p. 2745-52.

20. WHO, *Malaria Prevention Works - let's close the gap.* 2017.

21. Verhulst, N.O., et al., *Relation between HLA genes, human skin volatiles and attractiveness of humans to malaria mosquitoes.* Infect Genet Evol, 2013. **18**: p. 87-93.

22. Niels O Verhulst, P.A.M., Gabriella Bukovinszkine Kiss, Wolfgang R Mukabana, Joop JA van Loon, Willem Takken, Renate C Smallegange, *Improvement of a synthetic lure for Anopheles gambiae using compounds produced by human skin microbiota.* Malaria Journal 2011. **10**(28).

23. USDA, *Results of Screening Tests with Materials Evaluated as Insecticides, Miticides, and Repellents at the Orlando, Florida, Laboratory* 1947.

24. Zeng, F., P. Xu, and W.S. Leal, *Odorant receptors from Culex quinquefasciatus and Aedes aegypti sensitive to floral compounds.* Insect Biochem Mol Biol, 2019. **113**: p. 103213.

25. Haynes, R.B., et al., *Interventions for enhancing medication adherence.* Cochrane Database Syst Rev, 2008(2): p. CD000011.

26. Haehl, K., *Choosing the Optimal Drug Delivery System to Meet Patient Needs.* American Pharmaceutical Review, 2015.

27. Albert I. Werheimer, T.M.S., Albert J. Finestone, Richard A. Levy, *Drug Delivery Systems Improve Pharmaceutical Profile and Facilitate Medication Adherence.* Advances in Therapy, 2005. **22**(6).

28. Sameh Said Zaghloul, M.J.D.G., *Objective Assessment of Compliance With Psoriasis Treatment.* Arch Dermatol., 2004. **140**.

29. Barnes, T.M., et al., *Vehicles for Drug Delivery and Cosmetic Moisturizers: Review and Comparison.* Pharmaceutics, 2021. **13**(12).

30. Brown, M.B., et al., *Dermal and transdermal drug delivery systems: current and future prospects.* Drug Deliv, 2006. **13**(3): p. 175-87.

31. Kenneth A. Walters, M.S.R., *The Structure and Function of Skin* in *Dermatological and Transdermal Formulations* K.A. Walters, Editor. 2002, CRC Press: New York, United States.

32. Andrews, S.N., E. Jeong, and M.R. Prausnitz, *Transdermal delivery of molecules is limited by full epidermis, not just stratum corneum.* Pharm Res, 2013. **30**(4): p. 1099-109.

33. Elizabeth A. Grice, J.A.S., *The Skin Microbiome.* Nature Reviews, 2011. **9**.

34. R A Bojar, K.T.H., *Review: the human cutaneous microflora and factors controlling colonisation* World Journal of Microbiology and Biotechnology, 2002. **18**.

35. Lange-Asschenfeldt, B., et al., *Distribution of bacteria in the epidermal layers and hair follicles of the human skin.* Skin Pharmacol Physiol, 2011. **24**(6): p. 305-11.

36. Tiffany C. Scharschmidt, M.A.F., *What lives on our skin: ecology, genomics, and therapeutic opportunities of the skin microbiome.* Drug Discovery Today Disease Mechanisms 2013. **10**(3).

37. Elizabeth A. Grice, H.H.K., Sean Conlan, Clayton B. Deming, Jole Davis, Alice C. Young, NISC Comparative Sequencing Program, Gerard G. Bouffard, Robert W. Blakesley, Patrick R. Murray, Eric D. Green, Maria L. Turner, Julia A. Segre, *Topographical and temporal diversity of the human skin microbiome.* Science, 2009. **324**(5931).

38. Grice, E.A., et al., *A diversity profile of the human skin microbiota.* Genome Res, 2008. **18**(7): p. 1043-50.

39. Reichel, M., P. Heisig, and G. Kampf, *Identification of variables for aerobic bacterial density at clinically relevant skin sites.* J Hosp Infect, 2011. **78**(1): p. 5-10.

40. S A Malcolm, T.C.H., *The demonstration of bacteria on and within the stratum corneum using scanning electron microscopy* British Journal of Dermatology, 1980. **102**.

41. Nakatsuji, T., et al., *The microbiome extends to subepidermal compartments of normal skin.* Nat Commun, 2013. **4**: p. 1431.

42. Bay, L., et al., *Universal Dermal Microbiome in Human Skin.* mBio, 2020. **11**(1).

43. Wilson, M., *The skin and its indigenous microbiota*, in *Microbial Inhabitants of Humans*. 2010. p. 51-106.

44. Costello, E.K., et al., *Bacterial community variation in human body habitats across space and time.* Science, 2009. **326**(5960): p. 1694-7.

45. Julia Oh, A.L.B., Morgan Park, NISC Comparative Sequencing Program, Heidi H. Kong, Julia A. Segre, *Temporal stability of the human skin microbiome.* Cell, 2016. **165**.

46. Kong, H.H. and J.A. Segre, *Skin microbiome: looking back to move forward.* J Invest Dermatol, 2012. **132**(3 Pt 2): p. 933-9.

47. Katarina Chiller, B.A.S., George J. Murakawa, *Skin microflora and bacterial infections of the skin.* JID Symposium Proceedings 2001. **6**(3).

48. David J. Gonzalez, N.M.H., Andrew Hollands, Tinya C. Fleming, Matthew Hamby, Kit Pogliano, Victor Nizet, Pieter C. Dorrestein *Microbial competition between Bacillus subtilis and Staphylococcus aureus monitored by imaging mass spectrometry.* Microbiology 2011. **157**.

49. Lufton, M., et al., *Living Bacteria in Thermoresponsive Gel for Treating Fungal Infections.* Advanced Functional Materials, 2018. **28**(40).

50. Kumar, A. and N. Chordia, *Role of Microbes in Human Health.* Applied Microbiology: Open Access, 2017. **03**(02).

51. FDA, *GRAS Notices* 2020, US Food and Drug Administration.

52. Hedges, S.B., *The origin and evolution of model organisms.* Nat Rev Genet, 2002. **3**(11): p. 838-49.

53. Ogasawara, N., *Markedly unbiased codon usage in Bacillus subtilis* Gene, 1985. **40**.

54. Brockmeier, U., et al., *Systematic screening of all signal peptides from Bacillus subtilis: a powerful strategy in optimizing heterologous protein secretion in Gram-positive bacteria.* J Mol Biol, 2006. **362**(3): p. 393-402.

55. Bruckner, R., *A series of shuttle vectors for Bacillus subtilis and Escherichia coli.* Gene, 1992. **122**.

56. iGEM. *Registry of Standard Biological Parts*. Available from: http://parts.igem.org/Bacillus_subtilis#Ribosome_binding_sites.

57. Popp, P.F., et al., *The Bacillus BioBrick Box 2.0: expanding the genetic toolbox for the standardized work with Bacillus subtilis.* Sci Rep, 2017. **7**(1): p. 15058.

58. Moskovicz, V., et al., *Skin microbiota dynamics following B. subtilis formulation challengs.* 2020.

59. Stecher, B., et al., *Like will to like: abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria.* PLoS Pathog, 2010. **6**(1): p. e1000711.

60. Maldonado-Gomez, M.X., et al., *Stable Engraftment of Bifidobacterium longum AH1206 in the Human Gut Depends on Individualized Features of the Resident Microbiome.* Cell Host Microbe, 2016. **20**(4): p. 515-526.

61. Shepherd, E.S., et al., *An exclusive metabolic niche enables strain engraftment in the gut microbiota.* Nature, 2018. **557**(7705): p. 434-438.

62. Violle, C., et al., *Phylogenetic limiting similarity and competitive exclusion.* Ecol Lett, 2011. **14**(8): p. 782-7.

63. Wu, G., et al., *Metabolic Burden: Cornerstones in Synthetic Biology and Metabolic Engineering Applications.* Trends Biotechnol, 2016. **34**(8): p. 652-664.

64. Paul E Turner, V.S.C., Richard E Lenski *Tradeoff between horizontal and vertical modes of transmission in bacterial plasmids* Evolution, 1998. **52**(2).

65. Bertero, A., S. Brown, and L. Vallier, *Methods of Cloning*, in *Basic Science Methods for Clinical Researchers*. 2017. p. 19-39.

66. Watson, J.F. and J. Garcia-Nafria, *In vivo DNA assembly using common laboratory bacteria: A re-emerging tool to simplify molecular cloning.* J Biol Chem, 2019. **294**(42): p. 15271-15281.

67. Rai, P. and H. Arya, *Molecular cloning*, in *The Design & Development of Novel Drugs and Vaccines*. 2021. p. 135-163.

68. Gibson, D.G., et al., *Enzymatic assembly of DNA molecules up to several hundred kilobases.* Nat Methods, 2009. **6**(5): p. 343-5.

69. Liang, J., et al., *Twin-primer non-enzymatic DNA assembly: an efficient and accurate multi-part DNA assembly method.* Nucleic Acids Res, 2017. **45**(11): p. e94.

70. Friehs, K., *Plasmid Copy Number and Plasmid Stability*. Adv Biochem Engin/Biotechnol, 2004.

71. Ou, B., et al., *Techniques for chromosomal integration and expression optimization in Escherichia coli.* Biotechnol Bioeng, 2018. **115**(10): p. 2467-2478.

72. Lucht, J.M., *Public Acceptance of Plant Biotechnology and GM Crops.* Viruses, 2015. **7**(8): p. 4254-81.

73. George Gaskell, N.A., Wolfgang Wagner, Nicole Kronberger, Helge Torgersen, Juergen Hampel, Julie Bardes, *GM Foods and the Misperception of Risk Perception* Risk Analysis 2004. **24**.

74. Connor, M. and M. Siegrist, *Factors Influencing People's Acceptance of Gene Technology: The Role of Knowledge, Health Expectations, Naturalness, and Social Trust.* Science Communication, 2010. **32**(4): p. 514-538.

75. George Gaskell, N.A., Martin Bauer, John Durant, Agnes Allansdottir, Heinz Bonfadelli, Daniel Boy, Suzanne de Cheveigne, Bjorn Fjaestad, Jan M. Gutteling, Juergen Hampel, Erling Jelsoe, Jorge Correia Jesuino, Matthias Kohring, Nicole Kronberger, Cees Midden, Torben Hviid Nielsen, Andrzej Przestalski, Timo Rusanen, George Sakellaris, Helge Torgersen, Tomasz Twardowski, Wolfgang Wagner, *Biotechnology and the European Public* Nature Biotechnology, 2000. **18**.

76. Gupta, S., E. Allen-Vercoe, and E.O. Petrof, *Fecal microbiota transplantation: in perspective.* Therap Adv Gastroenterol, 2016. **9**(2): p. 229-39.

77. Stavropoulou, E. and E. Bezirtzoglou, *Probiotics in Medicine: A Long Debate.* Front Immunol, 2020. **11**: p. 2192.

78. Detmer, A. and J. Glenting, *Live bacterial vaccines--a review and identification of potential hazards.* Microb Cell Fact, 2006. **5**: p. 23.

79. Angelidou, A., et al., *Licensed Bacille Calmette-Guerin (BCG) formulations differ markedly in bacterial viability, RNA content and innate immune activation.* Vaccine, 2020. **38**(9): p. 2229-2240.

80. Vaccines, C., *Vivotif Package Insert USA.* 2013.

81. PaxVax, *Highlights of prescribing information* 2016.

82. Carina Kruger, Y.H., Qiang Pan, Harold Marcotte, Anna Hultberg, Dipu Delwar, Philip J. van Dalen, Peter H. Pouwels, Rob J. Leer, Charles G. Kelly, Craig van Dollenweerd, Julian K. Ma, Lennart Hammarstrom *In situ delivery of passive*

immunity by lactobacilli producing single-chain antibodies. Nature Biotechnology, 2002. **20**.

83. Chen, Z., et al., *Incorporation of therapeutically modified bacteria into gut microbiota inhibits obesity*. J Clin Invest, 2014. **124**(8): p. 3391-406.

84. Concetta Beninati, M.R.O., Maria Boccanera, Maria Rita Spinosa, Tiziana Maggi, Stefania Conti, Walter Magliani, Flavia De Bernardis, Giuseppe Teti, Antonio Cassone, Gianni Pozzi, Luciano Polonelli, *Therapy of mucosal candidiasis by expression of an anti-idiotype in human commensal bacteria.* Nature Biotechnology 2000. **18**.

85. Braat, H., et al., *A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease.* Clin Gastroenterol Hepatol, 2006. **4**(6): p. 754-9.

86. Vagesjo, E., et al., *Accelerated wound healing in mice by on-site production and delivery of CXCL12 by transformed lactic acid bacteria.* Proc Natl Acad Sci U S A, 2018. **115**(8): p. 1895-1900.

87. Schmidt, C., *Out of your skin.* Nat Biotechnol, 2020. **38**(4): p. 392-397.

88. Hyde, E. *Meet Staph epidermidis, gatekeeper of healthy skin*. 2018.

89. Koch, S., *Microbiome Gets Skinny.* Biocentury Innovations, 2016: p. 11.

90. Liu, X., et al., *Engineered vaginal lactobacillus strain for mucosal delivery of the human immunodeficiency virus inhibitor cyanovirin-N.* Antimicrob Agents Chemother, 2006. **50**(10): p. 3250-9.

91. Vargason, A.M. and A.C. Anselmo, *Clinical translation of microbe-based therapies: Current clinical landscape and preclinical outlook.* Bioeng Transl Med, 2018. **3**(2): p. 124-137.

92. B Appala Naidu, K.K., D P Santhosh Kumar, John W K Oliver, Zachary D Abbott *Lyophilized B. subtilis ZB183 spores: 90-day repeat dose oral (gavage) toxicity study in wistar rats* Journal of Toxicology 2019.

93. Crawford, F., *Athlete's foot* Clinical Evidence 2009.

94. Pearson, S.L. *These 5 popular antifungal creams are available over the counter* 2022.

95. Regine Maget-Dana, F.P., *Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties* Toxicology 1994. **87**.

96. Madewell, Z.J., *Arboviruses and Their Vectors.* South Med J, 2020. **113**(10): p. 520-523.

97. CDC. *Chikungunya Virus* 2022; Available from: https://www.cdc.gov/chikungunya/index.html.

98. The, L., *Malaria vaccine approval: a step change for global health.* Lancet, 2021. **398**(10309): p. 1381.

99. Mark S Fradin, J.F.D., *Comparative efficacy of insect repellents against mosquito bites* N Engl J Med, 2002. **347**(1).

100. EPA, *Reregistration Eligibility Decision (RED) DEET*. 1998.

101. Vanessa Chen-Hussey, R.B., James G Logan *Assessment of methods used to determine the safety of the topical insect repellent N,N-diethy-m-toluamide (DEET).* Parasites and vectors 2014. **7**(173).

102. Etschmann, M.M., et al., *Biotechnological production of 2-phenylethanol.* Appl Microbiol Biotechnol, 2002. **59**(1): p. 1-8.

103. Baryakova, T.H., et al., *Overcoming barriers to patient adherence: the case for developing innovative drug delivery systems.* Nat Rev Drug Discov, 2023. **22**(5): p. 387-409.

104. Berth-Jones, J., *Topical Therapy*, in *Rook's Textbook of Dermatology*, S.B. Tony Burns, Neil Cox, Christopher Griffiths, Editor. 2010, Blackwell Publishing Ltd. .

105. Ma, J., et al., *Engineered probiotics.* Microb Cell Fact, 2022. **21**(1): p. 72.

106. Vargason, A.M. and A.C. Anselmo, *Live Biotherapeutic Products and Probiotics for the Skin.* Advanced NanoBiomed Research, 2021. **1**(12).

107. Wang, F., et al., *Living Bacterial Microneedles for Fungal Infection Treatment.* Research, 2020. **2020**: p. 1-9.

108. Lee, N.K., W.S. Kim, and H.D. Paik, *Bacillus strains as human probiotics: characterization, safety, microbiome, and probiotic carrier.* Food Sci Biotechnol, 2019. **28**(5): p. 1297-1305.

109. Gary An, Q.M., Joyeeta Dutta-Moscato, Yoram Vodovotz, *Agent-based models in translational systems biology.* WIREs Systems Biology and Medicine, 2009. **1**.

110. Lin, C., et al., *GutLogo: Agent-based modeling framework to investigate spatial and temporal dynamics in the gut microbiome.* PLoS One, 2018. **13**(11): p. e0207072.

111. Sylvestre, J.P., et al., *Extraction and quantification of amino acids in human stratum corneum in vivo.* Br J Dermatol, 2010. **163**(3): p. 458-65.

112. Greene, R.S., et al., *Anatomical variation in the amount and composition of human skin surface lipid.* J Invest Dermatol, 1970. **54**(3): p. 240-7.

113. Zhan Gao, C.-h.T., Zhiheng Pei, Martin J Blaser, *Molecular analysis of human forearm superficial skin bacterial biota.* PNAS, 2007. **104**.

114. Meyer, F.M. and J. Stulke, *Malate metabolism in Bacillus subtilis: distinct roles for three classes of malate-oxidizing enzymes.* FEMS Microbiol Lett, 2013. **339**(1): p. 17-22.

115. Rudrappa, T., et al., *Root-secreted malic acid recruits beneficial soil bacteria.* Plant Physiol, 2008. **148**(3): p. 1547-56.

116. Antunes, L.C., et al., *Deciphering the multifactorial nature of Acinetobacter baumannii pathogenicity.* PLoS One, 2011. **6**(8): p. e22674.

117. Philippe J M Bouvet, P.A.D.G., *Taxonomy of the Genus Acinetobacter with the Recognition of Acinetobacter baumannii sp. nov., Acinetobacter haemolyticus sp. nov., Acinetobacter johnsonii sp. nov., and Acinetobacter junii sp. nov. and Emended Descriptions of Acinetobacter calcoaceticus and Acinetobacter lwoffii* International Journal of Systemic Bacteriology, 1986. **36**.

118. Brune, I., et al., *Under the influence of the active deodorant ingredient 4-hydroxy-3-methoxybenzyl alcohol, the skin bacterium Corynebacterium jeikeium moderately responds with differential gene expression.* J Biotechnol, 2006. **127**(1): p. 21-33.

119. Mounier, J., et al., *Growth characteristics of Brevibacterium, Corynebacterium, Microbacterium, and Staphylococcus spp. isolated from surface-ripened cheese.* Appl Environ Microbiol, 2007. **73**(23): p. 7732-9.

120. Tauch, A., et al., *Complete genome sequence and analysis of the multiresistant nosocomial pathogen Corynebacterium jeikeium K411, a lipid-requiring bacterium of the human skin flora.* J Bacteriol, 2005. **187**(13): p. 4671-82.

121. Oliveira, F., A. Franca, and N. Cerca, *Staphylococcus epidermidis is largely dependent on iron availability to form biofilms.* Int J Med Microbiol, 2017. **307**(8): p. 552-563.

122. Halsey, C.R., et al., *Amino Acid Catabolism in Staphylococcus aureus and the Function of Carbon Catabolite Repression.* mBio, 2017. **8**(1).

123. Liu, Y.K., et al., *Single amino acid utilization for bacterial categorization.* Sci Rep, 2020. **10**(1): p. 12686.

124. Bottinger, B., et al., *Regulated ploidy of Bacillus subtilis and three new isolates of Bacillus and Paenibacillus.* FEMS Microbiol Lett, 2018. **365**(4).

125. Kin-Ichi Sugae, E.F., *Requirement for Acetate and Glycine (or Serine) for Sporulation without Growth of Bacillus subtilis.* Journal of Bacteriology, 1970. **104**(3).

126. Boris R Belitsky, A.L.S., *Role and Regulation of Baillus subtilis Glutamate Dehydrogenase Genes* Journal of Bacteriology, 1998. **180**.

127. Chasin, L.A. and B. Magasanik, *Induction and Repression of the Histidine-degrading Enzymes of Bacillus subtilis.* Journal of Biological Chemistry, 1968. **243**(19): p. 5165-5178.

128. M N A Ansari, N.N., H C Fu, *Fatty acid composition of the living layer and stratum corneum lipids of human sole skin epidermis* Lipids 1970. **5**.

129. Albert Lehninger, D.N., Michael Cox *Lehninger Principles of Biochemistry*, in 2005, W. H. Freeman

130. Bender, D.A., *The metabolism of "surplus" amino acids.* Br J Nutr, 2012. **108 Suppl 2**: p. S113-21.

131. Hsieh, F.C., et al., *Comparing methods for identifying Bacillus strains capable of producing the antifungal lipopeptide iturin A.* Curr Microbiol, 2008. **56**(1): p. 1-5.

132. Qizhi Huang, R.A.D., David E. Pegg, John N. Kearney, Sheila Macneil, *Use of peracetic acid to sterilize human donor skin for production of acellular dermal matrices for clinical use.* Wound Repair and Regeneration, 2004. **12**.

133. Weyrich, L.S., et al., *The skin microbiome: Associations between altered microbial communities and disease.* Australas J Dermatol, 2015. **56**(4): p. 268-74.

134. Dreher-Lesnick, S.M., S. Stibitz, and P.E. Carlson, Jr., *U.S. Regulatory Considerations for Development of Live Biotherapeutic Products as Drugs.* Microbiol Spectr, 2017. **5**(5).

135. Guan, C., et al., *Engineering a "detect and destroy" skin probiotic to combat methicillin-resistant Staphylococcus aureus.* PLoS One, 2022. **17**(12): p. e0276795.

136. Dodds, D., et al., *Controlling the Growth of the Skin Commensal Staphylococcus epidermidis Using d-Alanine Auxotrophy.* mSphere, 2020. **5**(3).

137. Monod, M. and F. Lanternier, *Dermatophytes and Dermatophytosis*, in *Encyclopedia of Infection and Immunity*. 2022. p. 397-407.

138. R Lahlali, G.P., B D Gossen, L McGregor, F Q Yu, R K Hynes, S F Hwang, M R McDonald, S M Boyetchko, *Evidence that the biofungicide Serenade (Bacillus subtilis) suppresses clubroot on canola via antibiosis and induced host resistance.* Phytopathology, 2012. **103**(3).

139. Aranda, F.J., J.A. Teruel, and A. Ortiz, *Further aspects on the hemolytic activity of the antibiotic lipopeptide iturin A.* Biochim Biophys Acta, 2005. **1713**(1): p. 51-6.

140. Jin, H., et al., *Continuous enhancement of iturin A production by Bacillus subtilis with a stepwise two-stage glucose feeding strategy.* BMC Biotechnol, 2015. **15**: p. 53.

141. Tsuge, K., et al., *Horizontal transfer of iturin A operon, itu, to Bacillus subtilis 168 and conversion into an iturin A producer.* Antimicrob Agents Chemother, 2005. **49**(11): p. 4641-8.

142. Dang, Y., et al., *Enhanced production of antifungal lipopeptide iturin A by Bacillus amyloliquefaciens LL3 through metabolic engineering and culture conditions optimization.* Microb Cell Fact, 2019. **18**(1): p. 68.

143. Tsuge, K., T. Akiyama, and M. Shoda, *Cloning, sequencing, and characterization of the iturin A operon.* J Bacteriol, 2001. **183**(21): p. 6265-73.

144. Labs, B. *BWA Aligner*. 2020 [cited 2021; Available from: https://basespace.illumina.com/apps/2892890/BWA-Aligner?preferredversion.

145. Yuan, J., et al., *Quantification of the antifungal lipopeptide iturin A by high performance liquid chromatography coupled with aqueous two-phase extraction.* J Chromatogr B Analyt Technol Biomed Life Sci, 2011. **879**(26): p. 2746-50.

146. W C Nierman, T.V.F., *Genomic Library*, in *Encyclopedia of Genetics*. 2001, Academic Press.

147. Iwase Noriyasu, R.M.S., Ano Takashi, *Production of iturin A homologues under different culture conditions.* Journal of Environmental Sciences (China), 2009. **21**.

148. Hsieh, F.C., et al., *Rapid detection and characterization of surfactin-producing Bacillus subtilis and closely related species based on PCR.* Curr Microbiol, 2004. **49**(3): p. 186-91.

149. Ghelardi, E., et al., *Contribution of surfactin and SwrA to flagellin expression, swimming, and surface motility in Bacillus subtilis.* Appl Environ Microbiol, 2012. **78**(18): p. 6540-4.

150. Lilge, L., et al., *Expression of degQ gene and its effect on lipopeptide production as well as formation of secretory proteases in Bacillus subtilis strains.* Microbiologyopen, 2021. **10**(5): p. e1241.

151. CDC, *Malaria's Impact Worldwide*. 2021.

152. Verhulst, N.O., et al., *Differential attraction of malaria mosquitoes to volatile blends produced by human skin bacteria.* PLoS One, 2010. **5**(12): p. e15829.

153. Verhulst, N.O., et al., *Cultured skin microbiota attracts malaria mosquitoes.* Malar J, 2009. **8**: p. 302.

154. Guo, D., et al., *Metabolic Engineering of Escherichia coli for Production of 2-Phenylethanol and 2-Phenylethyl Acetate from Glucose.* J Agric Food Chem, 2018. **66**(23): p. 5886-5891.

155. Koma, D., et al., *Production of aromatic compounds by metabolically engineered Escherichia coli with an expanded shikimate pathway.* Appl Environ Microbiol, 2012. **78**(17): p. 6203-16.

156. Liu, J., et al., *Mimicking a New 2-Phenylethanol Production Pathway from Proteus mirabilis JN458 in Escherichia coli.* J Agric Food Chem, 2018. **66**(13): p. 3498-3504.

157. US Food and Drug Administration. *Substances added to food - phenethyl alcohol*. 2020; 01/14/2020:[Available from: https://www.accessdata.fda.gov/scripts/fdcc/?set=FoodSubstances&id=PHENETHYLALCOHOL.

158. National Center for Biotechnology Information. *2-PHENYLETHANOL*. Available from: https://pubchem.ncbi.nlm.nih.gov/source/hsdb/5002.

159. Peter Haima, S.B., Gerard Venema, *The effect of ristriction on shotgun cloning and plasmid stability in Bacillus subtilis Marburg.* Mol Gen Genet, 1987. **209**.

160. Geraldine C Hall, R.A.J., *Enzymological Basis for Growth Inhibition by L-Phenylalanine in the Cyanobacterium Synechocystis sp 29108.* Journal of Bacteriology, 1980. **144**.

161. Zhang, S., et al., *Chorismate mutase-prephenate dehydratase from Escherichia coli. Study of catalytic and regulatory domains using genetically engineered proteins.* J Biol Chem, 1998. **273**(11): p. 6248-53.

162. Li, S., J. Wen, and X. Jia, *Engineering Bacillus subtilis for isobutanol production by heterologous Ehrlich pathway construction and the biosynthetic 2-ketoisovalerate precursor pathway overexpression.* Appl Microbiol Biotechnol, 2011. **91**(3): p. 577-89.

163. Redondo-Salvo, S., et al., *Pathways for horizontal gene transfer in bacteria revealed by a global map of their plasmids.* Nat Commun, 2020. **11**(1): p. 3602.