Engineering Antimicrobial Probiotics for the Treatment of Vancomycin-Resistant *Enterococcus*

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Dedication

To my family

Abstract

The rise of antibiotic resistance in bacteria has become an urgent concern in global healthcare. There is now a strong drive for the preservation of our current antibiotics as well as for the rapid development of new antibacterial therapies.

Antimicrobial peptides (AMPs) are a vast collection of proteins naturally produced by living organisms as a defense mechanism against invading microbes. Unfortunately, though society has been aware of the therapeutic potential of AMPs for many years, their utility has been limited to topical applications because of toxicity and degradation in the body. Moreover, many bacterial infections originate in the gastrointestinal (GI) tract, which is largely unreachable for most AMPs by oral administration. To overcome this delivery challenge, we are engineering probiotic bacteria that can actively produce and deliver AMPs inside the GI tract.

Vancomycin-resistant enterococci (VRE) are among the most difficult to treat pathogens in hospital environments. These bacteria frequently reside in the GI tract, often in low counts because of competition from the surrounding microbiota. When patients are given broad-spectrum antibiotics, the competition is reduced and VRE dominate in numbers. The pathogen can then spread to other host organs or to the surrounding hospital environment.

Delivery of AMPs targeting VRE by probiotics may provide an alternative treatment or prevention option against these deadly pathogens. Importantly, the elimination of VRE from the GI tract using VRE-specific AMPs may allow removal of VRE while minimizing disruption of the surrounding bacteria.

In this thesis, we describe the development of two different probiotic delivery systems for the reduction of the two major causative species of VRE infections, *Enterococcus faecium* and *Enterococcus faecalis*. The first probiotic platform employs the Gram-positive species, *Lactococcus lactis,* for the production of three class IIa bacteriocins, AMPs endogenously produced by bacteria. We have developed a chloride-inducible expression vector for AMP delivery from *L. lactis,* which we show to be activated by physiologically-relevant chloride concentrations. Herein, we demonstrate the ability of this system to inhibit VRE, first in *in vitro* cultures. VRE intestinal colonization models in mice were then developed and used to test the

efficacy of our engineered *L. lactis* in the GI tract. Multiple trials showed statistically significant reduction of *Enterococcus faecium* in *L. lactis* treated mice compared to untreated mice.

The second probiotic delivery system uses probiotic *Escherichia coli* Nissle 1917 (EcN). Currently, no anti-enterococcal peptides are known to be naturally produced from *E. coli*. In this project, we developed a modular AMP expression system that can be used in *E. coli* to express and secrete a variety of AMPs derived from a wide range of producer strains. With this system, we are able to produce AMPs targeting not only Gram-positive pathogens like VRE, but also Gram-negative pathogens including *Salmonella* and diarrheagenic *E. coli*. We show this system can be used to simultaneously express multiple anti-enterococcal peptides *in vitro*. Lastly, we demonstrate the efficacy of Nissle producing Enterocin B, Enterocin A, and Hiracin JM79 in reducing VRE colonization in mice.

The final section of this thesis addresses the concern of bacterial resistance development to our antimicrobial probiotics. Class IIa bacteriocins are currently the most thoroughly-studied class of AMPs targeting enterococci. Though the mechanism of resistance to these peptides has been studied in *E. faecalis*, it has never been examined in *E. faecium*. In this project, we identified a mannose phosphotransferase in *E. faecium* that appears to be directly involved in *E. faecium* susceptibility to class IIa bacteriocins. We show that resistant mutants exhibit either downregulation or direct mutation of this transporter and that heterologous expression of this transporter in *L. lactis* confers susceptibility to the otherwise unsusceptible strain. We then include a brief discussion of the implications of this mode of resistance and potential methods for preventing resistance development in the future.

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Chapter 1: Introduction

1.1 The Rise in Antibiotic Resistance

The development of broad spectrum antibiotics is among the most influential scientific advances in modern human history. Prior to the discovery of penicillin in 1929, death commonly resulted from bacterial infections of what would now be considered trivial injuries. Penicillin and streptomycin changed the medical world by triggering the search for additional antibiotics and ultimately initiated the rise of the pharmaceutical industry [1].

Since the 1940's, we have become heavily reliant on these drugs. In 2010, 258 million courses of antibiotics were prescribed in the United States averaging over 8 prescriptions for every 10 persons [2]. Currently, 100,000 tons of antibiotics are produced each year for healthcare and agricultural applications [3]. Unfortunately, growth promotion in agriculture and ineffective medical administration account for a significant fraction of our antibiotic usage. In a 2016 study by the CDC, it was estimated that ~30% of the 154 million prescriptions administered each year in doctor's offices and emergency departments are unnecessary [4]. The ubiquity of antibiotic use has exerted a constant pressure for bacteria to evolve resistance to our drugs. Over time, these resistance mechanisms have accumulated and are now widely spread throughout both pathogenic and nonpathogenic species.

In 2013, there were over 2 million cases of antibiotic resistant infections reported in the United States with over 23,000 of these cases resulting in death [5]. Based on a 2014 study commissioned by the United Kingdom, antimicrobial resistant infections are estimated to result in over 700,000 deaths worldwide each year [6]. This same study projected that at the current rate of resistance development, antibiotic resistant infections (including bacterial infections, malaria, HIV/Aids and tuberculosis) will be responsible for over 10 million deaths each year by 2050. Based on these predicted mortality rates, resistant infections could soon be competing with cancer as one of the world's leading causes of death [7].

An increasing number of policies limiting the administration of antibiotics are now being proposed to reduce the unnecessary use of these drugs in attempts to preserve their activity. For example, in 2006 the European Union banned the use of all antibiotics as growth promoters

in animal feed [8]. Similar policies are now underway in the United States. For example, in October 2015, the Food and Drug Administration (FDA) introduced regulations regarding veterinary feed directive (VFD) drugs to reduce the unnecessary use of antibiotics in agriculture [9]. In December 2016, the FDA will implement a final rule to this regulation that will require animal producers to obtain veterinary approval for use of medically important antibiotics to ensure that their application is limited to control and treatment of specific diseases.

While reduction in the use of broad-spectrum antibiotics is an irrefutably important factor in slowing, and in some cases reversing the development of resistance, there is now a dire need for new antimicrobial technologies. Despite the continuous development of bacterial resistance, the discovery and approval of new antibacterial pharmaceuticals had been on a steady decline since the 1990's [10,11]. This long-term dry spell of antibiotic development is largely due to low economic incentive for pharmaceutical companies to develop new antimicrobials compared to other drugs [11]. For example, the net present value of an antimicrobial drug was estimated to be ~$50 million compared to $1 billion for a drug used to treat a neuromuscular disease [12].

The increasing occurrence of untreatable infections has sparked governments to provide economic incentives for the development of new antibiotic drugs. For example, the Generating Antibiotic Incentives Now (GAIN) Act of 2011 allows certain antibacterial drugs to undergo an expedited FDA approval process. Additionally, this act grants the drug an additional 5 years of market exclusivity thereby providing the company with additional time to earn funds without competition from generic brands [13]. Furthermore, in 2015, the White House released the National Action Plan for Combating Antibiotic-Resistant Bacteria (CARB). In this plan, specific goals were set to both reduce the excessive use of antibiotics, as well as to expedite the development new antimicrobial therapies [14].

The time is ripe for the development of new antimicrobial technologies. Though we are decades behind schedule, the area of antibacterial research has at last become a global priority. Without the immediate development and exploration of novel antibiotic therapies, we will be left without treatments for an inevitably increasing number of bacterial infections. As stated by

Tom Frieden, the director of the CDC, "If we are not careful, we will be in a post-antibiotic era. And in fact, for some patients and some bacteria, we are already there."

1.2 Antimicrobial Peptides: An Untapped Reservoir of Antimicrobial Activity

Antimicrobial peptides may offer an alternative to traditional small-molecule antibiotics. AMPs are short peptides, typically less than 10 kDa, with activity against microbes including viruses, fungi, and bacteria [15]. AMPs are produced by many organisms, both eukaryotic and prokaryotic, to eradicate invading pathogens [16]. Approximately 5,000 naturally derived and synthetic AMPs have been reported to date, many of which have established activity against specific pathogens [17].

Traditionally, AMPs are regarded as being cationic, amphipathic peptides with a relatively high fraction of hydrophobic residues (>30%) [15]. The cationic and amphipathic features of AMPs allow them to selectively interact with bacterial membranes over mammalian membranes, because bacterial membranes contain a high ratio of anionic phospholipids. In contrast, mammalian membranes are largely comprised of zwitterionic phospholipids [18]. Additionally, mammalian membranes contain cholesterol which has been shown to deter AMP-membrane interaction [19]. These modes of selectivity are thought to contribute to low toxicity of many AMPs against mammalian cells [19].

AMPs act via a variety of mechanisms against their target microbes. Direct membrane disruption via peptide insertion and pore formation has been well studied for a variety of eukaryotic-derived peptides [20–22]. In fact, this mode of action has sparked the successful design of numerous membrane-disrupting synthetic peptides [22,23].

These cationic, pore-forming peptides are highly beneficial in that low instances of resistance development are commonly observed compared to traditional antibiotics [24]. This is thought to be because of the significant changes that would need to be made to the fundamental structure of the pathogen's cell membrane to evade peptide interaction [24]. An additional quality of these cationic peptides is that they frequently target a wide array of both Gram-positive and Gram-negative bacteria [24]. While this attribute is useful for many applications, this off-target activity can be detrimental, for example in the case of gastrointestinal-derived infections where preservation of the native microbiome often proves crucial for patient recovery.

Bacteriocins are a broad class of AMPs produced by different species of bacteria, often as a means of eliminating competing species [25]. To date, the sequences and bacterial targets of nearly 300 bacteriocins have been reported [26]. Bacteriocins are typically more potent than eukaryotic-derived AMPs against their target species, often exhibiting activity at concentrations in the pico to nano-molar range [27]. Generally, bacteriocins most strongly target bacterial species that are phylogenetically similar to the producer strain [28]. For example, enterocins are a subgroup of bacteriocins derived from enterococcal species. Consequently, enterocins are most commonly active against other enterococci. Figure 1.1 provides examples of producer organisms and target genera for four different example bacteriocins. To avoid self-inhibition, producer strains typically express internal immunity proteins corresponding to a particular bacteriocin.

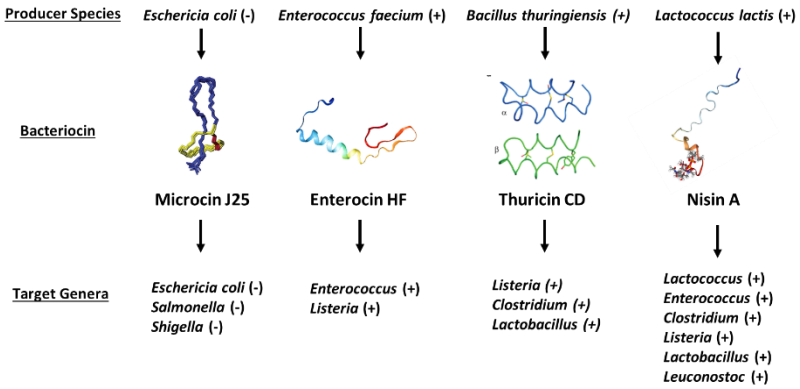

Figure 1.1 Producer species and target genera of four bacteriocins. (+) indicates the species or genera to be Gram-positive, (-) indicates the species of genera to be Gram-negative. Note that Gram-positive-derived bacteriocins generally target Gram-positive species and Gram-negative-derived bacteriocins target Gram-negative species.

Although anti-enterococcal peptides are often partially active against other particular Gram-positive species including *Listeria* and *Lactobacillus* ssp., they are rarely active against Gram-negative species [29,30]. This type of specificity may be beneficial for the treatment of infections originating in the gastrointestinal (GI) tract where the native microflora plays an essential role in patient recovery. When a patient is treated with broad spectrum antibiotics, their native gut bacteria are disturbed, freeing space for other bacteria to grow. These newly available niches provide opportunities for both the original and, in many cases, new antibiotic-resistant pathogens to take hold [31]. By using more specific antimicrobials, the pathogen could in theory be reduced while leaving the remaining microbiome intact. For example, the bacteriocin Thuricin CD from *Bacillus thuringiensis* has now been shown to eliminate the pathogen

Clostridium difficile in a human colon model with minimal disruption to the surrounding microbiome [32].

Interestingly, bacteriocins and bacteriocin-producing Gram-positive bacteria have long been used in the food industry for the prevention of other bacteria associated with food spoilage [33]. Due to the rising demand for novel antimicrobials, bacteriocins are now being recognized for their potential as alternatives to traditional antibiotics in both medical and agricultural applications. Nearly all bacterial isolates examined have been found to produce at least one bacteriocin [34,35]. Additionally, tens to hundreds of different bacteriocins have been found to be produced from a given bacterial species [35,36]. Though nearly 300 bacteriocins have been described, all with useful activity against different pathogens of interest, it appears that we have only begun to scratch the surface of this novel source of antimicrobial activity. If we are able to employ these peptides, we will have opened an unexplored arsenal of potential therapeutics.

1.3 Delivery of Antimicrobial Peptides using Engineered Probiotic Bacteria

Though many AMPs have been previously considered for use in medical applications, they have been limited to topical treatments because of problems with delivery, toxicity, and cost [24]. Many bacterial infections originate in the gastrointestinal (GI) tract and are thus largely unreachable by direct administration of AMPs. Due to their proteinaceous nature, most AMPs cannot be administered orally because they are digested during transit to the lower intestine where most GI tract infections reside. Intravenously injected AMPs are subject to substantial challenges in intestinal wall penetration and ample opportunities for peptide degradation during transit to the GI tract. Furthermore, systemic administration could result in toxic effects if high concentrations of AMPs are required.

Probiotic bacteria are bacterial species that are safe to consume, can survive transit through the gastrointestinal tract, and in some cases, confer some natural benefit to the host. Our lab proposes to overcome the AMP delivery challenge by engineering probiotic bacteria to produce the AMPs at the site of infection in the GI tract. In this technology, the probiotic would be orally administered to the host. The bacteria would then pass through the GI tract to the site in infection in the lower intestines where they will produce and secrete the AMP targeting the

pathogen of interest. Once the pathogen of interest has been cleared, the probiotic will be naturally or inducibly shed from the patient. A schematic of this proposed technology is shown in Figure 1.2.

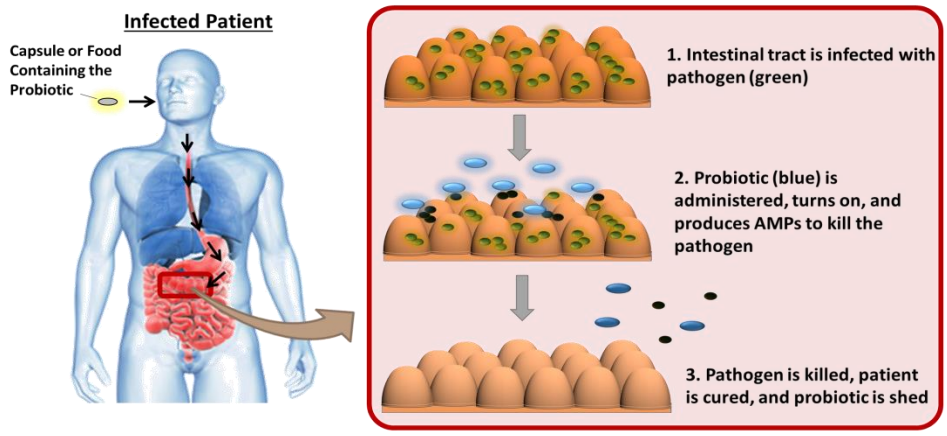

Figure 1.2. Proposed technology using probiotic bacteria to produce antimicrobial peptides at the site of infection. Genetically engineered bacteria will be orally administered. When they reach the gut, they will be induced by external signals to initiate the production of AMPs to combat the pathogen of interest. Once the patient is cured, the probiotic will leave the intestines.

This technology is inspired by the natural use of bacteriocin production by many bacteria to gain a competitive edge over invading species. Numerous studies exist in which probiotic species have been shown to reduce or prevent GI tract colonization with different pathogenic species including *Listeria spp.*, *Helicobacter pylori*, and *Salmonella spp.* [37–40]. There is now a growing body of evidence suggesting that the native AMP production of these probiotic species likely plays a key role in the efficacy of some to these probiotics against pathogenic bacteria [37,38,41]. For example, it was shown that *Lactobacillus* UCC118 became incapable of protecting mice from *Listeria* infection when the genes encoding the AMP ABP118 were eliminated [38]. Similarly, it was shown that *Pediococcus acidilactici* MM33 which produces pediocin PA1 was able to cure VRE colonization in mice, while the non-bacteriocin producing mutant was not [42].

Though these species provide evidence for the potential efficacy of bacterial AMP delivery, native AMP production systems are often weak or induced under particular, uncontrollable conditions making unmodified probiotics ineffective clinical treatments [31,43]. Additionally, most probiotic bacteria produce at most a few different types of AMPs meaning a

new organism would need to be characterized for each new peptide if one were to rely on native AMP producers. By engineering our own set of probiotic organisms to controllably produce a variety of AMPs, we can harness the therapeutic potential of this natural bacterial defense mechanism.

1.4 Scope and Organization of the Thesis

The purpose of this thesis is to describe our work on engineering probiotic bacteria to deliver AMPs targeting a particular pathogen of interest, Vancomycin-resistant *Enterococcus* (VRE). In the following chapter, we discuss the significance of VRE as the pathogen of interest and describe gut colonization, pathogenesis and their mode of infection. We then discuss the currently characterized anti-enterococcal bacteriocins available for use with our application. Previous work characterizing both native and synthetic AMP expression is then summarized to provide an understanding of the state of the technology.

In this thesis, we present two different probiotic platforms for the reduction of VRE in the intestines. In Chapter 3 we develop a potent, environmentally-inducible expression system for the production of AMPs from the Gram-positive host, *Lactococcus lactis*. We show that our engineered *L. lactis* is capable of reducing VRE by over three orders of magnitude in laboratory cultures. This study was published in the peer-reviewed journal, *Applied and Environmental Microbiology*[44].

In Chapter 4 we describe two VRE intestinal-colonization models in mice that can be used to evaluate the ability of *L. lactis* and other anti-enterococcal probiotics to reduce VRE colonization *in vivo*. The efficacy of our engineered *L. lactis* was tested using these infection models and the results are discussed.

Chapter 5 describes a second AMP delivery platform using probiotic *E. coli* Nissle 1917 (EcN). This system employs a powerful synthetic *E. coli* promoter to drive the expression of the peptides and the transporter of the *E. coli*-derived AMP, Microcin V, to secrete the peptides to the extracellular space. We show that this AMP production system is compatible for a variety of AMPs targeting VRE and other pathogens in vitro. The results from these studies are published in the peer reviewed journal *Pharmaceuticals* [45]. Finally, EcN expressing a combination of three

anti-enterococcal peptides is then tested in mice and shown to reduce VRE colonization compared to untreated mice.

Because bacterial resistance is an essential consideration for any new antimicrobial therapy, the final aim of this thesis was to address the concern of VRE resistance to the bacteriocins produced by our probiotics. In chapter 6, we elucidate the mechanism of resistance of *E. faecium* to class IIa bacteriocins, an important class of peptides used in both the *L. lactis* and EcN expression systems. This work is currently under review for publication in the peer-reviewed journal *Antimicrobial Agents and Chemotherapy*. We then discuss potential methods to prevent resistance development.

Chapter 7 summarizes the findings of the work and outlines future directions of the project. We speculate on potential applications and address concerns related to the use of genetically modified organisms as live biotherapeutics.

Chapter 2: Delivery of AMPs Targeting Vancomycin-Resistant Enterococci

2.1 Vancomycin-Resistant *Enterococcus*: A Pathogen of Interest

Vancomycin-resistant enterococci are currently the fourth most common cause of death by antibiotic resistant infection in the United States [1]. Over 20,000 VRE infections occur each year in the United States alone and over one in twenty patients infected with VRE die as a direct result of the infection [1]. These pathogens are considered a "Serious Threat" by the CDC because of their frequent high-level resistance to a wide array of antibiotics, including vancomycin, an antibiotic previously used as a last-resort for enterococci infections. Since the first known nosocomial outbreak of VRE in 1988, these pathogens have disseminated to five continents and now pose an escalating, global threat to our health care system [2].

Enterococci have become a flagrant infectious agent, particularly in nosocomial environments, where they act as opportunistic pathogens, due to their resistance to commonly used antibiotics [3]. Patients treated with antibiotics are particularly susceptible to VRE infections. Upon treatment with the drugs, a patient's native gut microbiome is drastically altered and becomes an easily-colonizable niche for the resistant enterococci. In some patients, particularly those with compromised immune systems, the densely-colonized VRE then translocate to other parts of the body, causing potentially lethal infections [3]. Frequently, even densely-colonized patients exhibit no symptoms and act as long-lasting reservoirs of the pathogen, making it difficult to eradicate the bacteria from the hospital environment [4].

Compounding the issue of antibiotic resistance is the fact that enterococci are naturally hardy bacteria, capable of surviving on nearly any surface and of resisting standard cleaning protocols [5]. These characteristics make them extremely difficult to contain once an outbreak has occurred. Additionally, enterococci are well known for their ability to transfer genetic material, including antibiotic resistant and virulence genes, to a wide array of other bacteria [6]. The expansion of VRE throughout the hospital environment thus results both in an escalated threat of infection to other patients as well as an increase in the potential for the rise of new antibiotic-resistant pathogens.

The common occurrence of VRE in the native microflora of patients plays a significant role in the spread of the pathogen. It is estimated that approximately 10% of intensive care units (ICUs) patients are colonized with VRE upon admission and that another 10% will become colonized during their stay [7]. The ability to selectively eliminate VRE from the GI tracts of densely-colonized patients or patients at risk of colonization could help prevent both lethal translocation events and reduce hospital contamination.

Two species, *Enterococcus faecalis* and *Enterococcus faecium* are responsible for nearly all enterococcal infections. *E. faecalis* comprises 80-90% of clinical isolates while *E. faecium* accounts for 5-10% of all clinical isolates [8]. Though *E. faecalis* is currently responsible for more enterococcal infections overall, nearly 80% of all *E. faecium* isolates are resistant to vancomycin compared to the 9% of *E. faecalis* isolates [1]. Additionally, vancomycin resistance in *E. faecium* is commonly also accompanied by high-level resistance to other antibiotics such as ampicillin [9,10]. Due to this high proportion of resistance, *E. faecium* result in approximately 50% of all VRE infections in the United States and is thus a primary species of concern [1].

Currently, VRE infections are most commonly treated using the broad-spectrum antibiotics linezolid and, in some cases, daptomycin [11,12]. Despite their relatively recent introduction to clinical settings, an increasing number of VRE infections resistant to both linezolid and daptomycin are being observed [11]. The antibiotics quinupristin-dalfopristin, tigecycline, teicoplanin, and telavancin offer last-resort treatment options, however these drugs are not approved in some countries, are ineffective against many enterococci, or exhibit frequent side effects [12]. New methods of VRE prevention and treatment must be developed as these pathogens continue to evade our even our most recently-developed drugs.

2.2 Antimicrobial Peptides Targeting VRE

Over 70 AMPs have been tested and reported to have activity against enterococci [13]. As mentioned above, enterocins are a widely-studied subgroup of bacteriocins derived from enterococcal species. Essentially all enterocins exhibit activity against enterococcal species making them a natural starting point in the development of anti-enterococcal AMP-based probiotics. Similarly, bacteriocins produced from other lactic acid bacteria, including *Lactobacillus* spp., *Lactococcus lactis*, and *Carnobacterium* spp. have been previously

characterized for their potential use in the dairy and meat industry and have been found to exhibit activity against enterococci [14]. These peptides may offer an additional benefit in that their corresponding immunity genes are not generally found among enterococcal species.

Bacteriocins derived from Gram-positive bacteria are generally divided into three major classes though these classifications and their subclasses are under continuous debate. Throughout this work, we will refer to the classification posed by Cotter et al and Yang et al [15,16]. Class I and class II bacteriocins are comprised of small post-translationally modified and unmodified peptides while class III bacteriocins encompass larger, generally heat-labile proteins. Class I bacteriocins, also known as lantibiotics, are short (<50 amino acids), post-translationally-modified peptides containing lanthionine or beta-methyllanthionine residues. Lantibiotics generally act via pore formation, often through interaction with lipid II, although other targets have been identified as well [14]. Lipid II serves as a docking molecule for many class I bacteriocins. Once bound, the peptides can insert themselves into the bacterial membrane resulting in pores. In other cases, the peptides have been shown to inhibit lipid II transport of peptidoglycan from the cytoplasm to the cell wall consequently inhibiting cell wall synthesis. The *L. lactis*-derived bacteriocin, Nisin, is the best-characterized class I bacteriocin and is known to act by both mechanisms of action [14].

Class II bacteriocins, sometimes termed “non-lantibiotic bacteriocins” are typically 30-60 amino acid peptides, are heat tolerant, lack major post-translational modifications, and are generally positively-charged. This class of bacteriocins is further divided into five subclasses (a through e). Class IIa are the most popularly studied subgroup of class II bacteriocins in Gram-positive bacteria. These AMPs are referred to as “*Listeria*-active” or “Pediocin-like” bacteriocins and contain the consensus amino acid sequence YGNGVXC (X = variable amino acid) in the N-terminus [15].

Nearly 40 different class IIa bacteriocins have been identified from a variety of producer species of enterococci, lactobacilli, carnobacteria, leuconostocs, and pediococci [13]. Extensive studies have been done in *Listeria* spp. and *E. faecalis* to elucidate both the mechanism of action and mechanisms of resistance against these peptides [17–20]. It has been established that this class of bacteriocins target a particular sugar transporter, more specifically a mannose

phosphotransferase, found in their target species [20]. It is thought that the interaction of the peptides with this protein target somehow disrupts the membrane integrity, ultimately causing cell death. Enterocin A, Enterocin P, and Hiracin JM79 are three class IIa bacteriocins discussed extensively throughout this thesis. These and several others have been previously shown to exhibit nanomolar inhibitory activity against both *E. faecium* and *E. faecalis*.

Class IIb-e bacteriocins are more diverse in their functions compared to class IIa peptides. For most of these peptides, the mechanisms of actions remain largely unknown, however, an increasing number of bacteriocin receptors are now being identified. Class IIb bacteriocins, or two-peptide bacteriocins, require the interaction of multiple peptides for antibacterial-activity. Recently, it was found that the class IIb bacteriocin Enterocin 1071 targets an undecaprenyl pyrophosphate phosphatase (Upp) on the surface of enterococci [21]. The *Lactobacillus*-derived class IIb bacteriocin ABP118 mentioned in chapter 1 is also known to be active against enterococcal species though to our knowledge, its mechanism of action remains uncharacterized.

Class IIc bacteriocins are cyclic peptides and include anti-enterococcal AMPs such as Enterocin AS-48, Carnocyclin A, and Garvicin ML. Garvicin ML targets a maltose transporter on enterococci and Enterocin AS-48 is known to result in membrane permeabilization, potentially through direct membrane-peptide interactions [21,22].

Class IId bacteriocins are comprised of the remaining linear, single-peptide bacteriocins which lack the defining YGNGVXaaC motif of class IIa bacteriocins. Enterocin B and Carnobacteriocin A both belong to this class of peptides along with Enterocin Q, Enterocin EJ97, and Enterocin K1. Currently, the mechanisms of action of these peptides remain unknown. It has been hypothesized however that Enterocin Q, EJ97, and K1 may target a zinc metallopeptidase based on homology with the anti-lactococcal bacteriocin LsbB [23].

Lastly, Class IIe bacteriocins are linear peptides containing a serine-rich C-terminus and non-ribosomal siderophore-type modifications. To date, the only known class IIe bacteriocins are derived from Gram-negative species and are not active against enterococci.

Table 2.1 summarizes the bacteriocins targeting enterococci discussed above. Included in the table are the name of the peptide, the producer strain, the class of the peptide, and the

mechanism of action (if known). It should be noted that numerous other peptides targeting enterococci are available and that these were simply selected based on their use in this thesis, prior use in gastrointestinal studies, and available knowledge regarding their mechanism of action.

Table 2.1. Examples of bacteriocins targeting *Enterococcus*.

Bacteriocin	Producer Species	Class	Target Receptor
Nisin A	*Lactococcus lactis*	I	Lipid II[14]
Enterocin A	*E. faecium*	IIa	ManPTS[24]
Enterocin P	*E. faecium*	IIa	ManPTS[24]
Hiracin JM79	*E. hirae*	IIa	ManPTS[24]
Enterocin 1071	*E. faecalis*	IIb	Upp (undecaprenyl pyrophosphate phosphatase)[25]
ABP118	*Lactobacillus salivarus*	IIb	Unknown[26]
Garvicin ML	*Lactococcus garvieae*	IIc	Maltose ABC transporter[27]
Enterocin AS-48	*E. faecalis*	IIc	Direct membrane disruption [22]
Enterocin B	*E. faecium*	IId	Unknown[28]
Carnobacteriocin A	*Carnobacterium piscicola*	IId	Unknown[29]
Enterocin Q	*E. faecium*	IId	Zinc-dependent metallopeptidase (putative) [23]

2.3 Natural and Heterologous Bacteriocin Production from Gram-Positive Species

As discussed above, most bacteria are capable of native bacteriocin production. Many of the gene clusters responsible for bacteriocin production in Gram-positive bacteria have been examined and found to contain several common features. Figure 2.1 shows the gene clusters of the four bacteriocins Enterocin A, Enterocin Q, Carnobacteriocin A, and Garvicin ML[29–32]. Most bacteriocin gene clusters contain the gene encoding the peptide shown in black, an immunity gene shown in yellow, genes encoding the necessary secretion machinery shown in green.

Commonly, the immunity and bacteriocin genes are expressed as an operon. In many cases, extensive regulatory components controlling AMP expression are also contained within the gene cluster.

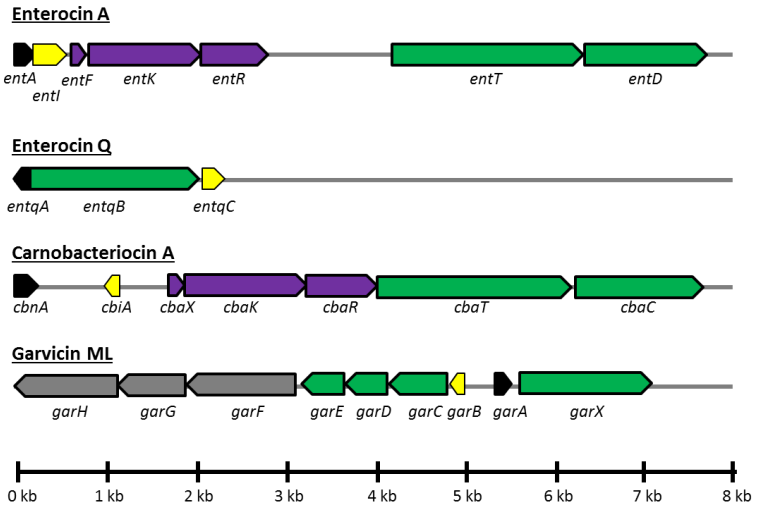

Figure 2.1 Examples of bacteriocin production gene clusters. Color code: AMP (black), immunity gene (yellow), secretion machinery (green), regulatory elements (purple), uncharacterized (grey).

As expected, most heterologous production of bacteriocins typically use standard plasmid-based expression systems containing the AMP and frequently the immunity gene. Secretion of the peptides however is often a challenge in these systems. The majority of heterologous bacteriocin secretion attempts from Gram-positive bacteria have used N-terminal Sec secretion tag fusions with the mature peptides to drive secretion through the general Sec-type secretion pathway of the host of interest. Heterologous expression of the enterocins Enterocin A, Enterocin P, and Hiracin JM79 has been previously demonstrated in a variety of Gram-positive hosts including *Lactococcus lactis*[68,69] and several species of *Lactobacillus* [33–35]. In all cases, the lactococcal general Sec-type signal peptide, Usp45, was fused to the mature peptides. In their native production organisms, Enterocin A is thought to be secreted via a dedicated transporter (shown in Figure 2.1) found within the bacteriocin gene cluster while both Enterocin P and Hiracin JM79 are believed to be secreted from the general Sec secretion machinery of their producer strains [30,36,37]. Interestingly, it has been observed that both Enterocin P and Hiracin JM79 are able to recognize the *L. lactis* general secretion pathway using their native signal peptides.

Similarly, the Divergicin A signal peptide has been shown to successfully drive the secretion of several diverse bacteriocins through the general secretion pathways of *Carnobacterium* spp., *L. lactis*, and *E. faecalis*. The class IIb peptide Brochocin-C, class IIa carnobacteriocin B2, class IId Enterocin B, and *E. coli*-derived Microcin V have all been successfully secreted from gram-positive bacteria using the divergicin A signal peptide [28,38–40]. The demonstrated flexibility of this heterologous production method suggests it may be a promising method for many class II bacteriocins.

The other methods of heterologous bacteriocin production have primarily used the dedicated secretion machineries of one bacteriocin to drive the secretion of another bacteriocin. For example, a study by van Belkum et al. showed that Divergicin A fused to the Leucocin A leader peptide could be secreted using the Leucocin A, Lactococcin A and Microcin V secretion machineries [41]. In the same study, it was shown that Microcin V could be secreted from *L. lactis* using the Lactococcin A secretion machinery and the Leucocin A secretion tag. Interestingly, Microcin V could not be secreted through the Leucocin A secretion machinery [41]. The class IIa bacteriocin, Pediocin PA-1 has now also been shown to be secreted from *L. lactis* using the Lactococcin A signal peptide and dedicated secretion machinery [42]. Lastly, it has also been shown that the Sakacin A secretion machinery can be used to produce sakacin P, pediocin PA-1, and Piscicolin from *Lactobacillus* [43].

The two general secretion methods discussed above provide much of the foundation for the development of the two probiotic platforms described in this thesis. In chapter 3, we employ the first method of peptide secretion (general Sec-type pathway) to express class IIa bacteriocins in our first probiotic host, *L. lactis*. In Chapter 5, the second general method using a dedicated AMP transporter from *E. coli* is used to secrete a wide array of peptides from *E. coli* Nissle 1917.

Chapter 3: Probiotic Platform 1 - *Lactococcus lactis*

3.1 *Lactococcus lactis* as a Delivery Organism

3.1.1 *Lactococcus lactis*

Lactococcus lactis was chosen as the first probiotic platform for the delivery of AMPs targeting VRE based on four primary considerations: safety, ease of genetic manipulation, prior use for delivery of therapeutic proteins, and prior use for the expression of heterologous AMPs. A brief history on *L. lactis* and the strains used in this study is provided, along with examples of the considerations listed above.

L. lactis is a Gram-positive, lactic acid bacterium widely used in the dairy industry for the fermentation of milk products. Because of their long-term use in the food industry, *L. lactis* are generally regarded as safe for consumption. Additionally, *L. lactis* are easily cultured and are among the most genetically well-characterized Gram-positive bacteria, making them logistically desirable protein expression hosts [1,2].

Throughout this work, we have employed the plasmid-free laboratory strain *L. lactis* NZ9000. *L. lactis* NZ9000 is a derivative of the dairy-starter strain, *L. lactis* NCDO712. To create *L. lactis* NZ9000, the *L. lactis* NCDO712 was cured of its native plasmid to facilitate genetic engineering thereby generating the strain *L. lactis* MG1363 [3,4]. The *nisRK* genes were then integrated into the genome of *L. lactis* MG1363 to generate *L. lactis* NZ9000. These genes were originally intended for use with the nisin-inducible gene expression system discussed in the following section [1]. The genome sequences of both *L. lactis* NZ9000 and MG1369 have been determined and a wide array of genetic expression tools have been developed for these strains (see section 3.2).

Importantly, both *L. lactis* MG1363 and NZ9000 are non-pathogenic, non-invasive species and have been used extensively for the production of therapeutic proteins [1]. These strains have been used in several animal studies for mucosal vaccination via the delivery of bacterial and viral antigens as well as for delivery of heterologous proteins to the human GI tract[5]. In a 2006 study, *L. lactis* was used in phase I clinical trials for the delivery of human interleukin-10 to human GI tract for the treatment of Crohn's disease [6]. This work provides an important precedent for the safe use of *L. lactis* as a therapeutic microbe in humans.

In comparison to other lactic acid bacteria such as Lactobacilli, *L. lactis* does not generally colonize the human GI tract. This is beneficial in that its presence can be easily controlled via discontinuation of the treatment. On the other hand, this lack of colonization may make it less effective at peptide delivery to the mucosal surface. Conveniently, multiple studies have been done to track *L. lactis* transit and survival through mouse GI tracts which may provide a foundation for future analysis of probiotic distribution in the gut [7,8].

Lastly, as mentioned in section 2.3, *L. lactis* has been previously used for the secretion of the three class IIa bacteriocins Enterocin A, Enterocin P, and Hiracin JM79 [9]. This prior work provided a valuable starting point for this project. Due to the genetic tractability, safety, and prior demonstration of protein delivery, *L. lactis* was a natural choice as a probiotic platform for the delivery of AMPs targeting VRE.

3.1.2 Lactococcal Gene Expression Systems

The selection of an appropriate gene expression system is essential in order to effectively produce AMPs at the site of infection from our bacterial host. Currently, the Nisin-inducible promoter is the most commonly employed inducible expression system used in *L. lactis*. The Nisin-inducible system is a tightly-regulated, inducible promoter that is activated by the addition of sub-inhibitory concentrations of the lactococcal-derived AMP, Nisin [1]. While the Nisin-inducible system is ideal for many industrial applications and *in vitro* studies, the exogenous addition of an inducer molecule (particularly a proteinaceous one like nisin) could prove difficult for the application at hand.

Multiple constitutive promoters have also been employed for *L. lactis* including P21, P23, P32, P44 and P59 [2]. Such systems are indeed useful when the addition of an inducer molecule is inconvenient, however, constitutive expression systems are often undesirable in industrial bacterial growth. The continuous high level production of the proteins of interest is both wasteful for this application and can in some cases result in toxicity to the producer cells.

The use of promoters which are induced by the environmental conditions of the GI tract offers the benefits of inducible gene expression while avoiding the need for exogenous supply of inducer. For example, promoters that respond to pH, temperature, salt, and infection-associated biomolecules have been previously identified in multiple bacterial species [9–13].

In a previous study from our lab, the class IIa bacteriocins Enterocin A, Enterocin P, and Hiracin JM79 were successfully produced in *L. lactis* NZ9000 under the *E. faecalis* responsive promoter, PrgX-PrgQ [9]. This regulatory system is activated by the sex pheromone peptide cCF10 which is produced by *E. faecalis*. Borrero and co-workers demonstrated that the expression system was highly effective at both targeting and decreasing *E. faecalis* [9]. However, while this system is theoretically intriguing for our application, it is unusable against pathogens lacking cCF10 production. Additionally, this type of system introduces an undesirable layer of complexity at this early stage of the technology. We thus sought to identify and employ a promoter which depends on a more robust parameter of the GI tract such as temperature or ion concentrations.

3.2 A Chloride-Inducible Expression Vector for AMP Delivery from *L. lactis*

3.2.1 The Chloride Inducible Promoter

The chloride ion inducible system was first discovered by Sanders and co-workers during a functional assay screen for the identification of new promoter systems in the chromosome of *L. lactis* MG1363. Figure 3.1 shows the annotated chloride-inducible promoter (CIP). The CIP is natively part of an acid resistance mechanism and contains a constitutively produced activator protein (gadR) which positively regulates the P_{gad} promoter. P_{gad} regulates the transcription of the genes encoding the proteins gadB and gadC which are believed to confer acid resistance [14].Previous work showed that gadR was constitutively produced and that production was not influenced by chloride. These results implied that the promoter's chloride sensitivity may be related to changes in gadR's ability to bind to DNA or to recruit the transcription complex. No other mechanistic studies have been done on P_{gad} activity [14].

In another study, the lacZ reporter gene which encodes β-galactosidase (β-gal) was fused downstream of the P_{gad} promoter region along with gadR, and P_{gadR} to form the plasmid PNS3Z. *L. lactis* containing PNS3Z grown in 0.5 M NaCl produced 1500 U/mg β-gal activity while cells grown with no additional NaCl produced only 1 U/mg β-gal activity [15]. When a variety of concentrations were tested for expression of cell lytic proteins it was found that 0.1 M NaCl proved to be the optimal chloride concentration for recombinant protein production by Pgad [15]. Based on these results, we deemed this system potentially relevant to drug delivery

applications, as typical chloride ion concentrations in the human digestive tract and blood range between ~0.05-0.15 M [16–18].

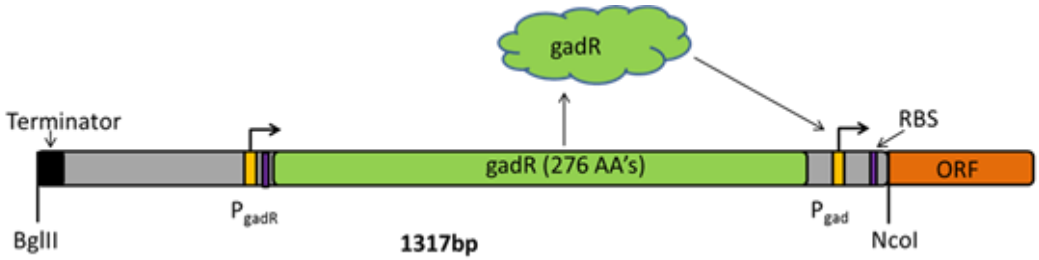

Figure 3.1. The Chloride-Inducible Promoter used in this study. Terminator region and restriction sites BglII, and NcoI were added for cloning in this study. PgadR is the constitutive promoter which regulates gadR production. GadR positively regulates Pgad promoter which controls production of the gene encoded in the open reading frame (ORF). RBS indicates the ribosomal binding site onto which the ribosome binds to initiate transcription of the protein in ORF. It is believed that chloride induces this system by allowing gadR to better activate Pgad thus increasing production of the protein encoded in the ORF.

3.2.2 Summary of the Study

In this study, we first characterized the chloride-inducible promoter and then compared it to the widely used Nisin-inducible promoter using reporter protein assays. The chloride-inducible promoter was then used to express the three bacteriocins Enterocin A, Enterocin P, and Hiracin JM79 in *L. lactis* NZ9000. The ability of the chloride-inducible AMP expression system to inhibit a variety of both *E. faecium* and *E. faecalis* strains was then tested using agar diffusion assays. *E. faecium* inhibition was further quantified using liquid co-culture tests. Last, as an extension of this project, combination treatments using traditional antibiotics and bacterial AMP delivery were explored as a means of reducing the development of AMP resistance.

3.2.3 Results

3.2.3.1 Characterizing Protein Expression from the Chloride-Inducible Vector

The primary motivation for using a chloride-inducible promoter to express antimicrobial peptides is its induction by physiological conditions, instead of exogenous induction. It was therefore necessary to characterize the promoter's dependence on chloride and to verify that it would be active within the range of chloride concentrations measured throughout the human GI

tract (~0.05-0.15 M) [16]. Additionally, it was desirable to compare the production under the chloride promoter to that of the widely-used nisin-inducible promoter to evaluate the strength of this expression system. To assess these parameters, the lacZ reporter gene was inserted under the control of the chloride-inducible promoter to create the plasmid pNZCL (Figure 3.2) or under the nisin-inducible promoter in pNZ8048 to create the plasmid pNZ8048L. *L. lactis* NZ9000-pNZCL and *L. lactis* NZ9000-pNZ8048L were then grown to an OD600 of ~0.45 and induced with chloride (0.01 M, 0.05 M, 0.1 M, 0.3 M, 0.5 M) or nisin (0 ng/mL, 5 ng/mL, 40 ng/mL). Beta galactosidase production under the two promoters was then measured over time.

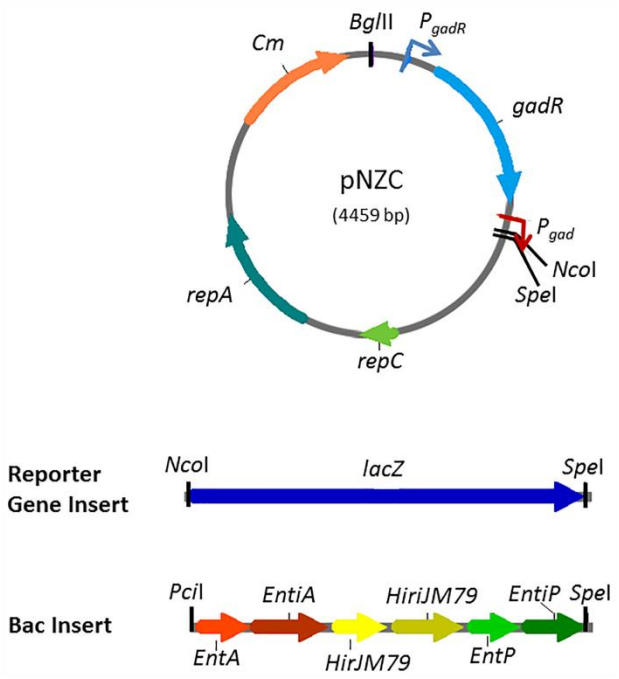

Figure 3.2. Maps of pNZC and inserts used in this study. Chloride-inducible promoter (CIP) was inserted between *Bgl*II and *Nco*I. P_{gadR} is a constitutive promoter controlling the production of the activator protein GadR. *LacZ* and AMP expression are controlled by chloride-inducible promoter P_{gad} (activated by GadR). *LacZ* and Bac are inserted between cut sites *Nco*I and *Spe*I in pNZC.

The results in Figure 3.3 indicate that the chloride-inducible system is highly responsive to the chloride levels in the media and that within the range of physiological conditions (0.05 M-0.1 M), the chloride-inducible system expresses significantly more protein than the fully-induced nisin-promoter. It also appears that the production does not vary much within the induction range found in the intestines. These results are promising for the use of this system for the delivery of proteins in the GI tract as they show high expression can be obtained from the promoter under the induction conditions naturally found within in the gut.

It should be noted that significant protein expression occurs even in GM17 medium without any added salt. The chloride concentration in GM17 was measured and found to be ~0.01 M Cl-. Based on the trends observed in Figure 3.3, we may conjecture that the expression under the chloride promoter could be further reduced if the chloride concentration was lowered by using a different medium. This approach is used in the agar diffusion tests discussed below. We note, however, that a fully uninduced state (no chloride ions) cannot be obtained because chloride is necessary for bacterial survival.

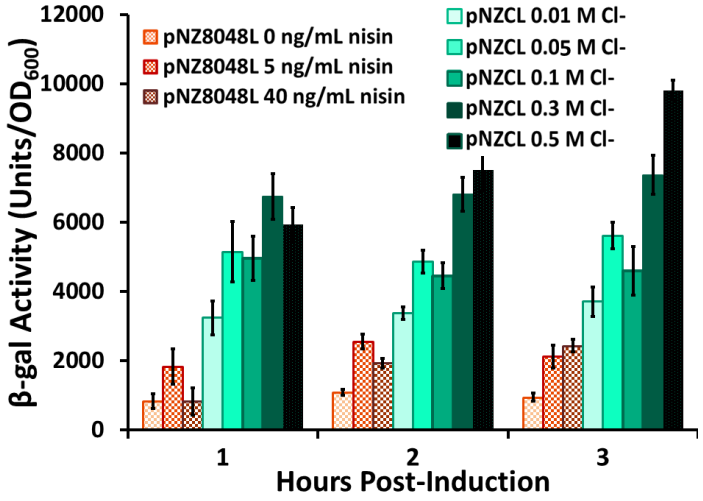

Figure 3.3. Comparison of β-galactosidase production under the nisin-inducible promoter and the chloride-inducible promoter in *L. lactis* NZ90000. The nisin-inducible promoter was induced with 0 ng/mL, 5 ng/mL, or 40 ng/mL nisin and the chloride-inducible promoter was induced with 0.01 M, 0.05 M, 0.1 M, 0.3 M, and 0.5 M NaCl. Cells were induced at an OD600 of ~0.45 then sampled at the designated time post-induction. Error bars represent +/- one standard deviation of assay triplicates.

3.2.3.2 Delivery of AMPs Using the Chloride-Inducible Promoter

Agar Diffusion Inhibition Tests

To apply the chloride inducible system for the production of AMPS, the three bacteriocin genes - enterocin A, enterocin P, and hiracin JM79 along with their immunity genes were inserted downstream of the chloride-inducible promoter to create the plasmid pNZCA3 (Figure 3.2). *L. lactis* NZ9000 was then transformed with this plasmid and its antimicrobial activity was tested using an agar diffusion test using *Enterococcus faecium* 8-E9 as the indicator strain.

Figure 3.4 shows the inhibition of *E. faecium* by *L. lactis*-pNZCA3 at different concentrations of chloride. For these studies, a modified GM17 medium was used which contains half the M17 in traditional GM17. This reduced the basal salt concentration from ~0.01 M Cl- to ~0.005 M Cl- which enabled a more nearly uninduced state. In this experiment, *L. lactis*-pNZC (chloride promoter, no AMPs) was used as a negative control.

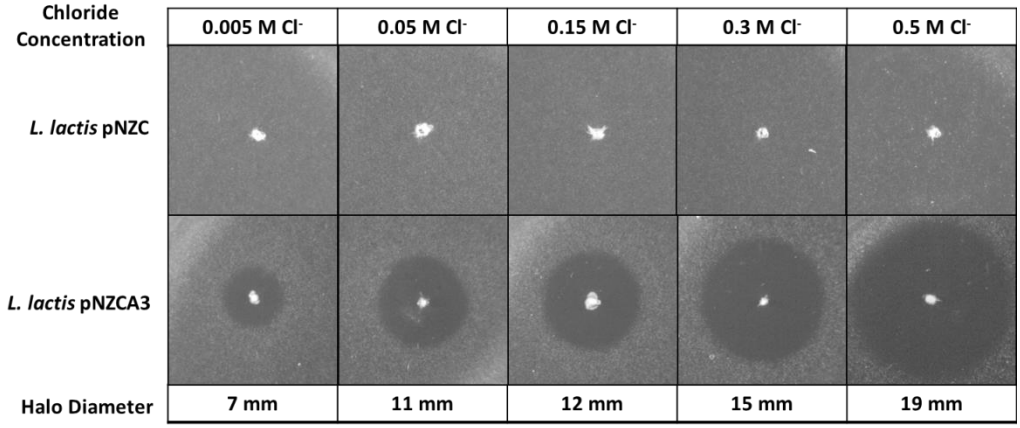

Figure 3.4 Agar Diffusion Inhibition Test of *L. lactis* NZ9000 producing Enterocin A, Enterocin P, and Hiracin JM79 under the CIP (pNZCA3) or with no AMPs under the CIP (pNZC) at different chloride concentrations. *E. faecium* 8-E9 was used as the indicator strain. Modified GM17 medium was used to obtain a lower basal level chloride concentration (0.005 M).

Based on the diameters of the halos produced, it appears that the overall AMP production is salt dependent as anticipated by the reported gene studies discussed above. Interestingly, there is a significant increase in diameter between 0.005 M and 0.05 M cultures but not between 0.05 M and 0.15 M. The halo sizes observed at 0.05 and 0.15 M in the modified GM17 are similar to those observed in traditional GM17 (10-12 mm). *E. faecium* and *L. lactis* growth curves in modified GM17 at different salt concentrations can be found in Figures 3.5a and 3.5b respectively. It should be noted that at 0.3 M and 0.5 M NaCl, *E. faecium* growth is significantly slowed (Figure 3.5a) which may contribute to the halo diameters at the higher salt concentrations.

The benefit of using an environmentally-inducible promoter for the delivery of AMPs is that it can be used against any type of pathogen, which may be necessary if the assaulting strain or species is unknown. To demonstrate that the chloride-promoter expressing the three AMPs Enterocin A, Enterocin P, and Hiracin JM79, can be used to target a broad range of enterococci, halo tests were performed against several strains of pathogenic, antibiotic-resistant strains of

both *E. faecium* and *E. faecalis*. Figure 3.6 shows the results of agar diffusion tests of *L. lactis*-PNZCA3 against 11 strains of enterococci (*E. faecium* 8-E9, not shown), all of which showed clear halos ~8 mm-13 mm in diameter. All halo tests shown in Figure 3.6 were done on BHI+agar (~0.15 M Cl-). Activity was also tested against two *E. faecalis* isolates from healthy patients (Com1 and Pan7) indicating this system could potentially impact commensal enterococcal species.

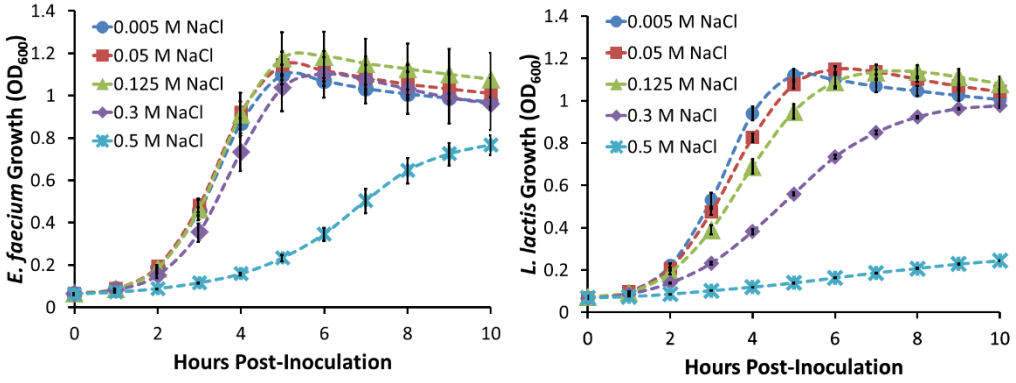

Figure 3.5 a) *E. faecium* 8-E9 growth in modified GM17 medium with 0.005 M, 0.05 M, 0.125 M, 0.3 M, and 0.5 M NaCl. b) *L. lactis* pNZCA3 growth in modified GM17 medium with 0.005 M, 0.05 M, 0.125 M, 0.3 M, and 0.5 M NaCl. Error bars represent +/- one standard deviation calculated from biological triplicates.

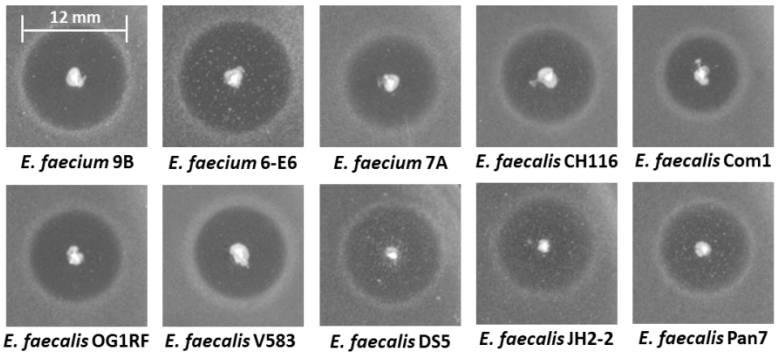

Figure 3.6. Agar diffusion inhibition test of *L. lactis* NZ9000 producing Enterocin A, Enterocin P, and Hiracin JM79 under the CIP (pNZCA3) against 10 different strains of *Enterococcus*. Indicator strain is shown below each halo. See Methods section for descriptions of strains. Halo diameters ranged from ~8-13 mm in diameter. Tests were done in BHI+agar (~0.15 M chloride).

Liquid Co-culture Inhibition Tests

To further quantify the effect of the chloride-inducible AMP expression cassette on *E. faecium* growth, co-culture inhibition tests were done using *E. faecium* 8-E9 and *L. lactis* expressing Enterocin A, Enterocin P, and Hiracin JM79 under the chloride-inducible promoter. Figure 3.7a shows the counts of viable *E. faecium* 8-E9 at different time points. *E. faecium* grown alone is shown in red and *E. faecium* treated with AMP-producing *L. lactis* in GM17 with 0.01 M Cl-, 0.1 M Cl-, or 0.3 M Cl- are shown in teal. *E. faecium* growth has also been tested in the presence of *L. lactis* producing no AMPs (pNZC) and has been found to be nearly identical to normal *E. faecium* growth (data not shown). Figure 3.7b shows the corresponding *L. lactis* counts in each culture. Interestingly, the highest inhibition of *E. faecium* by *L. lactis*-pNZCA3 co-culture was that of the 0.01 M culture. This is likely due to the faster growth of *L. lactis* at lower NaCl as seen in Figure 3.7b. 4 hours post-induction, the counts of viable *L. lactis* were approximately 2 and 4-fold higher in the 0.01 M cultures compared to the cultures supplemented with 0.1 and 0.3 M NaCl. Interestingly, the difference in growth at increasing salt concentrations is significantly more pronounced than that observed when *L. lactis* is grown alone rather than in co-culture with *E. faecium* (Figure 3.5b, 3.7b).

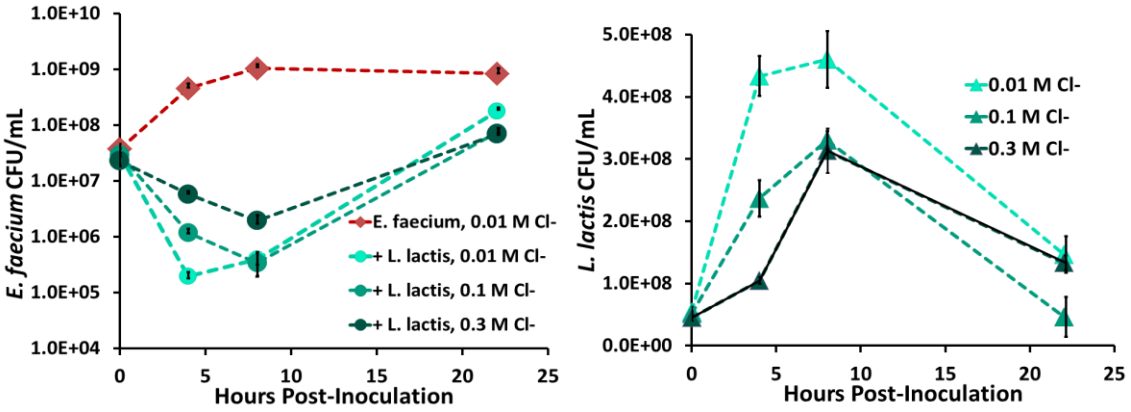

Figure 3.7 a) *E. faecium* growth when grown alone (red) or with (teal) *L. lactis* producing Enterocin A, Enterocin P, and Hiracin JM79 under the chloride-inducible promoter at 0.01 M, 0.1 M, and 0.3 M chloride. b) *L. lactis* PNZCA3 counts from the co-culture test in Figure 3.7 a. Note the reduced growth at higher salt concentrations. Error bars represent +/- one standard deviation calculated from the sample triplicates. Error bars represent +/- one standard deviation calculated from the sample triplicates.

It is clear from Figure 3.7a that even at non-optimal growth conditions, *L. lactis* is able to significantly inhibit *E. faecium* immediately upon treatment. It is clear however that within 10 hours the pathogen begins to regrow. To test whether this was truly due to the appearance of *E. faecium* with stable resistance to the peptides or due simply to decreased AMP concentrations over time, *E. faecium* that arose from the culture treated with AMPs were regrown in fresh GM17 for ~10 generations. Growth of the original culture and the re-grown resistant *E. faecium* was then monitored in GM17 with or without 10% *L. lactis* supernatant containing AMPs to determine if resistance was still present. Figure 3.8 shows the growth curves of both the original *E. faecium* culture and the supposed resistant culture with and without AMPs. Even after growing 10 generations in the absence of AMPs, the resistant culture is only mildly impacted by the AMPs. These results indicate that the surviving *E. faecium* from the co-culture experiments are in fact stably resistant to the AMPs for at least 10 generations. The resistance observed in these experiments was somewhat expected as the development of resistance to class IIa bacteriocins has been previously reported and studied in multiple bacterial species [19]. The proposed mechanisms of this resistance are further discussed below.

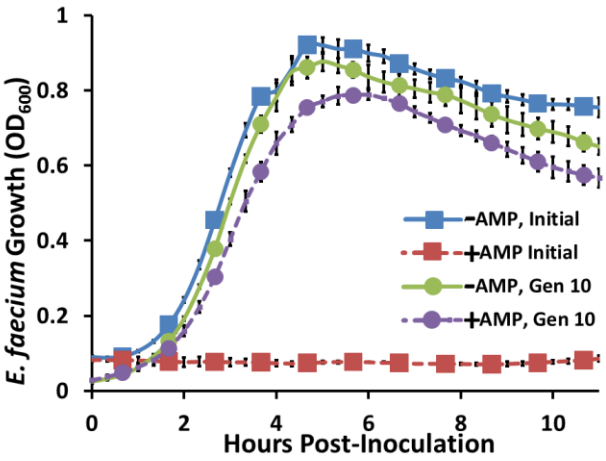

Figure 3.8. Stability of *E. faecium* resistance to AMPs after 10 generations of re-growth. Blue curve shows wild type *E. faecium* growth in the absence of AMPs and red curve shows wild type *E. faecium* growth in the presence of AMPs. After 15 hours, *E. faecium* grown in the presence of AMPs (red) were inoculated into GM17 with no AMPs and grown for 10 generations. The green curve shows the growth of the resulting *E. faecium* grown in the absence of AMPs and the purple curve shows the growth of this same *E. faecium* grown in the presence of AMPs. Results indicate resistance is maintained for at least 10 generations. Error bars represent +/- one standard deviation of biological triplicates.

Resistance Prevention using Class IIa Bacteriocins and Rifampicin Combined Treatment

Though *L. lactis* producing Enterocin A, Enterocin P, and Hiracin JM79 under the chloride-inducible promoter offers promise in temporarily decreasing *E. faecium* in co-culture, the rise in resistant mutants was concerning. To further demonstrate the potential of the AMP delivery system, we aimed to identify a means of combatting this resistance. It is well known that the combination of multiple antibiotics can help to postpone the development of antibiotic resistance [20]. Additionally, it has been observed in some cases that antibiotics as well as AMPs can act synergistically against the target pathogen [21,22]. One can thus imagine the potential in either producing additional AMPs from our currently engineered *L. lactis* or combining bacterial AMP delivery with traditional antibiotic treatments.

In an attempt to eliminate regrowth of *E. faecium*, several common antibiotics, including streptomycin, ampicillin, and rifampicin, were tested in combination with the three AMPs used in this study. Alone, none of these drugs showed significant activity against vancomycin-resistant *E. faecium* at clinically relevant concentrations [23] (Figure 3.9, and data not shown for streptomycin and ampicillin). It was found however that treating *E. faecium* with a combination of rifampicin and the supernatant containing the three AMPs both decreased *E. faecium* counts by nearly four orders of magnitude and prevented regrowth of the pathogen for over 24 hours (Figure 3.9). Synergy of the AMPs with streptomycin and ampicillin was also tested and was found to be minor with these antibiotics (data not shown). Because *L. lactis* used in these studies were not rifampicin-resistant, supernatant rather than co-culture was used with the antibiotic. A comparison between inhibition by 10% cell-free supernatant and 10% *L. lactis*-pNZCA3 demonstrates that similar stable inhibition might be obtained if rifampicin-resistant *L. lactis*-PNZCA3 rather than supernatant was used in combination with the antibiotic. In practice, these rifampicin-resistant *L. lactis* would be used in conjunction with the antibiotic.

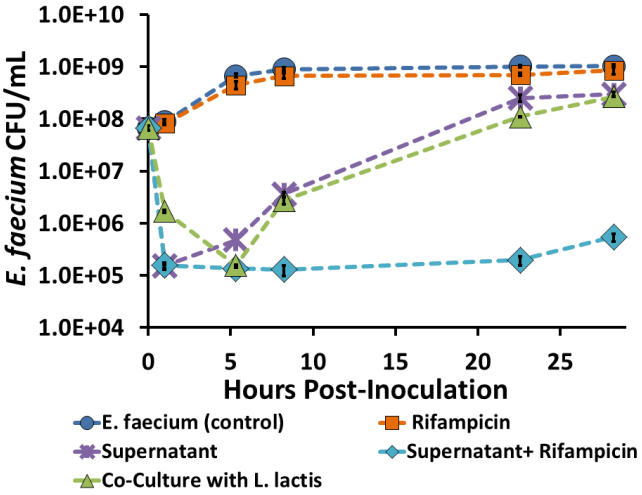

Figure 3.9. *E. faecium* inhibition by the addition of 30 µg/mL rifampicin, supernatant from *L. lactis* producing Enterocin A, Enterocin P, Hiracin JM79 under the CIP. (*L. lactis* pNZCA3), supernatant and rifampicin, or culture containing *L. lactis* pNZCA3 induced with 0.15 M chloride. Error bars represent +/- one standard deviation calculated from the sample triplicates.

3.2.4 Discussion

The focus of this study was to characterize and implement a chloride-inducible expression system for the bacterial delivery of antimicrobial peptides. Herein we have demonstrated the efficacy of a previously discovered chloride-inducible promoter as a potentially useful expression system for the delivery of antimicrobial peptides by *L. lactis*. In this paper we have focused on developing a system to eliminate antibiotic-resistant *E. faecium*, however this type of expression system can easily be expanded with different AMPs to target a wide variety of pathogens.

The first section of the study focuses on characterizing protein expression under the chloride-inducible promoter using reporter gene assays. By comparing β-gal production between the chloride-promoter and the commonly-used nisin-inducible promoter, we found the chloride promoter to be a powerful expression system which is strongly activated at typical chloride concentrations found inside the human GI tract. These results were promising because they indicated that this promoter could be used to express AMPs (or other proteins) in the GI tract without additional induction. The reporter studies also showed that significant promoter activity was observed in the lowest-attained induction state (0.01 M Cl-). This high expression observed in GM17 could be somewhat problematic in the production of more toxic proteins but

this issue could likely be diminished by further optimizing the medium to reduce chloride concentration.

In the future it may be of interest to further explore the responsiveness of the chloride-promoter to other environmental signals. For example, there is evidence that the promoter activity is also impacted by the glutamate concentration and pH of the culture [14]. Glutamate availability, pH, or other environmental conditions could thus play an important role in the delivery of AMPs in the body and should be examined in the future. These variables can also provide additional parameters for improving promoter control for manufacturing and growth purposes.

The second portion of this study was to implement the chloride-inducible promoter for the production of AMPs against VRE. In a recent study from our lab, the *E. faecalis*-responsive PrgX–PrgQ promoter was used to express Enterocin A, Enterocin P, and Hiracin JM79 to eliminate *E. faecalis*. While the PrgX-PrgQ system shows promise in targeting *E. faecalis*, it is not ideal for the treatment of *E. faecium* or other pathogens lacking the inducer pheromone cCF10 as these bacteria would not induce AMP production from the PrgX-PrgQ system. We thus sought an environmentally-inducible system that could be used for general AMP production against any pathogen.

To test the utility of this new expression system, the chloride promoter was used to express the same three AMPs (Enterocin A, Enterocin P, and Hiracin JM79) for the elimination of our indicator pathogen, *E. faecium* 8-E9, as well as a variety of other enterococcal strains at chloride concentrations obtainable in the intestines. It was shown that all 11 enterococcal strains tested were significantly inhibited by the lactococcal AMP delivery system. The wide-spectrum activity that can be obtained with this system demonstrates the potential benefit in using the chloride-inducible promoter for AMP delivery. Though this type of system will likely be effective in eliminating pathogenic enterococci, it was also observed that the two commensal *E. faecalis* strains were also inhibited by our AMPs. In the future, selection of AMPs with minimal impacts on the native gut microbiota is especially important when using the chloride-promoter since promoter activity will not be localized to the pathogen.

In liquid co-culture tests, the system proved to be extremely effective in reducing *E. faecium* counts. Interestingly, it was seen that increasing salt concentration did not result in increased killing of the pathogen. Based on the colony counts of the *L. lactis* from the 0.01 M,

0.1 M, and 0.3 M cultures, this is likely impacted by reduced growth (and productivity) of *L. lactis* early in the co-culture. These results contrasted those observed in the agar diffusion tests which showed increased halo diameters at increased salt concentrations.

It should be noted that the impact of chloride-concentration in 0.01 M and 0.1 M cultures on *L. lactis* growth was far more pronounced in co-culture than in *L. lactis* cultures grown alone. These differences may be due to the competition for nutrients between *L. lactis* and *E. faecium*. We recognize that the nutrient availability and environmental stresses found within the GI tract are significantly different from those in vitro and that only in vivo tests can tell the true utility of this system. Based on the studies performed thus far however, high AMP production under the chloride promoter appears robust to growth and induction conditions which is invaluable for the proposed application.

The liquid co-culture tests also revealed that while the AMPs produced under the chloride-inducible expression system were initially very effective against *E. faecium*, resistance began to overtake the culture within 10 hours. Resistance was verified by monitoring growth of surviving bacterial in the presence and absence of AMPs. These results were not surprising as resistance to bacteriocins by *L. lactis*, *E. faecalis*, and *Listeria* monocytogenes has been previously reported [19]. Several mechanisms of resistance for class IIa bacteriocins have been hypothesized and explored. Most of these proposed mechanisms involve the mannose-phosphotranspherase (Man-PTS) system which is believed to be the receptor of these class IIa bacteriocins. The Man-PTS is a major sugar uptake system found in many bacteria. The bacteriocins are thought to block open the Man-PTS, allowing free-flow of ions across the membrane, which ultimately leads to cell death [19]. Some of the major proposed resistance mechanisms include down-regulation of the Man-PTS, alterations to membrane composition and charge, and random mutations in the Man-PTS locus [19,24,25]. These mechanisms have been found to differ among species as well among mutants of the same species found to have varying levels of resistance [24].

Due to the rapid development of resistance to all three class IIa bacteriocins and the seemingly high fraction of resistant mutants in the unexposed culture, it is tempting to propose that the primary mode of resistance observed in this study relies on the down-regulation of the Man-PTS. As previously discussed by Kjos and co-workers, it is possible that this down-regulation could be the result of randomness in *E. faecium*'s metabolic gene regulation- a survival tactic

referred to as metabolic variability [19]. This hypothesis is further supported by a transcriptome analysis on Pediocin-resistant *E. faecalis* mutants that found mutants had altered transcription of approximately 200 genes, most of which related to metabolism and transport [24]. It is possible that *E. faecium* has a subpopulation that has switched their metabolism to paths not requiring the Man-PTS, relying on alternative carbon sources. At this point, this is only speculation and further, more extensive studies will be needed to determine the true cause of resistance.

As an extension of the delivery system developed in this study, we explored the combination of bacterial AMP delivery with traditional antibiotic therapies to help improve our current system by reducing the rise of resistant mutants [20]. This type of combination therapy is commonly used to avoid antibiotic resistance when using traditional antibiotics. Additionally, this type of combination therapy is conceptually similar to our future goal of adding alternative AMPs with orthogonal targets to those of the bacteriocins currently in our system. These combination studies successfully showed that the application of 30 μg/mL of rifampicin held off resistant mutants for over 24 hours when combined with the three AMPs. These results are interesting because VRE are often considered inherently resistant to rifampicin. It is possible that the AMPs help permeabilize the cell membrane as previous studies have found that cell membrane permeability likely plays a major role in bacterial susceptibility to rifampicin [26]. The reduction of bacterial resistance is essential and must be carefully considered for both traditional and new antibiotic technologies.

3.2.5 Concluding Remarks

With this study, we have successfully identified and implemented a chloride-inducible promoter for the production of AMPs. This new expression system shows promise for the production of a broad range of AMPs in GI tract environments without the need for added induction molecules. As an example of the application of the chloride-inducible promoter, we showed that the expression of AMPs under the new expression system drastically decreases counts of *E. faecium*. Furthermore, we showed that by combining the antibiotic rifampicin with three AMPs produced from this system, the inhibition *E. faecium* is longer-lasting, with limited re-growth of resistant mutants. This study gives promise that the chloride-inducible promoter can be used as a general expression system for the delivery of a wide array of AMPs targeting different pathogens.

In the future, we will explore the mechanisms of resistance to the AMPs Enterocin A, Enterocin P, and Hiracin JM79. Additionally we will test the survivability of the engineered *L. lactis* in more physiologically relevant conditions to further evaluate their effectiveness in this application. The need for new antimicrobial therapies is becoming increasingly urgent, and the in vivo production of AMPs may offer a new tool against even the most resistant pathogens.

3.2.6 Materials and Methods

Bacterial Strains and Growth Conditions

Bacteria used in this study are listed in Table 3.1. *L. lactis* NZ9000 was cultured at 30 °C in M17 broth (Oxoid Ltd., Basingstoke, U.K.) supplemented with 0.5% (w/v) glucose (GM17). *E. faecium* 8-E9 was grown in BHI broth (Oxoid) at 37 °C. *E. coli* MC1061 F' was grown in LB broth (Fisher Scientific, Fair Lawn, NJ, U.S.A.) at 37 °C, with shaking. Agar plates were made by the addition of 1.5% (wt/vol) agar (Oxoid) to the liquid media. When necessary, rifampicin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to the media at 5 µg/mL for *E. faecium*, and chloramphenicol (Mediatech Inc., Manassas, VA, U.S.A) at 5 µg/mL or 20 µg/mL, for *L. lactis* or *E. coli*, respectively.

Table 3.1 Bacteria Used in this Study

Bacteria Strain	Description	Source
Lactococcus lactis NZ9000	plasmid-free strain, derivative of MG1363; pepN::nisRK, nonbacteriocin producer	Mobitec
Enterococcus faecium 8-E9	Ampicillin/Vancomycin/Linezolid resistant	University Of MN
Enterococcus faecium 6-E6	Ampicillin/Vancomycin/Linezolid resistant	University Of MN
Enterococcus faecium 7A	Ampicillin/ Linezolid resistant	University Of MN
Enterococcus faecium 9B	Ampicillin/Vancomycin/Linezolid resistant	University Of MN
Enterococcus faecalis OGR1F	ATCC 47077; plasmid-free, Rifampicin/Fusidic Acid resistant mutant of OG1; common laboratory strain	University Of MN
Enterococcus faecalis V583	ATCC 700802; First isolated Vancomycin-resistant and first sequenced *E. faecalis* genome	University Of MN

Enterococcus faecalis Ch116	Gentamycin/Kanamycin/Streptmycin/Tetracycline/Erythromycin/ Penicillan resistant, β-lactamase-producing isolate	University Of MN
Enterococcus faecalis JH2-2	Rifampicin/Fusidic Acid resistant mutant; common laboratory strain	University Of MN
Enterococcus faecalis Pan-7	Panose 7; fecal sample of healthy volunteer	University Of MN
Enterococcus faecalis Com-1	fecal sample of healthy volunteer	University Of MN
Enterococcus faecalis DS5	ATCC 14508; pAD1, pAMα 1; Erythromycin/tetracycline resistant strain	University Of MN
Escherichia coli MC1061 F'	plasmid-free, recA+ , non-amber suppressor strain	Lucigen

Table 3.2 Plasmids and DNA Fragments Used in this Study

Plasmids	Description	Source
Geneart-chloride	*Kan*^{r}; source of the chloride-inducible promoter system (CIP)	Geneart
pNZ8048	*Cm*^{r}; inducible expression vector carrying the nisA promoter	(27)
pNZC	pNZ8048 derivative containing the CIP	This work
pNZ8048L	pNZ8048 derivative containing *lacZ* under the nisin-inducible promoter	This work
pNZCL	pNZC derivative containing *lacZ* under the CIP	This work
pBac	*Spc*^{r}; source of Bac fragment	(9)
pNZCA3	pNZC derivative containing Bac under the CIP	This work
pBK1	*Cm*^{r}; source of *lacZ*	(9)

Fragments	Description	Source
chloride-inducible promoter system (CIP)	1,317 bp fragment containing the chloride-inducible promoter (P_{gad}) and the gene encoding the activator protein (*gadR*) under the control of a constitutive promoter (P_{gadR})	(14)

Bac	1,610 bp fragment containing the enterocin A structural gene (*entA*) with its immunity gene (*entiA*), the enterocin P structural gene (*entP*) with its immunity gene (*entiP*), and the hiracin JM79 structural gene (*hirJM79*) with its immunity gene (*hiriJM79*)	(9)

Construction of plasmids

Plasmids were constructed using standard molecular cloning techniques. All restriction enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). Fragments obtained and plasmids used are listed in Table 3.2.

The chloride-inducible promoter sequence (CIP) used in this work was adapted from Sanders and coworkers (Genebank Accession number AF005098, base pairs 821-2,071) [14] and synthesized by Geneart. The sequence was then amplified using primers Chloride-F (5'-CGAATTGAAGGAAGGCCG-3') and Chloride-R (5'-GCAGTGAAAGGAAGGCC-3'). The PCR fragment and plasmid pNZ8048 were both digested with restriction enzymes *Bgl*II and *Nco*I (New England Biolabs) and ligated at 16°C for 16 hours. The resulting ligation was then transformed into electrocompetent *E. coli* MC1061 F' (Lucigen). Successful transformants were identified by colony PCR using the primers pNZ8048-F (5' –GCCCCGTTAGTTGAAGAAGG-3') and pNZ8048-R (5'-CAATTGAACGTTTCAAGCCTTGG-3') and further verified by sequencing analysis. The resulting plasmid, pNZC, was isolated from *E. coli* using a QIAprep Miniprep kit (Quiagen) and then transformed into electrocompetent *L. lactis* NZ9000 [28].

For β-gal reporter gene studies, the lacZ reporter gene (*lacZ*) was inserted downstream of the chloride-inducible promoter. *lacZ* was amplified using the primers LacZ-F (5'-GCTAAGCCATGGAAGTTACTGACGTAAGATTACGG-3') and LacZ-R (5'-TCGACTAGTTTATTATT ATTTTTGACACCAGACCAACTGG-3') from pBK1. Both the PCR product obtained and pNZC were then digested with restriction enzymes *Nco*I and *Spe*I, ligated, and transformed into *E.coli* MC1061 F' and *L. lactis* as described above. The resulting plasmid is referred to as pNZCL. The plasmid pNZ8048L was created for these studies using an identical procedure to that used to create pNZCL. In this case however, pNZ8048 rather than pNZC was used for the backbone.

For AMP production, the genes encoding Enterocin A, Enterocin P, and Hiracin JM79 along with their immunity proteins (Fragment Bac from Table 3.2) were inserted into pNZC. Fragment Bac was amplified using primers AMP-F (5'-CATAACATGTCTACTATGAAAAA

AAAGATTATCTC-3') and AMP-R (5'-CACTAGTTTATCAAAGTCCCGACC-3'), using pBac as the template. pNZC was then digested with *Nco*I and *Spe*I while Bac was digested using *Pci*I and *Spe*I. The digestion products originated were then ligated and transformed into *E.coli* MC1061 F'. The resulting plasmid, pNZCA3 was then transformed into electrocompetent *L. lactis* NZ9000.

Beta-Galactosidase Assays

L. lactis NZ9000 containing pNZCL or pNZ8048L were grown overnight in GM17. The following day, cells were re-inoculated into fresh GM17 at an OD_{600} of 0.15. Cells were then grown at 37°C to an OD_{600} of 0.4-0.5 at which point they were induced. *L. lactis* NZ900-pNZCL was induced by adding NaCl to the media to obtain final concentrations of 0.01, 0.05, 0.1, 0.3, and 0.5 M Cl^- and *L. lactis* NZ9000-pNZ8048L was induced by the addition of 5 ng/mL or 40 ng/mL nisin A as previously described [29]. After induction, OD_{600} readings and 1 mL samples were collected each hour. Upon collection, samples were centrifuged for 7 minutes at 5,600 x g, supernatant was removed, and the pellets were refrigerated until further analysis.

To measure β-gal activity we used the traditional Miller assay with some minor modifications[30]. First, pellets were resuspended in 990 µL of Z-buffer (60 mM $Na_2HPO_4.7H_2O$ (Sigma), 40 mM $NaH_2PO_4.H2O$ (Sigma), 10 mM KCl (Sigma), 1 mM $MgSO_4.7H_20$ (Sigma), 50 mM β-mercaptoethanol (Sigma), in DI water). 105 µL of each sample was transferred to a polypropylene 96 well plate. 20 µL of toluene was then added to each well and the plate was shaken for ~30 seconds. The plate was then covered and incubated for 15 minutes at 32 °C. 100 µL of 10 mg/mL ortho-Nitrophenyl-β-galactoside (ONPG) (Research Products Int'l. Corp., Mt. Prospect, IL, U.S.A) dissolved in Z-buffer was then added to each well. The plate was covered again then incubated at 32 °C for 5-15 minutes until sufficient color had developed. 125 µL of 2 M Na_2CO_3 was then added to the wells to stop the reaction. Lastly, the plate was centrifuged at 4 °C for 30 minutes at 6,130 x g to remove cell debris and the supernatant was transferred to a clean 96 well plate and the OD_{420} and OD_{550} was read using a plate reader (Synergy H1 Multi-Mode Reader; BioTek, Winooski, VT).

The following formula was used to convert to activity units:

1 unit= $1000*(Abs_{420}-1.75*Abs_{550})/(\text{incubation time (minutes)}*\text{sample volume (0.105 mL)}*OD_{600})$

In this equation, $Abs_{420}=OD_{420}$ sample*0.734+OD_{420} water*0.266 to adjust the final reaction concentration to that of the traditional Miller assay (30).

Supernatant production

L. lactis containing pNZCA3 was grown overnight in BHI. Cells were then re-inoculated in fresh BHI at an OD_{600} of 0.07-0.1. Because BHI contains ~0.15 M NaCl, no additional salt was added for induction. Cells were then grown ~6 hours then cell-free culture supernatant was obtained by centrifugation of culture at 12,000 × g at 4 °C for 10 min, filtered through 0.2 µm pore-size filters (Whatman Int. Ltd., Maidstone, UK), and stored at −20 °C until use.

Agar Diffusion Tests

L. lactis was grown on GM17 plates supplemented with chloramphenicol to produce single colonies while enterococci were grown overnight in BHI broth. The following morning, BHI supplemented with agar (0.8%) was inoculated with a 0.005% of the overnight enterococcal culture and poured into a petri dish. Once the plate had solidified, *L. lactis* colonies were stabbed into the semisolid media and the plates were incubated overnight at 37 °C. Inhibition was confirmed by the formation of clear zones around the recombinant *L. lactis* strains.

Co-culture and Supernatant activity assays

Salt concentration tests

E. faecium was grown overnight in GM17 at 37°C and *L. lactis*- pNZCA3 was grown in GM17 at 30°C. The following morning, 30 µL of *L. lactis* and *E. faecium* overnights were inoculated into 5 ml fresh GM17 supplemented with the specified concentration of sodium chloride. This resulted in ~1:2 ratio of *E. faecium* to *L. lactis*. GM17 was used for these experiments because of the lower salt concentration compared to BHI. Samples of the cultures were then taken at different times, serial diluted, and plated (10 µL of each dilution) on GM17 plates containing 5 µg/mL rifampicin (GM17-Rif) or 5 µg/mL chloramphenicol (GM17-Cm) and incubated overnight at 37°C for *E. faecium* and 32°C for *L. lactis*. The following day *E. faecium* or *L. lactis* colony forming units (CFU) were counted on GM17-Rif and GM17-Cm plates, respectively.

Combination with Rifampicin tests

E. faecium was grown overnight in BHI at 37°C and *L. lactis*-pNZCA3 was grown in BHI at 30°C. *E. faecium* was then inoculated at an OD_{600} of ~0.06 into 5 ml fresh BHI broth, and allowed to grow to an OD_{600} of ~0.1. For antibiotic experiments, 50 µg/mL streptomycin (Chem-Implex Int'l Inc., Wood Dale, IL, U.S.A.), 100 µg/mL ampicillin (Sigma), or 30 µg/mL rifampicin (23) were added to the fresh BHI broth. Cultures were then supplemented with 10% of the previously obtained supernatant or 10% of *L. lactis*-pNZCA3 culture at an OD_{600} of ~0.1 (time=0 hours). This resulted in ~5:1 ratio of *E. faecium* to *L. lactis*. Samples of the cultures were then taken at different times, serial diluted, and plated (10 µL of each dilution) on GM17 plates containing 5 µg/mL rifampicin (GM17-Rif) or 5 µg/mL chloramphenicol (GM17-Cm) and incubated overnight at 37°C. The following day *E. faecium* or *L. lactis* colony forming units (CFU) were counted on GM17-Rif and GM17-Cm plates, respectively.

Chapter 4: Testing Probiotic Efficacy in Mice

4.1 Introduction

The *in vitro* studies presented in Chapter 3 demonstrated that *L. lactis* producing Enterocin A, Enterocin P, and Hiracin JM79 under the chloride-inducible promoter was potent against both *E. faecium* and *E. faecalis* under laboratory growth conditions. To evaluate how these results translate under the physiological conditions of the GI tract, we tested the ability of *L. lactis* to reduce VRE counts in the GI tracts of mice. This work was done in collaboration with Professor Gary Dunny at the University of Minnesota, Professor Nita Salzman at the Medical College of Wisconsin, and her PostDoc, Dr. Sushma Kommineni. For these tests, we developed two *E. faecium* gastrointestinal colonization models in mice. *E. faecium*-colonized mice were fed sterile water or water containing the probiotic, and the *E. faecium* in the mouse feces was tracked for ~ 3 - 4 weeks. Success was determined based on whether the treated group exhibited a cumulative decrease in *E. faecium* in the feces during treatment with the probiotic.

Pathogen GI-tract Colonization Models in Mice

As discussed in chapter 2, the two primary bacterial species responsible for VRE infections are *E. faecalis* and *E. faecium*. In order to test the efficacy of the probiotic, VRE GI tract colonization models for these pathogens needed to be established. Previously, a VR *E. faecalis* infection model was developed in the Salzman lab in which *E. faecalis* was fed in the water at $5x10^8$ colony forming units (CFU)/mL for two weeks. It was shown that after this time, *E. faecalis* stably colonized the GI tract for over 6 weeks (personal communication). This original antibiotic-free model is beneficial in that it enables the study of pathogen colonization in the presence of a relatively undisrupted native microbiome.

Other VRE colonization models have also been developed in which antibiotics are administered in an attempt to mimic the microbiome disruption typically leading to VRE expansion in patient GI tracts.[90] These models are beneficial in that they may be more representative for the application at hand since the typical probiotic recipients will be those who have been recently treated with broad-spectrum antibiotics. Additionally, models employing antibiotics have been shown to at least temporarily stabilize *E. faecium* colonization in mice [1,2]. This is an extremely important logistical factor because reduction of the pathogen via

probiotic treatment is difficult to detect without sufficiently stable colonization of the control groups.

In the studies described herein, we employ both the original antibiotic free-colonization model from the Salzman lab as well as a new model in which mice are orally administered vancomycin-resistant *E. faecium* and 250 ug/mL vancomycin during the infection period.

4.2 Model 1: Antibiotic-free Colonization of *E. faecium*

4.2.1 Antibiotic-free Colonization Model Development

To establish an *E. faecium* infection model for the evaluation of our probiotics, it was first necessary to identify a strain of *E. faecium* which could remain colonized in the GI tracts of mice long enough to observe a result from the probiotic (preferably longer than 3 weeks). In collaboration with the Salzman lab, we tested the colonization of six different strains of *E. faecium*. The strains tested, their description, and their selective antibiotic resistances are shown in table 4.1.

For each strain, five male C57BL/6J mice were administered with $5x10^8$ CFU/mL *E. faecium* in their water for two weeks. After this two week period, the mice were provided with sterile drinking water and the *E. faecium* strains were enumerated on the appropriate selective agar. The results of these tests are shown in Figure 4.1.

Interestingly, *E. faecium* JL277 and *E. faecium* JL282 were the only strains to remain above the limit of detection (100 CFU/g feces) beyond two weeks after withdrawal of the pathogen from the water. This improved persistence may be explained by their ecological origin. For example, *E. faecium* JL282 is a derivative of a human GI tract commensal *E. faecium* Com12 and thus may be comparatively well suited for colonization in the intestines. Unfortunately, though the other strains are clinical isolates, we were unable to obtain further information on their origin (ex. GI tract vs. blood stream).

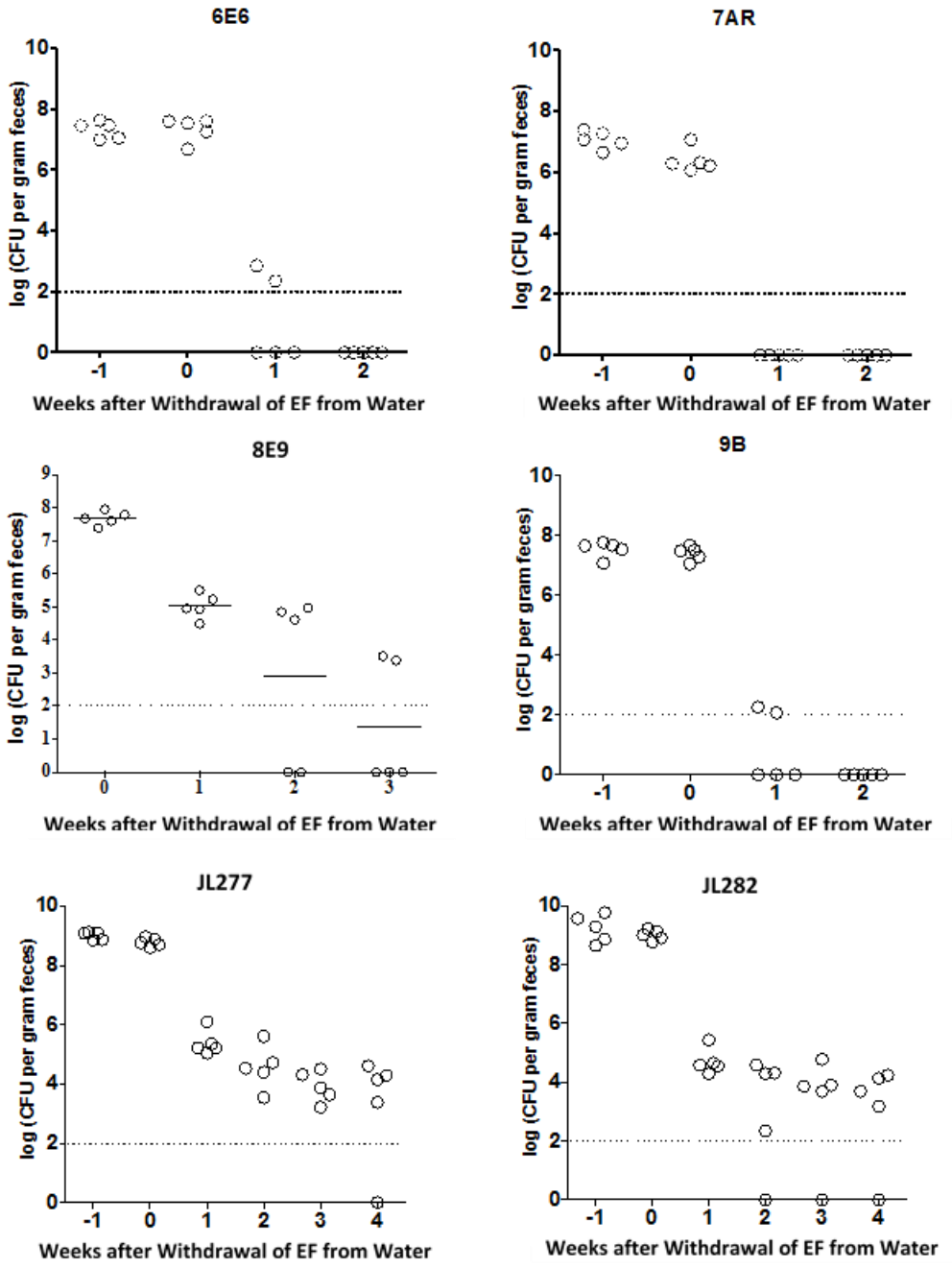

Figure 4.1 Persistence of Different *E. faecium* Strains in Mouse GI Tracts using an Antibiotic-free Colonization Model. Mice were supplied with water containing $5x10^8$ CFU/mL *E. faecium* 6E6, 7A, 8E9, 9B, JL277, or JL282 for two weeks. Mice were subsequently given sterile water starting at week 0. 5 mice were used to test colonization of each strain. *E. faecium* was enumerated in the feces over time by plating serial dilutions of feces. Circles indicate *E. faecium* counts of individual mice. Limit of detection was 100 CFU *E. faecium* /g feces.

4.2.2 *L. lactis* Efficacy Tests against *E. faecium* JL282 and *E. faecium* JL277

To test the efficacy of *L. lactis* in reducing colonization of the *E. faecium* strains JL282 and JL277, 10 male C57BL/6J mice were infected with *E. faecium* JL282 and another 10 with *E. faecium* JL277 for two weeks as described above. After this two week period, 5 mice from each group were administered $5x10^8$ CFU/mL *L. lactis* pNZCA3 and the remaining 5 mice were given sterile drinking water. After the initial infection period, mice were individually housed to avoid cross-contamination via coprophasia. *E. faecium* and *L. lactis* were enumerated in the feces throughout the experiments as described in the Materials and Methods Section. A timeline of this infection model is shown in figure 4.2a.

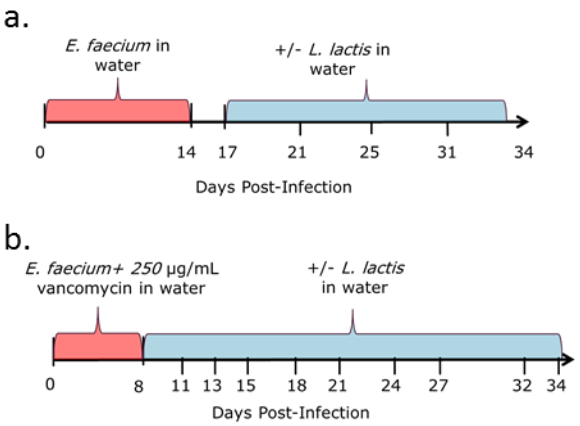

Figure 4.2 Timelines showing infection and treatment periods in a) the antibiotic-free model and b) the antibiotic-assisted model. In the antibiotic-free model (a), mice were supplied with water containing $5x10^8$ CFU/mL *E. faecium* JL277 or JL282 for two weeks. Mice were subsequently given sterile water starting at day 14. In the antibiotic-assisted model (b), mice were supplied with water containing $5x10^8$ CFU/mL *E. faecium* 8E9 for 8 days. On day 17 of the antibiotic-free model and day 8 of the antibiotic-assisted model, mice were given sterile water (untreated group), water containing $5x10^8$ CFU/mL AMP-producing probiotic, or water containing $5x10^8$ CFU/mL probiotic producing no AMPs. *E. faecium* and the probiotic were enumerated in the feces over time by plating serial dilutions of feces.

The results of the *E. faecium* JL277 and JL282 enumeration are shown in Figure 4.3 a and b respectively. *E. faecium* JL282 efficacy tests were then repeated to include an additional group of mice treated with *L. lactis* pNZC which produces no AMPs. This was done to determine if *L. lactis* had any impact on *E. faecium* colonization. The results of this second trial are shown in Figure 4.3 c. Throughout the treatment period, *L. lactis* was maintained at ~10^5 CFU/ g feces (data not shown).

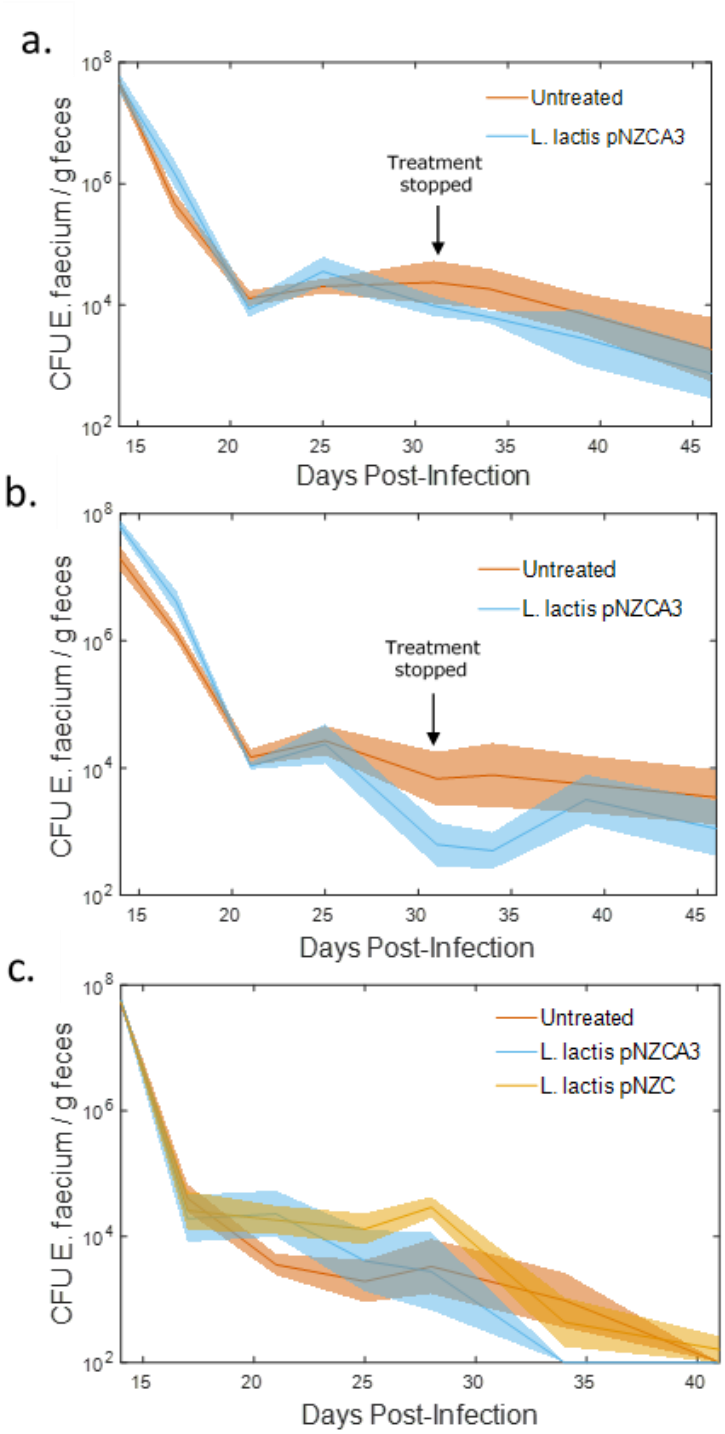

Figure 4.3 Efficacy of *L. lactis* pNZCA3 in reducing a) *E. faecium* JL277, b) *E. faecium* JL282 (trial 1), c) *E. faecium* JL282 (trial 2) in an antibiotic-free colonization model in mice. Mice were supplied with water containing *E. faecium* from day 0 to day 14. On day 17, groups received sterile water (untreated), water containing *L. lactis* producing Enterocin A, Enterocin P, and Hiracin JM79 (*L. lactis* pNZCA3), or *L. lactis* producing no AMPs (*L. lactis* pNZC, c only). Note, *L. lactis* treatment was stopped on day 31 in a and b. Solid lines indicate the mean CFU *E. faecium* / g feces across five mice, shaded region shows the standard deviation.

In all three trials, the average *E. faecium* CFU/(g feces) were below that of the untreated group approximately two weeks into treatment. Comparing figures 4.3 a and b, it becomes evident that *L. lactis* pNZCA3 was found to have a greater impact on *E. faecium* JL282 than *E. faecium* JL277. In the first trial of *L. lactis* pNZCA3 against *E. faecium* JL282, *L. lactis* reduced total *E. faecium* shedding with $p = 0.051$ based on the area under the pathogen count curve (AUC) shown figure 4.3 b. In comparison, the area under the curve for the *L. lactis*-treated group in the *E. faecium* JL277 was lower than the untreated group with $p = 0.170$. Based on these initial results, it appears that *L. lactis* does in fact reduce *E. faecium* colonization in the antibiotic-free model.

It should be noted that in these first trials, *L. lactis* treatment was stopped on day 31. At this point, the average *E. faecium* counts were an order of magnitude lower in the treated group than in the untreated group. *L. lactis* was undetectable in approximately half of the mice by day 34 (data not shown). Interestingly, after this point there is a drastic increase in *E. faecium* JL282 levels further implying *L. lactis* may have been suppressing the pathogen during treatment.

In the second *E. faecium* JL282 trial (figure 4.3 c), the *E. faecium* counts in the untreated group dropped more rapidly than in the previous trials. Because of the rapid decline in the first week, minimal differences could be observed between the AUCs of the untreated and *L. lactis* pNZCA3-treated groups. Interestingly though, *E. faecium* shedding from the *L. lactis* pNZCA3 group was significantly reduced compared to the *L. lactis* pNZC group ($p = 0.0211$). Furthermore, one can clearly see a decline in *E. faecium* JL282 later in the experiment in the *L. lactis* pNZCA3 group compared to the other treatments. Between days 28 and 41, the AUC of *L. lactis* pNZCA3 was less than that of the untreated and *L. lactis* pNZC groups with $p = 0.055$ and 0.015 respectively.

Collectively these results imply that *L. lactis* producing Enterocin A, Enterocin P, and Hiracin JM79 under the chloride-inducible promoter is in fact able to reduce *E. faecium* colonization to some extent. These results provide promising proof-of-concept evidence for the efficacy of AMP-based probiotics for the reduction of antibiotic-resistant enterococci. However, more work is needed to improve both the rate and extent of *Enterococcus* decolonization.

4.3 Vancomycin-Assisted Colonization of *E. faecium*

4.3.1 Antibiotic-Assisted Colonization Model Development

While the antibiotic-free colonization method appears relatively effective in establishing a detectablystable *E. faecium* population in the GI tracts of mice, it was desirable to develop an antibiotic-assisted model for two primary reasons; to better mimic the GI tracts of hospital patients susceptible to VRE infections, and to enable the use otherwise unstable *E. faecium* strains in probiotic efficacy tests. Recall from section 4.2 that neither *E. faecium* strain capable of antibiotic-free colonization is vancomycin-resistant.

Vancomycin was selected as the antibiotic of choice because it has been previously shown to enable VR-*E. faecium* stabilization the GI tracts of mice and because it is of significant medical importance [2]. Vancomycin is one of the most commonly-used antibiotics in acute care hostpitals [3]. Additionally, numerous studies have found that VRE colonization and infection is correlated with vancomycin-use for the treatment of both *Clostridium difficile* infections [4]. 250 µg / mL vancomcyin was selected as the concentration used in these trials based on the previous work by Whitman et al [2]. This concentration results in a daily dosage of ~ 35 mg vancomycin/kg body mass, which is a typical upper limit for oral vancomycin doses in humans [5]. This assumes an average patient weight of ~ 60 kg, average mouse weight of 0.02 kg, and a typical mouse water consumption rate of 3 mL/day [6].

E. faecium 8E9 was chosen as the model *E. faecium* strain because it appeared to be slightly more persistent in the colonization models compared to the other two vancomycin-resistant strains, *E. faecium* 6E6 and *E. faecium* 9B. Additionally, *E. faecium* 8E9 proved to be the most susceptible of the VR strains to *L. lactis* pNZCA3 in in vitro studies (see chapter 6).

4.3.2 *L. lactis* Efficacy Tests against *E. faecium* 8E9

For the vancomycin-induced model, mice were infected from day 0 to day 8 by providing them with water containing 250 µg / mL vancomycin and 5 x 10^8 CFU/mL *E. faecium* 8E9. On day 8, mice were provided with sterile water (untreated), water containing *L. lactis*

producing Enterocin A, Enterocin P, and Hiracin JM79 (*L. lactis* pNZCA3), or water containing *L. lactis* producing no AMPs (*L. lactis* pNZC, trial 2 only). A timeline of this infection model is shown in figure 4.2 b.

The resulting *L. lactis* levels in the feces were significantly higher ($5x10^8$ CFU/g feces vs 10^5 CFU/ g feces) throughout the duration of these experiments than those described in the previous section. We are currently unsure as to why this may have occurred. The impact of the antibiotic treatment on the mouse GI environment, the differences in UMN versus Wisconsin Medical College mouse facilities, and the differences in the strains or sexes of the mice are all potential contributing variables.

Figure 4.4 shows two trials in which mice colonized with *E. faecium* 8E9 were treated with *L. lactis*. We observe that the starting density of *E. faecium* in the feces is over two orders of magnitude higher than that in the antibiotic-free colonization model (10^{10} vs 10^8 CFU *E. faecium*/g feces). After withdrawal of *E. faecium* from the water, *E. faecium* counts drop to ~ 10^6 CFU/g feces and eventually decline to ~10^4 CFU / g feces 3-4 weeks after removing *E. faecium* from the water. This was a significant improvement over the antibiotic-free colonization stability results for *E. faecium* 8E9 in which over half the mice had undetectable levels of *E. faecium* 8E9 in the feces 3 weeks after withdrawal. We note that the effect of the different mouse strains and sexes on colonization of either *L. lactis* or *E. faecium* was not explored and could certainly be a contributing factor in these colonization results.

L. lactis pNZCA3 efficacy tests yielded sporadically promising results. In the first trial (Figure 4.4a), treatment of mice with *L. lactis* pNZCA3 significantly decreased *E. faecium* shedding with $p = 0.020$. After two days of treatment, *E. faecium* counts were ~5-10x lower in the treated group than in the untreated group at all of the time points. By the end, *E. faecium* counts were ~100x lower in the treated group. In the second trial (Figure 4.4 b) however, *L. lactis* pNZCA3 appeared to have no impact on *E. faecium* colonization compared to the untreated group. Oddly, mice treated with *L. lactis* pNZC exhibited significantly reduced carriage of *E. faecium* compared to the untreated group ($p = 0.023$).

The unexpected results of the second *E. faecium* 8E9 trials may have been impacted by housing two mice to a cage rather than using individual housing during the treatment period.

We expect that during this trial there was a constant transfer of native microbiota species and *E. faecium* between co-housed mice. Although the impact of this added variable cannot be readily predicted, this trial is irregular compared to the other *L. lactis* pNZCA3 efficacy trials. An additional vancomycin-assisted efficacy trial in which mice are housed individually may be repeated in the future to verify the results seen in trial 1.

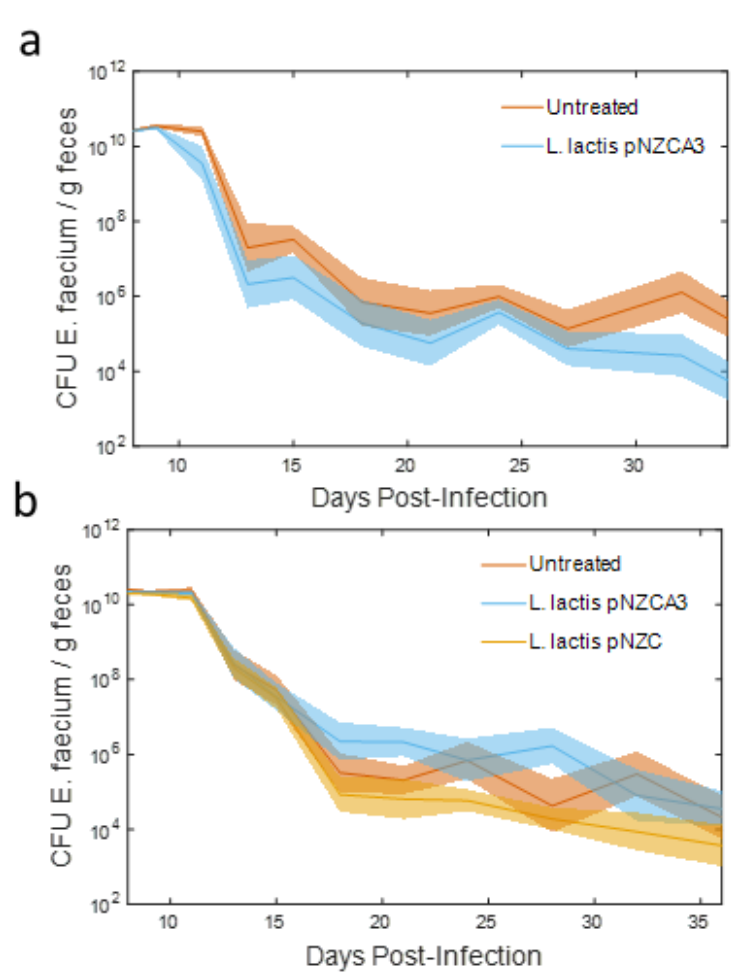

Figure 4.4 Efficacy of *L. lactis* pNZCA3 in reducing *E. faecium* 8E9 in a vancomycin-assisted colonization model in mice. Mice were fed *E. faecium* and 250 µg / mL vancomycin in their water from day 0 to day 8. a) Trial 1, b) Trial 2. On day 8, groups received sterile water (untreated), water containing *L. lactis* producing Enterocin A, Enterocin P, and Hiracin JM79 (*L. lactis* pNZCA3), or *L. lactis* producing no AMPs (*L. lactis* pNZC, c only). Solid lines indicate the mean CFU *E. faecium* / g feces across five mice, shaded region shows the standard error.

4.4 Concluding Remarks

In collaboration of the Dunny and Salzman labs, we have now developed both antibiotic-free and an antibiotic-assisted *E. faecium* colonization in mice. These models resulted in detectable levels of *E. faecium* for up to four weeks thus providing sufficient time to observe an impact of our probiotics. In four of the five trials, average *E. faecium* carriage was reduced by treatment with *L. lactis* expressing the three class IIa bacteriocins, Enterocin A, Enterocin P, and Hiracin JM79 under the chloride-inducible promoter.

In the future, it may be useful to test other antibiotics, such as ampicillin which has been previously shown to more thoroughly and more uniformly reduce the microbiome diversity of mice which may reduce the variability in *E. faecium* colonization across mice [1]. Additionally, microbiome analysis of mouse fecal samples should be performed as these data may provide important information regarding the specificity of our probiotics.

4.5 Materials and Methods

Mouse Species and Housing Conditions

Eight-week old male C57BL/6J mice were used in all antibiotic-free colonization models and were individually housed starting from day 0 of the experiments. Eight-week old female Balb/cJ mice were used in all antibiotic-assisted colonization models. Mice were fed standard food and were provided with the designated water treatments. Water was exchanged every 3-4 days and housing was changed once per week.

In all antibiotic-assisted models, mice were housed 3-4 to a cage during the infection period. With the exception of the second *L. lactis* efficacy trial against *E. faecium* 8E9, mice were housed individually upon treatment administration to avoid contamination between mice. In *L. lactis* trial 2, mice were housed two to a cage. This was done primarily for logistical reasons and to better suit the animals' social behavior. In *E. coli* Nissle 1917 efficacy trials (see chapter 5), mice were housed in autoclaved cages. All antibiotic-free colonization experiments were performed by Dr. Sushma Kommineni at the Wisconsin Medical College. The Wisconsin Medical College Institutional Animal Care and Use Committee has reviewed and approved the protocol involving all live vertebrate animals described herein, ensuring compliance with federal

regulations, inspecting animal facilities and laboratories and overseeing training and educational programs. All vancomycin-assisted tests were performed by Kathryn Geldart at the University of Minnesota. The University of Minnesota Institutional Animal Care and Use Committee has reviewed and approved protocol 1508-32931A involving all live vertebrate animals described herein, ensuring compliance with federal regulations, inspecting animal facilities and laboratories and overseeing training and educational programs.

Preparation of E. faecium and Probiotic Water Treatments

All *E. faecium* were grown overnight in BHI medium (ResearchProducts Intl, Mt. Prospect, IL) in a stationary flask at 37°C. *L. lactis* was cultured overnight at 30 °C in M17 broth (Oxoid Ltd., Basingstoke, U.K.) supplemented with 0.5% (w/v) glucose (GM17). *E. coli* Nissle 1917 (see chapter 5) was grown overnight in LB broth (Fisher Scientific, Fair Lawn, NJ, U.S.A.) at 37 °C, with shaking. All cultures were initiated with < 24 hour-old colonies. Cultures were prepared in sufficient volume to allow 100 mL culture / mouse

Fully grown cultures were centrifuged at 3000 rcf for 10 minutes to pellet cells. *E. faecium* and *L. lactis* cells were then immediately resuspended in sterile tap water in a 2:1 water to culture ratio. *E. faecium* and *L. lactis* treatments were prepared fresh for each water exchange.

E. coli pellets were resuspended in 1/100 culture volume of 15% sterile glycerol and PBS. Suspensions were then aliquoted into vials for each water exchange day in the experiment. Aliquots were frozen at -80^{o}C and stored until use. On water-exchange days, aliquots were thawed and resuspended in a 2:1 water to culture ratio.

Bacterial Enumeration in Feces

During fecal collection, mice were placed in individual autoclaved containers where they were left to defecate for ~5-10 minutes. Mice were then returned to their cages and the feces were transferred to sterile 0.6 mL sample tubes. Feces were ground into a homogenous paste. Half of the sample was resuspended in 300 µL PBS and the remaining half was transferred to another sample tube and stored at -80^{o} for later analysis. Serial dilutions of the resuspended feces were then plated on the appropriate selective agar and allowed to grow overnight at 37^{o}C (*E. coli* and *E. faecium* plates) or at 32^{o}C (*L. lactis* plates).

Table 4.1 lists the selective agar used to enumerate all bacteria strains used in these studies. Note, Rifampicin-resistant mutants of *L. lactis* and EcN strains were used for all mouse trials discussed in this thesis. Thus throughout this chapter and section 5.4 we refer to *L. lactis* NZ9000R as "*L. lactis*" and all references to *E. coli* Nissle 1917 imply *E. coli* NissleR.

Table 4.1. *E. faecium* Strains tested in an Antibiotic-free Mouse Colonization Model

Strain	Description	Selective Agar Used	Source
E. faecium 6E6	Ampicillin/Vancomycin/Linezolid resistant clinical isolate	m-Ent + Erm	UMN Hospital
E. faecium 7AR	Spontaneous Rifampicin-resistant mutant of Ampicillin/Linezolid resistant clinical isolate,	m-Ent + Rif	UMN Hospital
E. faecium 8E9	Ampicillin/Vancomycin/Linezolid resistant clinical isolate	m-Ent + Erm	UMN Hospital
E. faecium 9B	Ampicillin/Vancomycin/Linezolid resistant clinical isolate	m-Ent + Erm	UMN Hospital
E. faecium JL277	Rifampicin-resistant derivative of *E. faecium* 1,231,501 (Ampicillin resistant clinical isolate[93])	m-Ent + Rif	92
E. faecium JL282	Rifampicin-resistant derivative of *E. faecium* Com12 (human GI tract commensal[94])	m-Ent + Rif	92
L. lactis NZ9000R	Spontaneous CNR-resistant *L. lactis* NZ9000 mutant	gM17 + CNR	This study
E. coli NissleR	Spontaneous NR-resistant EcN mutant	LB + NR	This study

Abbreviations:
m-Ent: m-*Enterococcus* agar (BD Difco, Franklin Lakes, New Jersey)
Erm: 20 μg/ml Erythromycin
Rif: 100 μg/ml Rifampicin
CNR: 20 μg/ml Colistin Sulfate, 30 μg/ml Nalidixic Acid, 100 μg/ml Rifampicin
NR: 30 μg/ml Nalidixic Acid, 100 μg/ml Rifampicin

Graphing and Statistical Analysis

To produce graphs, the $\log_{10}$(CFU/g feces) was taken for all mice at all of the time points. These values were then averaged at each time point within a group and the standard deviation

was determined. The average was then re-transformed and plotted as the mean. Upper and lower error bounds shown in the figures were calculated by adding or subtracting the standard deviation to the $\log_{10}$ mean then re-transforming the resulting bounds. Statistical significance was based on the area under the curves of the log-transformed CFU/g feces data. The trapezoidal rule was then used to calculate the area under the curves (AUC) of individual mice beginning on the first day of treatment. The Lilliefors test was then completed to test the normality of AUC distributions within a group. No groups were found to have non-normal distributions. A single iteration of Grubbs test for outliers was used to determine if any AUC outliers were present. No outliers were found with the exception of one mouse in the EcN efficacy trials. Data for this mouse was not included in the statistical analysis. The differences in mean AUCs across treatment groups were then evaluated using a one-tailed Student's t-test with unequal variance.

Chapter 5: Probiotic Platform 2 - *Escherichia coli* Nissle 1917

5.1 *Escherichia coli* Nissle 1917 as a Delivery Organism

5.1.1 Nissle 1917: a Probiotic Species of *Escherichia coli*

In the previous chapter, we developed and tested an anti-enterococcal probiotic based on the Gram-positive species, *L. lactis*. In this chapter, we aim to expand our therapeutic toolbox by developing an additional probiotic platform that uses *E. coli* Nissle 1917, a well-established probiotic strain of *E. coli*.

E. coli Nissle 1917 (EcN) is a non-pathogenic species of *E. coli* discovered by the physician and bacteriologist, Alfred Nissle in 1917[1]. EcN was originally isolated from the feces of a World War I soldier who exhibited a unique immunity to the infectious diarrhea affecting his colleagues. Alfred Nissle was drawn to EcN because of its natural antibacterial activity against a variety of enteropathogens and hypothesized that it was the agent responsible for the soldier's immunity. After verifying its efficacy though laboratory and "self-experimentation" tests, Alfred introduced EcN as treatment against infectious diarrhea[1]. Since its original introduction to the medical field, EcN has been shown to exhibit numerous beneficial features making it useful as a probiotic organism. Notably, EcN has been found to be therapeutically effective in treating a variety of GI tract ailments including infectious diarrhea, ulcerative colitis, and constipation [2–4]. To this day, EcN is commercially available as the active ingredient in the microbial probiotic supplement Mutaflor [2,4].

Several unique physiological aspects of EcN make this organism a suitable delivery vehicle for therapeutic proteins. The demonstrated and well-characterized safety features of EcN are perhaps the most important consideration for its use as an AMP-delivery organism. EcN is non-invasive, exhibits no virulence factors, and is sensitive to mammalian blood serum [5]. These properties prevent possible translocation of the probiotic from the GI tract to the blood stream, which is an essential safety feature of our probiotic platforms. Additionally, unlike many *E. coli* strains, EcN does not impart immunotoxic effects to humans or other animal hosts [5].

In order to be effective, it is essential that the probiotic survive GI tract passage. The numerous studies demonstrating EcN's impact on the GI tract convincingly demonstrate its functionality post-ingestion. Additionally, EcN has been shown to be highly bile-tolerant

compared to some potential gram-positive species [6]. This is important for this application because bile is one of the primary obstacles for the survival of probiotics during oral administration.

In *in vitro* studies comparing EcN and *L. lactis* growth in the presence of porcine bile, it was found that bile concentrations required to inhibit EcN growth were over ten times higher those required to inhibit *L. lactis* growth (data not shown). Additionally, while *L. lactis* is known to pass transiently through the GI tract, EcN is a natural colonizer of the human GI tract. Several fitness factors have been identified that are thought to assist EcN's ability to colonize its hosts including mucosal adhesion factors, iron uptake systems, and competitive bacteriocin production [1]. The improved durability and persistence of EcN compared to *L. lactis* may improve its effectiveness as a peptide-delivery host.

In addition to the essential considerations of safety and survival, EcN exhibits several bonus features. For example, EcN has been shown in numerous studies to secrete factors that protects against the invasion of pathogenic bacteria across the endothelial cell wall. Though this has not been tested for VRE in particular, EcN is known to prevent invasion from a variety of pathogens including *Salmonella*, Shigella, pathogenic *E. coli*, *Listeria*, Yersinia, and Legionella [7,8]. Furthermore, EcN has been shown to exhibit anti-inflammatory effects in the human GI tract and to fortify epithelial tight junctions [1]. Both of these features are important for healing of the epithelial barrier and prevention of pathogen invasion. Since VRE lethality often depends on the translocation of the pathogen from the GI tract to the blood stream, this protective feature of EcN could vastly improve its efficacy in preventing VRE infection.

In addition to its direct pathogen inhibition via bacteriocin production, EcN is also known to recruit host defense mechanisms against bacterial infections. For example EcN has been shown to induce the production of defensins and other cationic AMPS from the GI tract epithelium. Lastly, several studies have been done demonstrating EcN's ability to upregulate the gut immune response [1].

5.1.2 Bacteriocins from Gram-Negative Bacteria

As discussed above, Gram-negative bacteriocins are generally inactive against Gram-positive organisms like *Enterococcus* and are thus not of immediate relevance to this thesis. However, in this work we constructed a system that employs the secretion machinery of

Microcin V, a Gram-negative bacteriocin, to produce anti-enterococcal peptides. A brief discussion of Gram-negative bacteriocins with a particular focus on their secretion is thus included for reference.

Gram-negative bacteriocins are divided into two primary classifications, the colicins and the microcins. Colicins are high-molecular weight (25-80 kDa) proteins and typically contain three structural domains; an N-terminal translocation (T) domain which enable translocation across the outer membrane and periplasmic space, a central receptor-binding domain (R) that recognizes the target-cell's outer membrane receptor, and a C-terminal cytotoxic domain (C) which is responsible for the final cytotoxcic activity [9]. To be active, colicins must first bind a receptor protein on the cell surface. Several of these receptors have been identified for different colicins and include BtuB, FepA, Cir, FhuA, among others. Once bound, the colicin is transported into the target bacterium via a translocator complex, typically either the Tol or Ton system [10]. Once inside the outer membrane, colicins exibit cytotoxic activity via a variety of mechanisms which are divided into three primary groups; inner-membrane pore formation, nuclease activity to non-specifically degrade DNA and RNA, and degradation of peptidoglycan precursors to inhibit membrane synthesis. The mechanisms of over 20 colicins have been characterized thus far [10].

The second major class of Gram-negative bacteriocins are the microcins. Microcins are a diverse class of hydrophobic, low molecular weight peptides (<10 kDa) [11]. Like many Gram-positive bacteriocins, microcins are frequently expressed from gene clusters containing the structural AMP gene, an immunity gene, and a dedicated Type I ABC (ATP binding cassette) transporter secretion system [11]. Microcins are currently divided into two major classes, however, due to the diversity and low number of identified microcins (14 identified as of 2012),the logical division into subclasses is somewhat vague.

Class I microcins are extremely diverse, low molecular weight (<5 kDa) peptides and contain extensive post-translational modifications [10]. This class includes Microcin B17, Microcin C, and Microcin J25. Class II microcins exhibit many similarities to the class II bacteriocins of Gram-positive bacteria and are of particular interest in this thesis. Class IIa microcins include the plasmid-encoded Microcin V, Microcin L, and Microcin N (i.e. Micrcoin 24) [11,12]. These microcins are all assembled and transported using a three-protein secretion system. The secretion system of Microcin V will be discussed in detail in the following section. Class IIb microcins are currently

comprised of the chromosomally-encoded Microcin E492, Microcin M, and Microcin H47. These are all linear peptides and in some cases, contain a C-terminal siderophore [13]. Microcins act via a wide array of mechanisms including pore formation, inhibition of DNA polymerase, inhibition of RNA polymerase, and DNAase and RNAse activity [11].

5.1.2 Gram-Positive Bacteriocin Production in *E. coli*

Since no native *E. coli* bacteriocins are known to exhibit anti-enterococcal activity, the heterologous production of Gram-positive bacteriocins is essential for the use of EcN in targeting VRE. While some work has been done on the internal production of Gram-positive bacteriocins in *E. coli* for production and purification purposes, very few studies exist regarding secretion of mature heterologous AMPs from *E. coli* systems. In section 2.3 we described the two primary methods of Gram-positive bacteriocin secretion using either general Sec-type pathways or dedicated ABC transporters. While it seems numerous studies have shown relatively reliable secretion from the general Sec-type pathways of Gram-positive bacteria, secretion from Gram-negative bacteria has been more challenging.

Many heterologous secretion attempts from *E. coli* use Type II secretion systems, which transport the protein in two steps; first across the inner membrane to the periplasm then across the outer membrane to the extracellular environment [14]. In many cases, the protein passes through the inner membrane but becomes trapped in the periplasm [14,15].

Other studies have successfully secreted heterologous proteins using Type I secretion systems in *E. coli*, most notably the haemolysin (HlyA) transporter [16]. This transporter has successfully been used to secrete a variety of proteins ranging in size and origin making it a promising option for protein secretion [16]. Type I transporters are beneficial in that they span both the inner and outer membrane thus allowing a single step secretion pathway [14]. However, the HlyA transporter is unsuitable for the secretion of active AMPs because it does not cleave the signal peptide from the N-terminus of the protein upon exit from the cell [16]. The presence of the signal peptide (or other N-terminal extensions) would likely drastically reduce, if not eliminate the activity of the AMP of interest.

A promising work examining Gram-positive bacteriocin secretion from *E. coli* was a 1997 study by Van Belkum et al. which demonstrated glycine-glycine type leader peptides of Gram-

positive and Gram-negative bacteriocins could in some cases be exchanged to drive secretion from alternative dedicated secretion systems [17]. In this study, it was shown that the Gram-positive bacteriocin, Divergicin A could be secreted from *E. coli* using the Microcin V secretion machinery and either the Leucocin A or Microcin V secretion tag (98). Despite the success of this original study, little further work has been done to assess this secretion method.

5.2 pMPES: A Modular Peptide Expression System for the Delivery of AMPs from *E. coli*

5.2.1 Introduction and Summary

In the previous sections, we discussed the many merits of *E. coli* Nissle 1917 (EcN) as a potential AMP delivery organism but explain that current *E. coli* secretion systems are highly limited in their ability to secrete active bacteriocins targeting VRE. The development of a bacterial vector that can powerfully express and secrete a wide array of heterologous AMPs would enable use of EcN to deliver peptides targeting a wide array of pathogens regardless of their phylogenetic diversity. Ideally, these vectors should be well-defined and contain all of the necessary components needed for both AMP expression and secretion.

In this study, we have created a powerful AMP expression vector, pMPES, which employs the Microcin V secretion system to produce and secrete a wide-array of AMPs from probiotic *E. coli* Nissle 1917. This study is separated into the following three sections.

In the first section, we focus on testing the breadth of the Microcin V secretion machinery in secreting a variety of AMPs derived from a diverse set of bacteria. The primarily purpose of this section is to provide a proof of concept for the use of the Microcin V secretion system in EcN AMP delivery. We start by briefly discussing the Microcin V secretion machinery as a candidate for flexible AMP secretion. We then describe our incorporation of this secretion system into an easily-manipulated *E. coli* vector which we refer to as pMPES. pMPES was then tested with seven different AMPs derived from both gram-positive and gram-negative bacteria to evaluate the versatility of the Microcin V secretion machinery.

In the second section, we describe the further development of the pMPES expression system into a more potent, well-defined construct termed pMPES2. We demonstrate pMPES2's improved efficacy over pMPES in vitro. We then use this improved system to simultaneously express three anti-enterococcal peptides from the vector pMPES2:BHA. In the final section, the

efficacy of EcN containing this three-peptide construct in reducing VR-*E. faecium* colonization is then tested in mouse GI-tract colonization model. Results from this work clearly depict a reduction in VRE in the treated versus untreated mice giving promise to the probiotic platform developed herein. Collectively, the system described herein appears to be effective both in vitro and in vivo and has the potential to drastically facilitate future testing and optimization for the rapid advancement of AMP-based therapies.

5.2.2 Modular Peptide Expression Proof of Concept

5.2.2.1 Development of pMPES

The Microcin V Secretion Machinery

As stated above, Microcin V (MccV) is a class IIa Microcin naturally produced by *E. coli* species. The MccV secretion system was selected as the transporter in the modular peptide expression system because it is compatible with *E. coli*, it is among the most well-characterized AMP secretion systems and because it recognizes a glycine-glycine type signal peptide, a common class of signal peptide used by many AMPs [17].

To date, three proteins have been identified as essential components of MccV secretion machinery. The ABC transporter CvaB and the accessory protein CvaA are expressed from plasmid-encoded genes found within the MccV gene cluster [18]. The third protein, TolC, is a pore-like outer membrane protein encoded in the *E. coli* chromosome and is an essential component of many *E. coli* efflux systems [14,19]. As is typical of all ABC transporters, CvaB is a cytoplasmic membrane-bound protein and contains a transmembrane domain (TMD) and a C-terminal nucleotide-binding domain (NBD) responsible for ATP binding [20,21]. It is hypothesized that ATP binding to the NBD of CvaB results in dimerization of CvaB that ultimately drives Microcin V transport from the cytoplasm to the the inner membrane [22].

CvaA belongs to the membrane fusion protein (MFP) family and spans the periplasm, acting as a bridge between CvaB and TolC [21]. The hydrophobic N-terminus of CvaA is anchored to the inner membrane while the C-terminus is thought to interact with TolC [21]. Figure 5.1 a shows a general schematic of this proposed assembly.

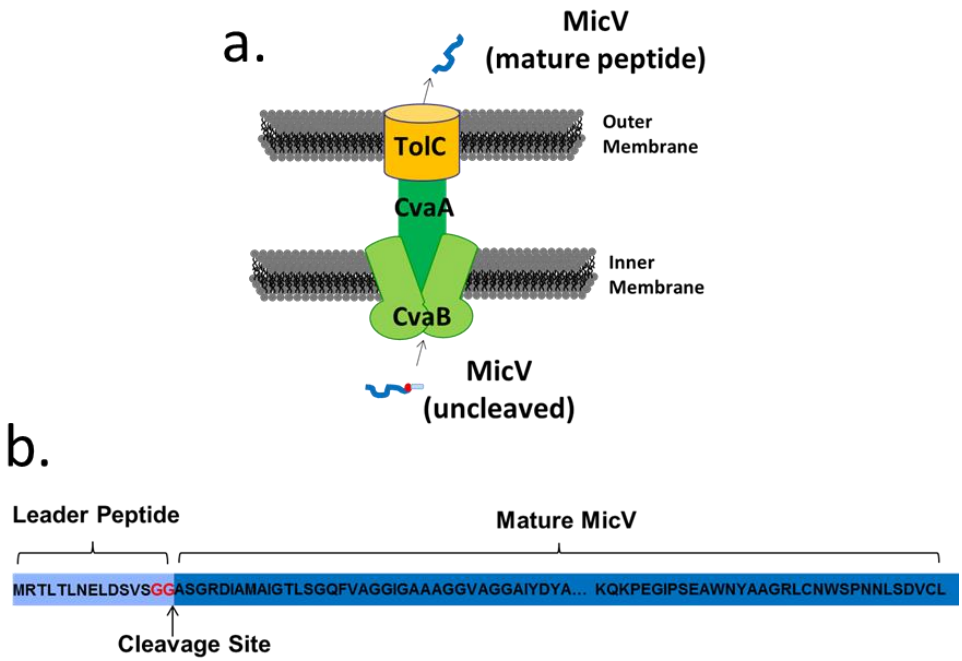

Figure 5.1 a. A general schematic of the Microcin V secretion machinery. Machinery employs an ABC transporter protein, CvaB; a membrane fusion protein, CvaA, and an outer membrane protein, TolC to process and secrete active Microcin V. b. The Microcin V precursor containing the leader peptide and the mature MicV sequence. The glycine-glycine leader peptide is cleaved from the mature peptide upon transit through the secretion system. Cleavage occurs immediately downstream of the glycine-glycine motif.

Like many secreted proteins, MccV is encoded with an N-terminal secretion tag which directs secretion from the dedicated transporter. As mentioned above, MccV uses a glycine-glycine leader peptide. These leader peptides contain a glycine-glycine motif directly upstream of the peptide to be secreted. Figure 5.1.b shows a schematic of the Microcin V precursor containing the leader peptide and the mature MicV sequence. The leader peptide is cleaved from the mature peptide by a peptidase domain within the ABC transporter protein (CvaB) upon secretion as shown in Figure 5.1 a [23]. The use of a glycine-glycine type leader peptide was a promising feature of the MccV secretion apparatus because this class of leader peptides is common across numerous AMPs from a variety of bacterial species. As stated above, the MccV secretion machinery had in fact already been shown to secrete other AMPs with glycine-glycine motifs. However, its full potential had remained unexplored for nearly a decade since this seminal study.

Assembly of the pMPES Vector

The purpose of this study was to create a bacterial vector for the production and secretion of a variety of AMPs from a single probiotic organism. To do this, we remodeled a MccV production plasmid, pHK22, in order to create a vector that contained the entire MccV secretion machinery, as well as a strong DNA promoter system and an AMP cloning site.We refer to this final vector as pMPES, for Modular Peptide Expression System.

Figure 5.2 shows a diagram of this vector. Vector pHK22 contains a 9.1-kb fragment originally isolated from the native MccV production plasmid, pColV-K30. This region, located between the HindIII and SalI restriction enzyme cut sites shown in Figure 5.2, was previously found to contain all of the necessary components for native MccV production, immunity and secretion [18]. The sequence of this region is reported under GenBank Accession Number X57524.1 [19].

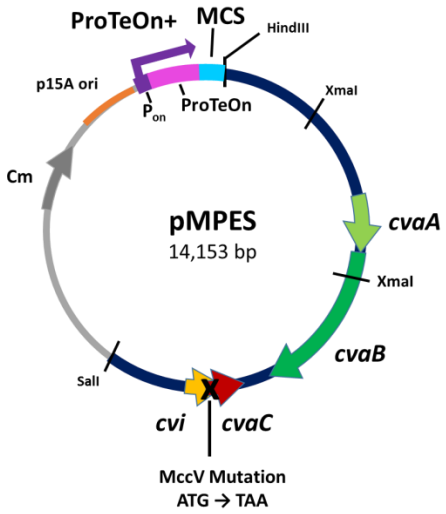

Figure 5.2. Diagram of pMPES (Modular Peptide Expression System). ProTeOn+: synthetic DNA promoter; Pon: promoter region; ProTeOn: activator protein; cvaA/cvaB: MccV section machinery; cvaC: MccV peptide (native); cvi: MccV immunity protein; Cm: Chloramphenicol resistance; MCS: Multiple Cloning Site.

For the development of pMPES, we made three primary modifications to pHK22: (1) we mutated the MccV structural gene to remove native AMP activity; (2) we added a well-characterized synthetic DNA promoter; (3) we added a multiple cloning site and a terminator site to facilitate insertion and removal of AMPs.

The mutation of the native *mccV* gene was essential for testing of pMPES, since MccV production would interfere with the characterization of other peptides. This mutation was

introduced into the start codon of the native *mccV* gene, *cvaC*, to encode a stop codon as shown in Figure 5.2. This new vector is referred to as pHK22Δ. Mutation of the stop codon was selected over complete removal of *mccV* in order to minimize the risk of disrupting any unknown secretory components. To verify that MccV or any potentially active truncated peptides were no longer being produced, *E. coli* MC1061 F' containing pHK22Δ was tested against the indicator strain, *E. coli* DH5α, and shown to have no activity (data not shown).

Next, a strong DNA promoter was incorporated into the vector to enable high-level protein expression. We have previously developed a synthetic DNA promoter for *E. coli,* which relies on a synthetic hybrid activator protein, ProTeOn [24]. ProTeOn was constructed by physically linking the reverse tetracycline repressor protein to the activating domain of the *Vibrio fischeri* transcription factor, LuxR. ProTeOn makes strong contacts within the engineered DNA promoter site (P_{on}), which contains optimally-spaced tetracycline and LuxR operator binding regions. This system recruits RNA polymerase and strengthens the holoenzyme-DNA interactions to up-regulate gene expression.

ProTeOn has been further modified in this study to include a positive feedback loop by inserting the gene encoding ProTeOn downstream of Pon. By using this feedback loop, we are able to amplify promoter expression and obtain high levels of the proteins of interest compared to the original ProTeOn promoter. Herein, we will refer to this new expression construct as ProTeOn+.

A multiple cloning site (MCS) containing the five restriction sites SacI, ApaI, AvrII, NotI and PciI and a rho-independent terminator sequence was then inserted downstream of ProTeOn+.

5.2.2.2 Testing pMPES Peptide Production

In this study, we evaluated the production of seven different peptides from the MccV secretion machinery; MccV, Microcin L (MccL), Microcin N (McnN), Enterocin A (EntA), HirJM79, Enterocin P (EntP) and Enterocin B (EntB). These peptides were selected to represent a wide range in percent sequence identity to MccV and its production system. We note that these tests were not intended to be comprehensive, but only indicative of combinatorial possibilities.

MccV, MccL and McnN are natively produced by *E. coli* while the remaining peptides are produced by *Enterococcus,* a Gram-positive genus of bacteria [25–29]. Like MccV, the studied enterocins are considered class II bacteriocins, which generally lack major post-translational

modifications and are commonly secreted by ABC-transporters using N-terminal secretion tags [30].

With the exception of EntP and HirJM79, all of the peptides tested herein are naturally encoded with glycine-glycine leader peptides [25,26,29,31]. EntP and HirJM79 are believed to be secreted via the general Sec-type secretion pathways of their native producers [27,32].

Table 5.1 shows the percent identity and similarity of the signal peptides, the mature peptides and the two primary secretion genes associated with MccL, McnN and EntA compared to MccV. The EntB, HirJM79 and EntP transporter genes are not compared because they are either unknown as in the case of EntB or they belong to a different class of transporters [32–34]. Identity and similarity values were calculated using the EMBOSS Needle (European Molecular Biology Open Software Suite version 6.6.0.0, Needleman-Wunsch global alignment application) global sequence alignment program with default parameters [35].

Based on the alignment results, one can see that the signal peptides of all three AMPs share significant similarity to MccV's signal peptide. These similarities are further discussed below. Importantly, the transporters for MccL and MccV are nearly identical despite the differences in the mature peptides.

Table 5.1 Comparison of the MccL, McnN, EntA, EntB, HirJM79 and EntP components to MccV.

AMP	Signal Peptide	Mature Peptide	Transporter (CvaB)	Accessory (CvaA)
MccL	60%/86.7%	44.1%/51.0%	95.4%/97.3%	97.6%/99.3%
McnN	41.2%/47.1%	20.8%/26.4%	71.5%/85.0%	69.4%/84.5%
EntA	22.2%/50%	10.3%/14.0%	24.8%/43.4%	18.5%/37.0%
EntB	22.2%/50%	14.9%/18.8%	NA	NA
HirJM79	10.0%/16.7%	6.0%/6.9%	NA	NA
EntP	6.5%/19.4%	5.5%/11.0%	NA	NA

NA: Comparisons for EntB, HirJM79 and EntP transporters were not applicable.

Figure 5.3 shows an alignment of the secretion signal peptides and the first ten amino acids of the mature peptides. The first ten amino acids were included in the alignment because it has been previously hypothesized that the amino acids adjacent to the signal peptide may significantly impact secretion and processing [36]. The multiple sequence alignment program Clustal Omega was used for alignment results [37]. Amino acid conservation scores are based on the Gonnet PAM250 substitution matrix. Residues with a score >0.5 are considered highly conserved in similarity, and those with scores <0.5 are considered to have low conservation.

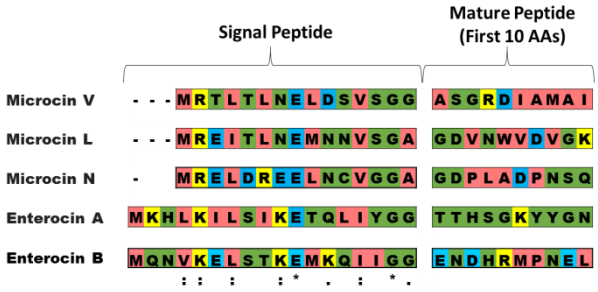

Figure 5.3. Alignment of signal peptides and first ten amino acids of class II peptides selected for initial activity tests. Pink residues indicate small and hydrophobic amino acids (AVFPMILW); yellow indicates basic amino acids (RK); blue indicates acidic amino acids (DE); and green indicates hydroxyl/sulfhydryl/ amine/G amino acids (STYHCNGQ). * means fully conserved residues; : means high conservation (scoring >0.5 in the Gonnet PAM250 matrix);. means low conservation (scoring <0.5).

In addition to testing the capacity of the MccV secretion system with seven different AMPs, another objective of this study was to compare the effect of different signal peptides on active peptide secretion. Consequently, in addition to testing peptide production using the MccV signal peptide (denoted Vsp), we also tested the production of MccL, McnN and EntA using their own, native signal peptides. For EntA, the impact of fusing the MccL signal peptide to EntA (denoted LspA) was also tested because of the high level of similarity between MccV and MccL secretion tags and transporters. Ultimately, the combination of distinct AMPs and of separate secretion signal tags resulted in eleven systems, listed in Table 5.2.

Table 5.2. AMP constructs tested in this study.

Construct (pMPES:)	Signal Peptide	Mature Peptide	Immunity Gene Included
V	MccV	MccV	Yes
L	MccL	MccL	Yes
VspL	MccV	MccL	Yes
N	McnN	McnN	Yes
VspN	MccV	McnN	Yes
VspA	MccV	EntA	Yes
LspA	MccL	EntA	Yes
A	EntA	EntA	Yes
VspH	MccV	HirJM79	No
VspP	MccV	EntP	No
VspB	MccV	EntB	No

In order to reduce the toxicity of the constructs to the producer strain, the immunity genes MccV, MccL and McnN were included in constructs encoding these peptides. Immunity genes were not included for HirJM79, EntP or EntB because these peptides were previously found to be inactive against the producer strain, *E. coli* MC1061 F'.

Agar diffusion assays with appropriate indicator strains were used to screen for peptide activity from the different constructs. Figure 5.4 shows the agar diffusion assay results of *E. coli* Mc1061 F' containing the nine AMP constructs listed in Table 5.2, as well as the empty control, pMPES. For these tests, 3 µL of overnight producer strain culture were spotted on agar containing the indicator strain and then incubated overnight. The white spots are the producer strain, and the dark regions surrounding the producer are zones of inhibition.

Inhibition is likely the result of expressed and secreted AMPs. We acknowledge that in this study, despite our efforts, no peptides were isolated and quantitatively measured as direct proof that inhibition was due to their production and secretion. This may be due to the small culture sizes used (40 mL), which may not produce sufficient peptide amounts for detection using canonical protein isolation methods. It may be useful to scale up culture sizes to one liter or more and to test alternative concentrating, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and purification methods for each of the tested systems, but this was beyond the scope of this study.

Nevertheless, all negative controls, which are included for all studies for activity comparison, suggest that antimicrobial activity is indeed the result of secreted AMPs. This conclusion that AMPs are expressed and secreted is further supported by the fact that in all cases, activity was observed only against the expected indicator strain. For example, MccL constructs were inactive against the Gram-positive *E. faecium*, whereas EntA constructs were inactive against the Gram-negative *E. coli* DH5α.

For these assays, MccV, MccL and McnN were tested against *E. coli* DH5α (Figure 3a) because it has previously been used as the indicator strain for MccV and McnN [17,28]. EntA, HirJM79, EntP and EntB were tested against *E. faecium* 8E9 (Figure 3b) because these peptides were previously shown to have activity against this strain [38,39]. Note, *E. faecium* 8E9 is a multidrug-resistant pathogenic isolate.

MccL and MccV producers were also tested against foodborne pathogen *E. coli* O157:H7 (Figure 3c, top), and McnN producers were tested against foodborne pathogens *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar 4,[5],12:i:- (Figure 3c, bottom) [40,41].

In Figure 5.4a, one can see clear zones of inhibition around *E. coli* MC1061 F', pMPES:V, L, VspL and VspN. These results strongly suggest that all of these strains are producing and secreting inhibitory levels of their respective peptides.

Similarly, results in Figure 5.4 b suggest the production and secretion of EntA, HirJM79, EntP and EntB using all signal peptides tested. The activity depicted in Figure 5.4c demonstrates that the V, L and VspN constructs are potent enough to exhibit activity against pathogenic *E. coli* and *Salmonella* strains.

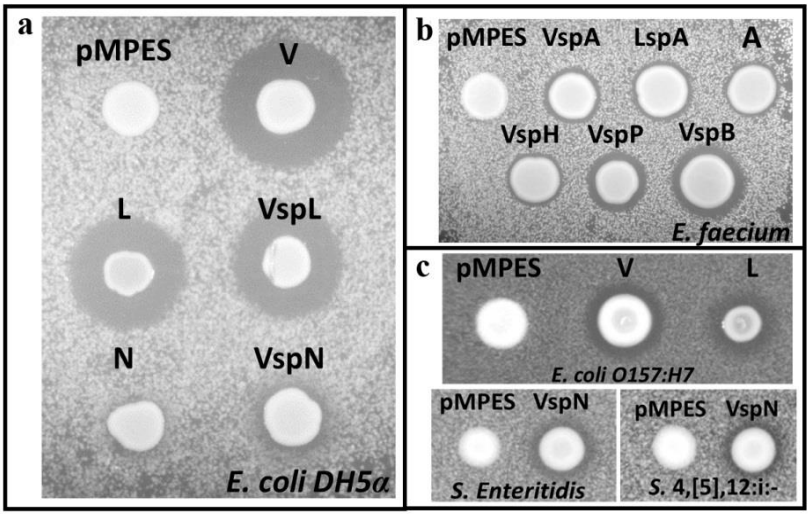

Figure 5.4. (a) Activity of MccV, MccL and McnN from *E. coli* MC1061F' using the pMPES production system. *E. coli* DH5α was used as the indicator strain on this agar plate. (b) Production and secretion of EntA, HirJM79, EntP and EntB from *E. coli* MC1061F' using pMPES. *E. faecium* 8E9 was used as the indicator strain on this agar plate. (c) Inhibition of *E. coli* O157:H7 by *E. coli* MC1061F' pMPES:V and L (top) and *Salmonella* enterica serovar Enteritidis and *Salmonella* enterica serovar 4,[5],12:i:- by pMPES:VspN. In the figure, Vsp and Lsp indicate the use of the MccV or MccL signal peptide rather than naturally-encoded signal peptide.

In order to further validate the hypothesis that the peptides were in fact being secreted using the MccV secretion machinery, negative controls were made for VspA, L and N. For these controls, *cvaA* and *cvaB* were removed from the vector to abolish MccV secretion by digesting pMPES with XmaI (see Figure 5.2) then relegating the digestion. Using agar diffusion tests, no activity was detected from any of these negative controls.

In the future, we aim to use this type of AMP production system to deliver peptides using probiotic bacteria. We therefore sought to test pMPES's compatibility with probiotic *E. coli* Nissle 1917. The pMPES:V, L, VspN, and VspA constructs were transformed into *E. coli* Nissle 1917 and tested using agar diffusion assays (Figure 5.5). To improve the sensitivity of this assay, wells were cut into BHI agar plate and were filled with BHI agar seeded with the producer strain. The plates were incubated overnight to allow peptide production and diffusion into the surrounding agar prior to the introduction of the indicator strain. Agar containing the indicator strain was then poured on top of the overnight plate and the plate was incubated again.

Figure 5.5 a shows *E. coli* Nissle 1917 expressing MccV, MccL, and MccN against *E. coli* DH5α. 5.5 b shows *E. coli* Nissle 1917 expressing EntA against *E. faecium* 8E9. Definitive activity could be detected from all three constructs compared to the negative control, suggesting that the pMPES expression and secretion systems are compatible with Nissle. Figure S3 shows an additional agar diffusion assay, the results of which are consistent with those shown in Figure S2. Note that, unlike *E. coli* MC1061 F', *E. coli* Nissle 1917 naturally produces AMPs, Microcin H47 and Microcin M, which could account for the activity observed in the negative controls against *E. coli* DH5α [42]. In Figure S3, it appears that VspN shows a more defined halo than pMPES, implying, but not definitively determining activity.

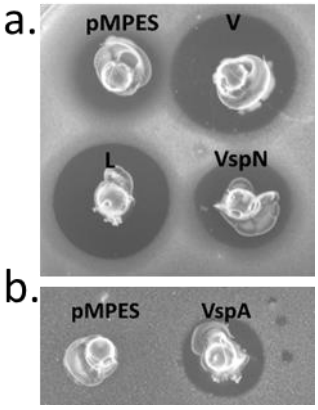

Figure 5.5. Agar diffusion assay showing MccV, MccL, McnN and EntA production from pMPES:V, L, VspN, and VspA in *E. coli* Nissle 1917. *E. coli* DH5α was used as the indicator strain for V, L, and VspN and *E. faecium* 8E9 was used as the indicator strain for VspA. Note *E. coli* Nissle 1917 pMPES has native activity against *E. coli* DH5α but not *E. faecium* 8E9.

Simultaneous Expression of Multiple AMPs

One of the primary benefits of a flexible secretion system for AMP production is the potential to simultaneously produce multiple peptides from a single construct. To verify this potential with the pMPES secretion system, we assembled a construct containing VspA and L. Note that this construct employs a different Ribosomal Binding Site (RBS) upstream of the peptides. We therefore refer to this backbone as pMPESb.

Figure 5.6 shows agar diffusion assays of *E. coli* MC1061 F' pMPESb:VspAL on both *E. coli* DH5α and *E. faecium* 8E9. This figure suggests simultaneous secretion of both EntA and MccL. We recognize that the zones of inhibition presented here are less prominent than those observed using pMPES. Based on comparisons of the VspA supernatant from pMPES versus pMPESb, we believe the alternative RBS drastically reduces AMP expression compared to pMPES. This statement is supported by translation rates estimated using the Ribosomal Binding Site calculator [43]. Nevertheless, we present these results as a proof-of concept, albeit it a rather weak one, for multiple peptide production.

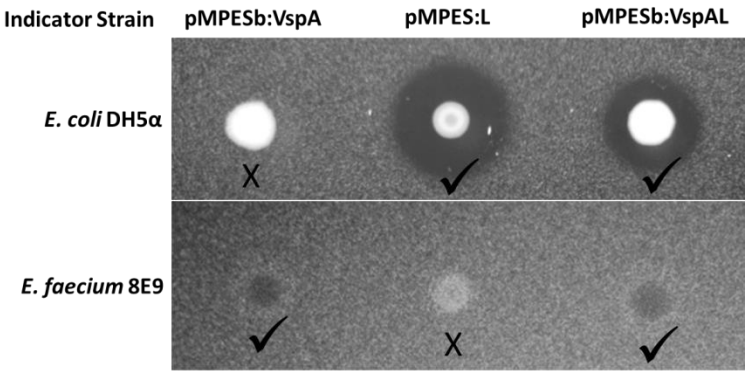

Figure 5.6. Simultaneous production of EntA and MccL from *E. coli* MC1061 F' pMPESb:VspAL and comparison to single AMP systems. Note, pMPESb has a different Ribosomal Binding Site (RBS) and exhibits reduced peptide activity.

Supernatant Activity Assays

To quantitatively compare the activities of the different constructs, we performed liquid supernatant inhibition assays. Figure 5.7 shows the growth curves of the two indicator strains in the presence of 75% supernatant from the constructs discussed above. These growth curves are averaged over three biological replicates. On the left, *E. coli* DH5α growth curves are shown in

the presence of supernatant from *E. coli* MC1061 F' pMPES (negative control), the MccV, MccL and McnN constructs. On the right, *E. faecium* 8E9 growth is shown in the presence of supernatant from pMPES, VspA, LspA, A, VspH, VspP and VspB constructs.

Note that V, L, VspL, VspA, LspA and VspH curves remain at OD_{600} = 0 on their respective graphs. No regrowth was observed in any of these cultures after 48 h, indicating complete killing of the indicator strains, which were originally inoculated at ~5 × 10^3 CFU/mL. In this study, an OD_{600} between ~0.05 and 1 is approximately linearly correlated with CFU/mL.

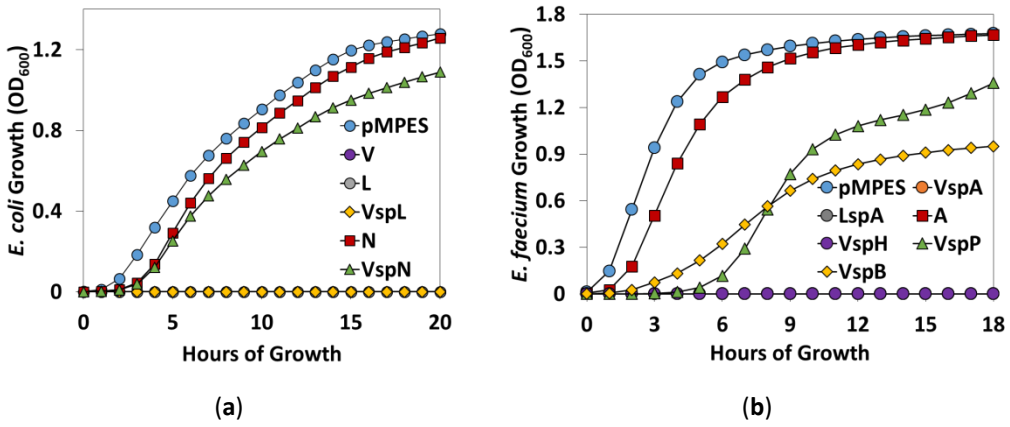

Figure 5.7. Liquid inhibition assays of the pMPES construct supernatants against: (a) *E. coli* DH5α and (b) *E. faecium* 8E9. Cultures are grown in 75% supernatant, 25% rich medium. Note, the V, L, VspL, VspA, LspA and VspH curves remain at OD_{600} = 0 on their respective graphs.

To further quantify the effectiveness of the constructs, the activity of the supernatants containing the AMPs was evaluated based on liquid growth assays. We stress that without the specific activities of the individual peptides, peptide production cannot be quantitatively compared across the different AMPs. Unfortunately, we were unable to isolate the peptides in any of the supernatants after multiple SDS-PAGE and HPLC attempts (see Materials and Methods). Additionally, it is important to consider peptide stability, particularly when comparing supernatant activity in which the calculated activity depends on peptide accumulation over several hours. Nevertheless the tests herein are potentially useful in comparing the efficacy of different signal peptides for a given AMP. In the case of the enterocins, which were tested against a pathogenic strain, these tests provide insight into which constructs may be the most potent for future application in probiotics.

Table 5.3 reports the inhibitory activities of the supernatants in terms of Bacteriocin Units (BUs) [27]. One BU is defined as the reciprocal of the highest dilution of supernatant required to

reduce the growth of the indicator strain, with $p < 0.05$ compared to growth with pMPES supernatant. For these studies, supernatant was diluted in 2x dilutions from 0.75 down to 0.0059 (1.3–170 BUs). Reported values are the average of three biological replicates. Error represents the standard deviation of these replicates. Note, a higher number of supernatant BUs indicates greater potency against the indicator strain.

Table 5.3. Inhibitory activities of supernatants produced by *E. coli* MC1061 F' with the pMPES AMP constructs.

Indicator	Construct (pMPES:)	Bacteriocin Units (BU) [1]	Previously Reported MIC
E. coli DH5α	V	120.9 ± 86.2	0.1 nM (*E. coli* MH1) [44]
	L	>170.7	160 nM (*E. coli* ML-35p) [12]
	VL	142.2 ± 49.3	160 nM (*E. coli* ML-35p) [12]
	N	1.3 ± 0	150 nM (*S. Enteriditis*) [31]
	VspN	1.3 ± 0	150 nM (*S. Enteriditis*) [31]
E. faecium 8E9	VspA	7.2 ± 3.4	129 nM (*E. faecium* TUA 1344L) [45]
	LspA	7.2 ± 3.4	129 nM (*E. faecium* TUA 1344L) [45]
	A	1.3 ± 0	129 nM (*E. faecium* TUA 1344L) [45]
	VspH	21.3 ± 0	~0.2 nM (*E. faecium T136*) [27]
	VspP	2.7 ± 0	~0.4 nM (*E. faecium* T136) [32]
	VspB	2.7 ± 0	43.4 nM (*E. faecium* TUA 1344L) [45]

[1] One Bacteriocin Unit (BU) is defined as the reciprocal of the highest dilution of supernatant required to reduce the growth of the indicator strain; error represents the standard deviation of three biological replicates.

Table 5.3 also contains previously-reported minimum inhibitory concentrations (MICs) of the peptides in nM. We acknowledge however that these values were obtained by a variety of methods using different indicator strains than those used here. These values only are provided to give some idea of the potential activity of the different peptides, but should not be directly compared to this study.

Inhibition with $p < 0.05$ was observed for all supernatants tested in 75% supernatant. Interestingly, pMPES:L was more potent than pMPES:VspL, implying that the naturally-encoded signal peptide was more effective than Vsp for this particular AMP. This is in contrast to what was observed with EntA and McnN (see Figure 5.4 and Figure 5.7).

It should be noted that in multiple trials, VspA slightly out-performed LspA, but the difference is not observable in the BU calculations provided here.

5.2.2.3. Discussion

Delivery of AMPs to intestinal sites of infection poses a major challenge in their application as therapeutic antimicrobial agents. By engineering probiotic bacteria to produce AMPs at the site of infection in the GI tract, we can enable the delivery of otherwise unusable peptides, reduce the amount of peptide required and eliminate the need for protein purification. Furthermore, cocktails of peptides may result in synergistically higher activities and plausibly reduce the occurrence of resistance emergence.

To date, most AMP production systems have been created or tested with at most two or three distinct peptides. Ultimately, we aim to develop a library of probiotics that can be rapidly modified to produce a wide array of AMPs targeting different pathogens of interest. A more general AMP expression and secretion system would facilitate the development and testing of these new AMP-based probiotics. In this study, we have developed an AMP-production vector, pMPES that can be used to produce a variety of AMPs from a single delivery organism.

Secretion has proven to be a major hurdle and area of interest in studies of heterologous AMP production. In this study, we evaluated the flexibility of the MccV secretion machinery contained in pMPES for heterologous AMP production by testing the production of seven different AMPs ranging in similarity to MccV. Note that for this study, AMP production was measured indirectly in the form of AMP activity. As shown in the Results section, all seven peptides tested with the pMPES vector could be detected at some level using inhibition assays. We acknowledge however that future improvements will likely be necessary to achieve levels of pathogen inhibition required for therapeutic applications.

The use of alternative signal peptides is a common approach for improving heterologous secretion [17,46–48]. We thus compared the activities of MccL, McnN and EntA constructs employing their naturally-encoded signal peptides versus the MccV signal peptide. Interestingly, while Vsp improved EntA and McnN construct activity, it actually hindered MccL activity compared to the naturally-encoded signal peptides. These results imply that the Vsp signal peptide may be more reliable for dissimilar AMPs, while the naturally-encoded signal peptide may be more effective when significant homology exists between the peptide/secretion machinery of the AMP and MccV.

Numerous other studies have also examined heterologous secretion of AMPs from other secretion pathways. Among the most interesting was a study in which Divergicin A (DivA) was

shown to be secreted from the Leucocin A (LeuA), Lactococcin A (LcnA) and MccV secretion machineries using their respective signal peptides [17]. Additionally, this study also showed that the LeuA signal peptide could drive DivA secretion from the MccV machinery, but not the LcnA machinery. Furthermore, it was shown that MccV could be secreted from the LeuA machinery using the LeuA signal peptide. These peptides and secretion machineries are highly diverse, making this study particularly insightful.

In another study, chimeras of MccV and Microcin H47 (MccH47) were shown to be secreted from the MccH47 secretion machinery using either the MccV or MccH47 signal peptides [49]. It was believed that this capability was due to the similarity in transport proteins. The ABC transporter of MccH47 is 89% identical (93% similar) to that of MccV, and the accessory proteins are 42% identical (62% similar). Similarly, the high level of homology between the signal peptides and primary secretion components of MccL and MccV may explain why the Lsp signal peptide appears to have been efficiently recognized by the MccV machinery. Interestingly however, very little homology exists between MccL and MccV in the first 50 amino acids following the signal peptides, a region previously hypothesized to be of potential importance to secretion efficiency [36]. Collectively, these studies imply that while homology can in some cases predict successful AMP secretion, lack of homology does not necessarily result in secretion failure.

We are still in a period of attempts largely based on trial-and-error. However, we can imagine that this increasing body of knowledge can facilitate the rational design of AMP expression and secretion systems. In the future, we can explore additional secretion systems, AMPs and signal peptides. Additionally, high-throughput screening methods can be used to select peptides from mutagenesis libraries exhibiting increased activity. Such a method could also in theory select for mutants with increased secretion efficiency.

In the future, in addition to improving peptide secretion, we will also explore possible improvements in gene expression and protein translation to increase overall peptide production. As mentioned in Section 2.3, RBS optimization offers a promising next step, since we have previously observed that even small differences in the RBS can drastically alter observed activity. Additionally, though we have previously observed ProTeOn and ProTeOn+ promoters to be highly active in *E. coli*, there may still be some room for improvement. It may also be of use to explore alternative origins of replication and to remove unnecessary components in the 9.1-

kb MccV production region to reduce the burden of the vector on the host and to improve plasmid stability.

Though much work remains to be done, the study herein provides a foundation for a general AMP expression system. With this foundation, studies may be launched on the efficacy, safety and ADME (adsorption, distribution, metabolism and excretion) properties of antimicrobial probiotics, as well as on questions related to the use of genetically-modified live biotherapeutic bacteria, including environmental release and DNA transfer.

5.3 pMPES2: An Improved Modular Peptide Expression System for *E. coli* Nissle 1917

5.3.1 Summary

In the previous section we demonstrated the versatility of the Microcin V secretion machinery in secreting class II AMPs derived from and targeting a variety of bacterial species. In this section, we describe several improvements made to the original pMPES vector to improve peptide production, create a more well-defined system, and to facilitate cloning. We refer to this vector as pMPES2. We start by describing these improvements then demonstrating the resulting increase in enterocin production. We then use this vector to simultaneously express three anti-enterococcal bacteriocins; Enterocin B, Hiracin JM79, and Enterocin A from *E. coli* Nissle 1917.

5.3.2 Development of pMPES2

Although pMPES, the original modular peptide expression vector, was promising as a proof of concept for modular peptide production, several improvements were necessary to enable its use in our probiotic application. Firstly, we desired a more well-defined vector that contained only the essential components of the MccV secretion system rather than the entire 9.4 kb region which contained several uncharacterized protein coding regions [18]. It was anticipated that only the genes encoding the transport components CvaA and CvaB would be necessary to enable secretion of our peptides. These two genes were thus cloned into the vector FFusion_BF which contained the ProTeOn+ promoter, a ColEI origin of replication, and a spectinomycin resistance marker. The isolation and transfer of the CvaA and CvaB into FFusion was performed by Brittany Forkus, another member of the Kaznessis lab. By isolating this region,

the size of the expression vector was decreased from 14.1 kb to 7.6 kb. Figure 5.8 depicts the two pMPES vectors.

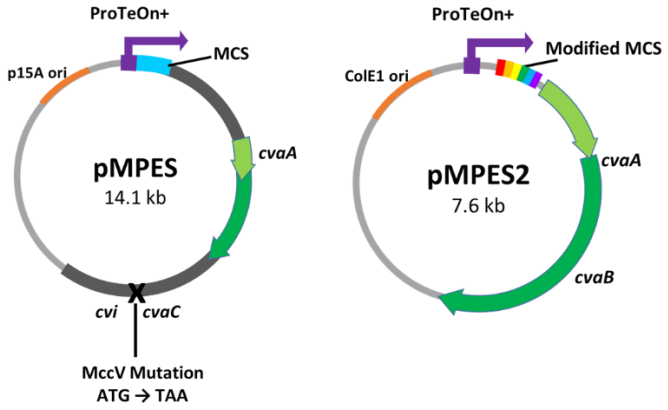

Figure 5.8. Comparison of the pMPES and pMPES2 vector components. ProTeOn+: synthetic DNA promoter; Pon: promoter region; ProTeOn: activator protein; cvaA/cvaB: MccV section machinery; cvaC: MccV peptide (native); cvi: MccV immunity protein;; MCS: Multiple Cloning Site.

In the previous work with the original pMPES, we observed that alterations in the ribosomal binding site (RBS) significantly impacted the AMP expression. We thus sought to improve the RBS using the Salis Lab Ribosome Binding Site Calculator [43,50]. This program attempts to optimize the sequence of the RBS based on the sequence of the surrounding DNA sequences. For this study, we opted to encode MccV secretion tag on the N-terminus of all AMPs to be produced. Using the same DNA code for the secretion tag ensured that the sequence downstream of the RBS remains the same for all of the AMPs. We thus hypothesized that a single optimized RBS sequence could be used effectively for all AMPs tested.

The final improvement included in pMPES2 was the development of a molecular cloning site to facilitate modular insertion and removal of AMPs from the vector. The cloning process is outlined in Figure 5.9.

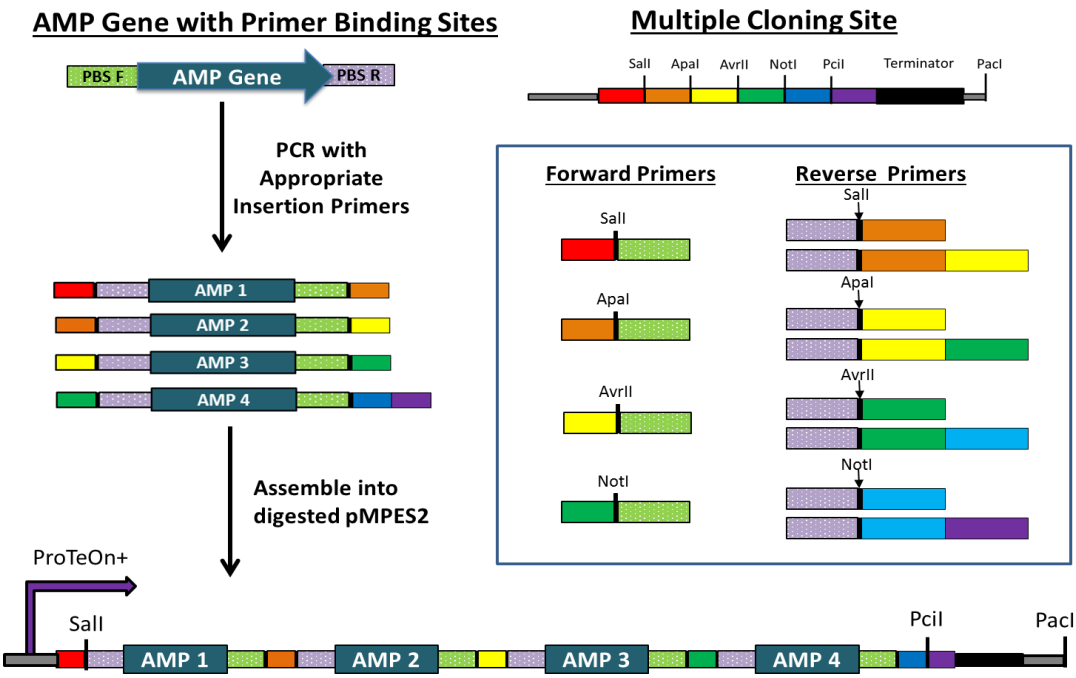

Figure 5.9. Modular AMP cassette assembly using the pMPES multiple cloning site. Molecular cloning site contains five restriction enzymes cute sites followed by a terminator sequence. Between each cut site is an "overlap region" which is used with the forward and reverse primers shown to enable assembly of the final construct. AMPs and their immunity genes will be ordered so as to include the primer binding sites PBS F and PBS R. The appropropriate F/R primers are then selected and fused to the AMP via PCR to attach the necessary overlap region. Reverse primers also introduce an additional restriction site following each AMP (identical to that found in the forward primer) to later allow seamless removal of individual AMPs from the assembly. AMPs with their attached overlap regions are then assembled with the linearized pMPES2 backbone to give the final construct.

Essentially, a forward and reverse primer binding site (PBS F, PBS R) is directly fused to the start codon of the AMP of interest and the stop codon of the AMP or the immunity gene if it is included. The optimized RBS is included in PBF F. These primer sites are the same for all AMPs. The set of twelve primers can then fused to any AMP containing the forward and reverse PBS using PCR. These primers contain regions that overlap with each other and with the multiple cloning site (MCS). For each cloning procedure, primers are selected to allow the overlap between the exposed upstream end of the MCS and the first AMP, the first AMP and the second AMP, the second AMP and the third AMP, etc. and the final AMP and the downstream end of the MCS. Each reverse primer also adds a restriction enzyme site identical to that found directly upstream of the AMP. This was done to add flexibility to the design by enabling scarless removal of individual AMPs by single restriction enzyme digest. Once the appropriate primers have been fused to the AMPs using PCR, the AMPs and linearized pMPES2 can then be assembled using a

one-pot seamless cloning reaction (ex. NEB Hifi Assembler or Gibson). Leftover AMP inserts and linearized pMPES2 can then be stored and directly used in subsequent assembly reactions to save both time and resources.

5.3.3 Comparison of Enterocin Production from pMPES and pMPES2

To evaluate the collective effect of the modifications made between pMPES and pMPES2 on peptide production, we compared activities of *E. coli* producing four different enterocins from the two vectors. Figure 5.10 shows agar diffusion assays of *E. coli* MC1061 F' producing Enterocin A, Hiracin JM79, Enterocin P, and Enterocin B from pMPES (top row) and pMPES2 (bottom row). *E. faecium* 8E9 was used as the indicator strain. Qualitatively, one can see that pMPES2 produces more pronounced zones of inhibition compared to pMPES implying an increased rate of peptide production. We note that *E. coli* MC1061 F' (the cloning host) was used in these preliminary tests rather than *E. coli* Nissle 1917 (EcN).

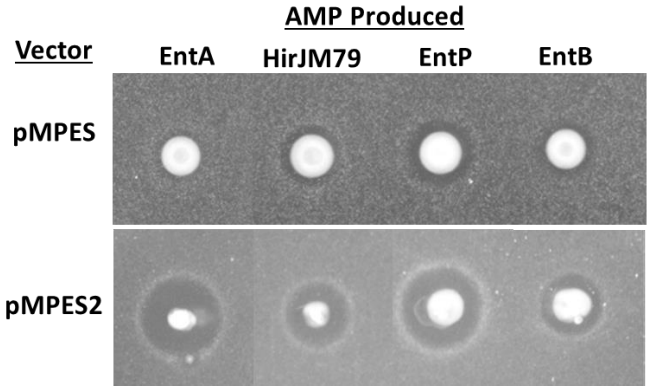

Figure 5.10. Agar diffusion assay showing Enterocin A, Hiracin JM79, Enterocin P, and Enterocin B production from pMPES versus pMPES2 in *E. coli* MC1061 F'. *E. faecium* 8E9 was used as the indicator strain.

To more quantitatively evaluate the efficacy of the pMPES2 vector in secreting anti-enterococcal peptides from EcN, supernatant inhibition assays were performed on *E. faecium* 8E9 liquid cultures. Supernatant from *E. coli* MC1061 F' expressing Enterocin A, Enterocin H, Enterocin P, and Enterocin B from pMPES were compared to supernatants from EcN pMPES2 expressing the same AMPs. Table 5.4 shows the supernatant activities in terms bacteriocin units (BUs) [27]. One BU is defined here to be the reciprocal of the minimum volumetric fraction of

supernatant required to suppress *E. faecium* growth by 50% compared growth in the absence of AMPs. Higher BUs indicate higher supernatant activity.

Table 5.4 Comparison of pMPES and pMPES2 Supernatant Activity

Enterocin Tested	Supernatant Activity (BUs)[1]	
	pMPES (*E. coli* MC1061 F')	pMPES2 (*E. coli* Nissle 1917)
Enterocin A	5	100
Enterocin H	10	>100
Enterocin P	2.5	50
Enterocin B	2.5	50

[1]One Bacteriocin Unit (BU) is defined as the reciprocal of the lowest volumetric fraction of supernatant required to suppress the growth of the indicator strain by 50%.

As can be seen from Table 5.4, over a 20x improvement in peptide production was observed between pMPES and pMPES2 expression systems for all four enterocins tested. We acknowledge that the use of different *E. coli* strains in these tests prevents an exact comparison between the constructs. EcN was not used for pMPES expression in these results because we frequently observed low and sporadic supernatant activities from EcN pMPES enterocin producers. We believe this is due to a combination of the relatively low production from pMPES and peptidase activity of non-laboratory *E. coli* strains. The comparisons shown above thus provide a highly conservative estimate of the overall improvement between pMPES and pMPES2. Importantly, the activities observed for the EcN pMPES2 enterocin producers are comparable to the supernatant activity of *L. lactis* pNZCA3 which we have generally observed to be 350 BUs in an overnight *L. lactis* culture. Note the supernatants tested here were collected after 7 hours of production.

5.3.4 Simultaneous Production of Enterocin B, Hiracin JM79, and Enterocin A

Previous work with AMP production in *L. lactis* has shown that the simultaneous production of multiple AMPs can significantly improve the potency of probiotics targeting VRE. One of the benefits of using a modular peptide expression system is that it can be used to express and secrete multiple AMPs from a single construct as demonstrated in section 5.2.2.2.

We thus chose to express the following three AMPs in a single pMPES2 construct; Enterocin B, Enterocin A, and Hiracin JM79. We refer to this construct as pMPES2:BHA. A simplified schematic of the peptide operon in pMPES2:BHA is shown in figure 5.11. Note that ribosomal binding sites are located upstream of each AMP gene but are not depicted in the figure.

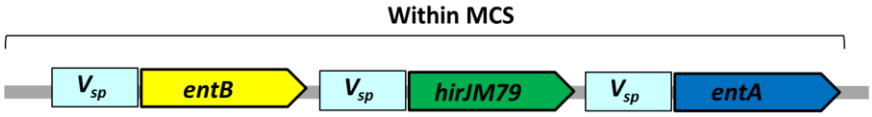

Figure 5.11. Simplified schematic of the pMPES:BHA peptide operon. Mature peptides of Enterocin B, Hiracin JM79, and Enterocin A are shown in yellow, green and dark blue respectively. All peptides are fused to the Microcin V secretion tag (V_{sp}) shown in light blue.

As mentioned earlier, Enterocin A and Hiracin JM79 are both class IIa bacteriocins with nanomolar activity against *E. faecium*. These bacteriocins were both previously used in *L. lactis* pNZCA3 and were selected for use in pMPES2 because they exhibited the highest activity based on EcN supernatant activity assays. Enterocin B was selected because it belongs to a different class of bacteriocins (class IId). Though not much is known about mechanism of action of Enterocin B, we have observed that *E. faecium* 8E9 mutants resistant to class IIa bacteriocins remain susceptible to this peptide. This implies that Enterocin B likely employs a different mechanism of action than the class IIa bacteriocins which means their simultaneous application may reduce the likelihood of resistance development. Additionally, other studies have shown combinations of Enterocin A with Enterocin B against *E. faecium* to be more effective than treatment with the individual peptides [29].

Supernatant activity assays were performed to compare the potency of single or multipeptide production from EcN pMPES2 constructs. Figure 5.12 shows the growth curves of *E. faecium* 8E9 grown in medium containing 0.8 % (v/v %) supernatant from EcN pMPES2 (no AMPs produced), EcN pMPES2:H (producing Hiracin JM79), and EcN pMPES2:BHA (producing Enterocin B, Hiracin JM79, and Enterocin A). pMPES2:H was chosen for comparison to pMPES:BHA because pMPES2 expressing Hiracin JM79 was found to be the most potent single-peptide system against *E. faecium*. Although the activities of the two systems were not radically different, it is clear from Figure 5.12 that pMPES:BHA was noticeably more potent than pMPES:H.

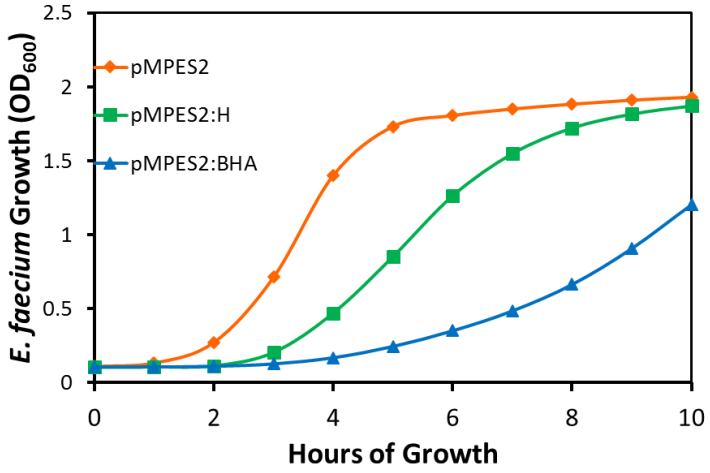

Figure 5.12. Liquid inhibition assays of the EcN pMPES2, EcN pMPES2:H, and EcN pMPES2:BHA supernatants against *E. faecium* 8E9. Cultures are grown in 0.8 % supernatant, 99.2 % rich medium. EcN pMPES2 produces no AMPs, EcN pMPES2:H produces Hiracin JM79 only, EcN pMPES2:BHA produces Enterocin B, Hiracin JM79, and Enterocin A.

Importantly, we have observed that the order of the AMP genes in the polycistronic construct can significantly impact the observed activity of multipeptide constructs. All six combinations of Enterocin B (B), Hiracin JM79 (H), and Enterocin A (A) were tested and BHA was found to the most effective peptide order. Table 5.5 reports the supernatant activities of the different polycistronic constructs.

As shown in table 5.5, the activity of pMPES2:BHA was nearly an order of magnitude higher than the poorest-performing constructs. These results are consistent with similar studies using fluorescence reporter genes[51]. These differences are thought to be largely attributed to changes in mRNA structure that can either interfere or assist with translation initiation. While much remains to be explored, these results demonstrate that the order of our peptide genes offers an additional important optimization variable in future constructs.

Table 5.5 Comparison of Supernatant Activities of Enterocin B, Enterocin A, and Hiracin JM79 Polycistronic Constructs

Order of Genes	Supernatant Activity (BU)
HAB	128
BHA	256
ABH	32
HBA	32
BAH	64
AHB	32

[1]One Bacteriocin Unit (BU) is defined as the reciprocal of the lowest volumetric fraction of supernatant required to suppress the growth of the indicator strain by 50%. H = Hiracin JM79, A = Enterocin A, B = Enterocin B

5.4 Testing *E. coli* Nissle 1917 Efficacy against *E. faecium* 8E9 in a Mouse Model

The efficacy of EcN pMPES2:BHA in reducing VRE in the GI tracts of mice was tested using the vancomycin-assisted colonization model described in Chapter 4. In this model, 18 mice were infected with *E. faecium* 8E9 in their drinking water for 8 days. On the 8th day of treatment, six mice were provided with sterile water (untreated group), six mice were provided with water containing 5 x 10^8 CFU/mL EcN pMPES2:BHA (producing AMPs), and six mice were provided with water containing 5 x 10^8 CFU/mL EcN pMPES2 (producing no AMPs). *E. faecium* was enumerated in the feces as described in Chapter 4. Figure 5.13 shows the average *E. faecium* 8E9 counts in the feces of untreated mice, mice treated with EcN producing Enterocin B, Hiracin JM79, and Enterocin A (Nissle pMPES2:BHA), and mice treated with EcN producing no AMPs (Nissle pMPES2). Solid lines show the average *E. faecium* counts across six mice. Shaded regions represent the mean ± 1 standard deviation.

The results shown in figure 5.13 clearly show a reduction in *E. faecium* carriage in mice treated with EcN pMPES2:BHA compared to the untreated group. The cumulative *E. faecium* shed throughout the treatment in mice treated with EcN pMPES2:BHA was shown to be lower than that of both the untreated group and the group treated with EcN producing no AMPs with $p = 0.011$ and $p = 0.098$ respectively. Interestingly, it appears that EcN itself may have some minor impacts on *E. faecium* colonization though further testing with larger groups would be necessary to establish an acceptable level of confidence ($p = 0.158$ for this study).

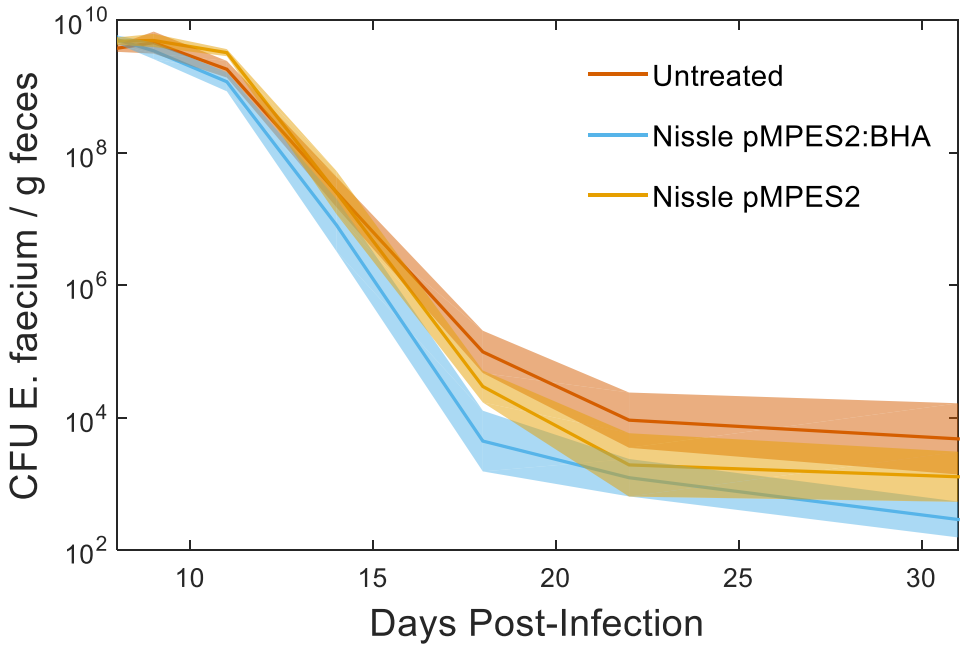

Figure 5.13 Efficacy of EcN pMPES2:BHA in reducing *E. faecium* 8E9 in a vancomycin-assisted colonization model in mice. Mice were fed *E. faecium* and 250 µg / mL vancomycin in their water from day 0 to day 8. On day 9, groups received sterile water (untreated), water containing EcN producing Enterocin A, Enterocin B, and Hiracin JM79 (Nissle pMPES2:BHA), or EcN producing no AMPs (Nissle pMPES2). Solid lines indicate the mean CFU *E. faecium* / g feces across five mice, shaded region shows the standard error.

5.5. Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 5.6.

Table 5.6. Bacteria and plasmids used in this study.

Strain	Description	Source
E. coli MC1061 F'	Plasmid-free, recA+, non-amber suppressor strain	Lucigen
E. coli DH5α PRO	Derivative of *E. coli* DH5α; PN25/tetR, Placiq/laci, cloning host	Clontech
E. faecium 8E9	Ampicillin/vancomycin/linezolid resistant hospital isolate	UMN[1] collection
E. coli O157:H7 472	Common pathogenic species	UMN[1] collection
S. Enteritidis Mh91989	Chicken isolate; common pathogenic species	UMN[1] collection
S. 4,[5],12:i:- Mh06225	Chicken isolate; common pathogenic species	UMN[1] collection

Plasmid	Description	Reference
pHK22	pACYC184 derivative containing 9.1-kb MccV production fragment	[9]
pHK22Δ	pHK22 derivative with mutated *cvaC* gene	This study
pMPES	pHK22Δ derivative containing the ProTeOn+ promoter	This study
pMPES:V, L, VspL, N, VspN, VspA, LspA, A, VspH, VspP, VspB	See Table 5.2	This study
pMPESΔ	pMPES digested with XmaI to eliminate cvaA and cvaB expression	This study
pMPESΔ:V	pMPESΔ with V and MccV immunity gene	This study
pMPESΔ:L	pMPESΔ with L and MccL immunity gene	This study
pMPESΔ:VA	pMPESΔ with VspA and EntA immunity gene	This study
pMPESb	pMPES with alternative ribosomal binding site	This study
pMPESb:VspA	pMPESb with VspA	This study
pMPES_b:VspA_L	pMPESb with VspA and L with their immunity genes	This study
[1]University of Minnesota (UMN)		

Bacteria Growth Conditions

E. coli and *Salmonella* strains were grown with agitation in Luria-Bertani (LB) broth at 37 °C. *E. faecium* was grown in Brain Heart Infusion (BHI) medium at 37 °C in static conditions. When appropriate, chloramphenicol was added to the medium at a concentration of 20 μg/mL for *E. coli*.

Construction of Plasmids

Column or gel purification of digested vector backbone was performed using the Qiagen QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) or the Gel Extraction Kit (Qiagen), respectively. Column purification of all PCR-amplified inserts and insert digests was done using the Qiagen Minelute DNA purification kit (Qiagen). Aside from colony PCRs, all PCRs used NEB Phusion® High-Fidelity DNA Polymerase (New England Biolabs Inc., Ipswich, MA, USA). Colony PCRs used Promega GoTaq® Green Master Mix (Promega, Madison, WI, USA). Ligations were done using NEB T4 DNA ligase, and assemblies were done using NEBuilder® HiFi DNA Assembly Master Mix (NEB). Electrocompetent *E. coli* MC1061 F' from Lucigen (Lucigen, Middleton, WI, USA) were used in all transformations unless otherwise stated. Electrocompetent *E. coli* Nissle 1917 was made as previously described [52]. Nissle was electroporated in a 2-mm cuvette under standard conditions [53]. All restriction enzymes were purchased from NEB. All procedures were

done according to the manufacturer's protocol unless stated otherwise. Primer and DNA fragment sequences are provided in Table 5.7. Successful transformants for pMPES, pMPESb and all AMP vectors were first screened using colony PCR with primers pHK22 HindIII Seq F and pHK22 HindIII Seq R and were then verified with Sanger sequencing.

pHK22Δ *cvaC* mutation: The start codon of the *cvaC* gene (sequence provided below) in pHK22 was mutated from ATG to TAA to create pHK22Δ. Note that the gene is encoded on the reverse strand, such that the mutation was CAT to TTA. The mutation was introduced in a piecewise fashion via PCR. First, two fragments were amplified from pHK22, so as to introduce the mutation at their overlap. The fragments were then fused and reinserted into pHK22. Fragment A (~1.9 kb), which sits between the BssHII restriction site and the *cvaC* start codon, was generated by PCR using forward primer SDML and reverse primer SDM R. The primer-introduced mutation is underlined in Table 5.7. Fragment B (~0.5 kb), which sits between the *cvaC* start codon and the BglII restriction site, was generated by PCR using forward primer SDM F and reverse primer SDML R. Purified Fragments A and B were then fused using a PCR reaction with primers SDML F and SDML R to give Fragment C (~2.4 kb). Purified Fragment C and pHK22 were then digested with BssHII and BglII. Fragment C digest was column purified, and pHK22 was gel purified to isolate the ~10.5 kb fragment generated. The pHK22 backbone and Fragment C were then ligated using T4 DNA ligase to form pHK22Δ, and the resulting ligation was transformed into electrocompetent *E. coli* MC1061 F'. Successful transformants were first screened using colony PCR with primers SDM_seq_F and SDM_seq_R. Correct mutation was then verified with Sanger sequencing.

pMPES ProTeOn+ insertion: The ProTeOn+ DNA fragment was first amplified using Proteon_Assembler_F and Proteon_Assembler_R to give the ProTeOn Plus fragment provided in Table 5.7. Purified HindIII-digested pHK22Δ and ProTeOn Plus insert were then fused using the NEB HiFi assembly kit.

AMP Insertion into pMPES:MccL, Vsp:MccL, McnN, Vsp:McnN, EntA, Vsp:EntA, Lsp:EntA, Vsp:HirJM79, Vsp:EntP and Vsp:EntB gblocks were ordered from Integrated DNA Technologies (IDT), and the MccV fragment was ordered from Geneart. The sequences of these DNA fragments are provided in Table 5.7. McnN, Vsp:McnN and Vsp:EntA were inserted directly into SacI-PacI digested pMPES using the NEB HiFi assembly kit. In contrast, MccL,Vsp:MccL and Lsp:EntA were first amplified using the forward primer AMP F, and the reverse primer AMP R.

MccV was amplified using MccV_SacI_F and MccV_PacI_R. EntA was amplified using EntA_pHK22ΔP F/R. Vsp:HirJM79, Vsp:EntP and Vsp:EntB were amplified using pMPES transition_F/R. The resulting MccL, Vsp:MccL, Lsp:EntA and MccV inserts were then digested using SacI and PacI restriction enzymes, column purified, then ligated into SacI-PacI-digested pMPES using T4 DNA ligase. EntA, VspHirJM79, VspEntP and VspEntB PCR products were column purified then assembled into SacI-AvrII-digested pMPES:N.

Negative controls of MccV, MccL, VspL, McnN and VspA were generated by digesting the pMPES vectors with XmaI restriction enzyme to remove the essential MccV secretion genes *cvaA* and *cvaB* (see Figure 1). Vectors were then reclosed via ligation. Successful transformants were first screened using colony PCR with primers MccV SeC F SpeI and CvaB seq R and were then verified with Sanger sequencing using CvaB seq R.

pMPESb: The original purpose of creating pMPESb was to create a more modular multiple cloning site. However, we later found the RBS to be less effective, and thus, pMPESb was abandoned for the majority of this study. The gblock encoding EntA was made so as to include the pMPESb multiple cloning site, the sequence of which is provided below. The AMP-free pMPESb was thus generated by first assembling the EntA gblock into SacI-PacI-digested pMPES. The resulting vector was then digested with SacI to cleave out EntA and the immunity gene. The purified digestion was re-ligated with T4-DNA ligase to produce pMPESb.

AMP insertion into pMPESb: To create pMPES_VA_L, VA and L inserts were amplified from pMPES:VspA and pMPES:L using PBS VA_For/Rev and PBS L_For/R. The resulting VEntA fragment was then amplified using R_For and O_Rev, and the MccL fragment was amplified using O_For and YG_Rev. The resulting fragments were then assembled into SacI-AvrII-digested pMPESb using the NEB HiFi-Assembler. To create pMPESb:VspA, the Vsp:EntA gblock for pMPESb was assembled directly into SacI-digested pMPES.

Table 5.7. Primers and DNA fragments used in this study.

Primer	Sequence
SDML	CTGCGCGCATGGTCTTC
SDM R	GATAAAAAGGAGATCATTAAAGAACTCTGACTCTA
SDM F	TAGAGTCAGAGTTCTTTAATGATCT CCTTTTTATC
SDML R	TTGAGATCTGTTGAG AGGGGTTTT
SDM_seq_F	CAGCAGCTGCTCCAAT

SDM_seq_R	ATTGCATTAGCTATATATGATTGTGT
Proteon_Assembler_F	GACAGCTTATCATCGATACTAGATAGATGACCTCGGG
Proteon_Assembler_R	CTGGCATTATGGTGGAAAGCTTCAGTTTAATTAAG TGAGCTCACAGCTGTTTC
pHK22 HindIII Seq F	ATGTAGCACCTGAAGTCAGCCC
pHK22 HindIII Seq	GGTAATAGCGGTAAAGTGGCTAAACGG
MccV_SacI_F	TGCTAGAGCTCAAGTCAA GGCCGCATCG
MccV_PacI_R	CATAGTTAATTAATCTACAGTGAG CGGAAGGCC
AMP F	ATCCGGAGCTCTTAGCA
AMP R	TTGACTTAATTAATCGTAGGGGG
EntA_pHK22ΔP F	GAGAATTCAAGGAGGAAACAGCTGTGAGCTCTTAGCAGGAGGTGAACATGAAGCATTTGAAG ATCCTGAGC
EntA_pHK22ΔP R	GGACATGTCCGTAGCGGCCGCACTGACCTAGGTTAGAACTGACTTTTATCTCCATAGTCG
pMPES transition_F	GAGAATTCAAGGAGGAAACAGCTGTGAGCTCTTAGCAGGAGGTGAACATGAGAACTCTGACT CTAAATGAATTAGATTCT
pMPES transition_R	ACATGTCCGTAGCGGCCGCACTGACCTAGGCTCATGGGCCCGTCAACTGTACGATCCTCTGAGC TTCG
MccV SeC F SpeI	CATAACTAGTATAGGAGGTTGTTTCGCCAGGAT
CvaB seq	ACAATCTGATCACAGGCGGT
PBS L_For	CTGATCATGCTCAAGGAGGAGTCAGATGAGAGAAATAACGTTAAATGAAATG
PBS L_Rev	CTGTACGATCCTCTGAGCTTCGACCTCATTTGCCACGGTTGAA
PBS VA_For	CTGATCATGCTCAAGGAGGAGTCAGATGAGAACTCTGACTCTAAATGAATTAG
PBS VA_Rev	CTGTACGATCCTCTGAGCTTCGACCTTAGAACTGACTTTTATCTCCATAGTCG
R_For	AAGGAGGAAACAGCTGTGAGCTCCTGATCATGCTCAAGGAGGAGTCAG
O_Rev	GGGCCCTCTGTAACTACTGTGGGAGCTCCTGTACGATCCTCTGAGCTTCGACC
O_For	CCACAGTAGTTACAGAGGGCCCCTGATCATGCTCAAGGAGGAGTCAG
YG_Rev	GCGGCCGCTATATGTTCTAGGCCTAGGGTCGCTATAGCTGCAGGGGCCCCTGTACGATCCTCTG AGCTTCGACC

DNA Fragments	Sequence [1]
ProTeOn+	gacagcttatcatcgatactagatagatgacctcggggagcccgcctaatgagcgggctttttgcgcgccactctatcattgac tctatcattgatagagtacttaacataagcacctgtaggatcgtacaggtttagcgaagaaaatggtttgttatagtcgaataa acctcgagttatctcgagtgagatattgttgacgcaccaaggaggaagcttctatgatgagccgtctggataaaagcaaagt gattaatagcgcactggaactgctgaatgaagttggtattgaaggtctgaccacccgtaaactggcccagaaactgggtgtt gaacagccgaccctgtattggcatgtgaaaaataaacgtgcactgctggatgcactggccgttgaaattctggctcgccatca tgattatagcctgcctgcagcaggcgaaagctggcagagctttctgcgtaataatgccatgagctttcgtcgtgccctgctgcg ttatcgtgatggtgcaaaagaacatctgggcacccgtccggatgaaaaacagtatgataccgttgaaacccagctgcgtttta tgaccgaaaatggttttagcctgcgtgatggtctgtatgcaattagcgcagttagccattttaccctgggtgccgttctggaaca gcaggaacataccgcagcactgaccgatcgtcctccggcaccggatgaaaatctgcctccgctgctgcgtgaagcactgatg attatggattctgatgatggtgaacaggcatttctgcatggtctggaaagcctgattcgtggttttgaagttcagctgaccgca ctgctgcagattgttggtggtggtggtgcacgtacccagtatagcgaaagcatgggtgcccgtacacagtattctgaatctatg ggtgctcgcacccagtattcagaaagtatgggtgcaagaacacagtatagcgagtctatgggagcgcgtactcagtatagtg aatcaatgggaggtggtatgccgagcctggttgataattaccgcaagattaatattgccaataataaaagcaacaacgatct gaccaaacgtgaaaaagaatgtctggcctgggcatgtgaaggtaaaagcagctgggatattagcaaaattctgggttgtag cgaacgtaccgtgacctttcatctgaccaatgcccagatgaaactgaataccaccaatcgttgtcagagcattagcaaagca attctgaccggtgccattgattgtccgtatttcaaaaactgagaattcaaggaggaaacagctgtgagctcacttaattaaact gaagctttccaccataatgccag
MccV	gtcaaggccgcatcgaggaggaaacagct**atgagaactctgactctaaatgaattagattctgtttctggtggtGCTTCA** **GGGCGTGATATTGCGATGGCTATAGGAACACTATCCGGGCAATTTGTTGCAGGAGGAATTG** **GAGCAGCTGCTGGGGGTGTGGCTGGAGGTGCAATATATGACTATGCATCCACTCACAAACCT** **AATCCTGCAATGTCTCCATCCGGTTTAGGGGGAACAATTAAGCAAAAACCCGAAGGGATACC** **TTCAGAAGCATGGAACTATGCTGCGGGAAGATTGTGTAATTGGAGTCCAAATAATCTTAGTG** **ATGTTTGTTTATAA**gataccaggaggaaactgct*ATGGATAGAAAAAGAACAAAATTAGAGTTGTTAT* *TTGCATTTATAATAAATGCCACCGCAATATATATTGCATTAGCTATATATGATTGTGTTTTTAGA* *GGAAAGGACTTTTTATCCATGCATACATTTTGCTTCTCTGCATTAATGTCTGCAATATGTTACTTT* *GTTGGTGATAATTATTATTCAATATCCGATAAGATAAAAAGGAGATCATATGAGAACTCTGACT* *CTAAATGA*aggtccatggtacgtacccatagataggcgccgttatcgactgggcctcatgggccttccgctcactg
MccL	atccggagctcttagcaggaggtgaac**atgagagaaataacgttaaatgaaatgaataatgtctctggtgctGGTGATG** **TCAATTGGGTTGATGTCGGGAAAACTGTAGCAACAAACGGTGCAGGAGTGATTGGCGGTGC** **ATTCGGAGCTGGTCTGTGCGGCCCTGTTTGTGCTGGTGCCTTCGCTGTTGGATCTTCTGCCGCT** **GTTGCTGCTTTGTATGATGCAGCAGGAAATTCCAACTCAGCGAAACAAAAACCAGAAGGACT** **ACCTCCAGAAGCATGGAACTACGCTGAAGGTAGAATGTGTAATTGGAGTCCAAATAATCTTA** **GTGATGTTTGTTTATAA**actggaggaggtgcac*ATGAAAACTTGGCAGGTTTTCTTCATCATCCTTCC* *GATCTCCATCATTATCTCTCTGATTGTTAAACAGCTTAACAGCTCCAACCTTGTACAGAGCGTTGT* *AAGCGGCATTGCTATCGCACTCATGATCTCCATCTTTTTCAACCGTGGCAAATGA*ttgacgggcccat gagcctaggtcagtgcggccgctacggacatgtccgta*gctagcgaaaaaaaaacccgcccctgacaggacgggttttttt* *tttaatttaacccccc*tacgattaattaagtcaa
Vsp:MccL	atccggagctcttagcaggaggtgaac**atgagaactctgactctaaatgaattagattctgtttctggtggtGGTGATGT** **CAATTGGGTTGATGTCGGGAAAACTGTAGCAACAAACGGTGCAGGAGTGATTGGCGGTGCA** **TTCGGAGCTGGTCTGTGCGGCCCTGTTTGTGCTGGTGCCTTCGCTGTTGGATCTTCTGCCGCTG** **TTGCTGCTTTGTATGATGCAGCAGGAAATTCCAACTCAGCGAAACAAAAACCAGAAGGACTA** **CCTCCAGAAGCATGGAACTACGCTGAAGGTAGAATGTGTAATTGGAGTCCAAATAATCTTAG** **TGATGTTTGTTTATAA**actggaggaggtgcac*ATGAAAACTTGGCAGGTTTTCTTCATCATCCTTCCG* *ATCTCCATCATTATCTCTCTGATTGTTAAACAGCTTAACAGCTCCAACCTTGTACAGAGCGTTGTA* *AGCGGCATTGCTATCGCACTCATGATCTCCATCTTTTTCAACCGTGGCAAATGA*ttgacgggcccatg agcctaggtcagtgcggccgctacggacatgtccgta*gctagcgaaaaaaaaacccgcccctgacaggacgggttttttttt* *taatttaacccccc*tacgattaattaagtcaa

McnN	gagaattcaaggaggaaacagctgtgagctcttagcaggaggtgaac**atgcgtgagctcgatcgtgaagaacttaactgcg tgggtggcgcgGGGGACCCACTCGCAGATCCTAACAGTCAGATCGTTCGCCAGATCATGTCCAAT GCGGCCTGGGGGGCCGCCTTTGGTGCCAGAGGCGGTTTAGGGGGCATGGCGGTTGGTGCGG CTGGGGGCGTGACTCAAACAGTATTGCAGGGTGCTGCGGCGCATATGCCGGTCAACGTGCCG ATTCCTAAGGTGCCGATGGGTCCTAGCTGGAACGGGTCCAAAGGCTAA**actggaggaggtgcac*A TGAGCTTTTTGAACTTTGCCTTTAGCCCCGTGTTTTTTTCCATCATGGCCTGCTATTTCATCGTATG GCGTAATAAACGCAATGAATTTGTTTGCAACCGCCTGCTGTCGATTATCATCATCTCATTTTTAAT CTGCTTCATTTACCCATGGCTTAACTATAAAATTGAAGTGAAATACTACATCTTTGAACAGTTTTA CTTGTTCTGCTTCCTGTCGAGCCTGGTTGCGGTAGTCATTAACCTGATCGTATACTTCATTCTGTA CCGTCGCTGTATTTGA*ttgacgggcccatgagcctaggtcagtgcggccgctacggacatgtccgta*gctagcgaaa aaaaaacccgcccctgacagggcgggttttttttttaatttaacccccc*tacgattaattaaactgaagctttccaccataatg cc
Vsp:McnN	gagaattcaaggaggaaacagctgtgagctcttagcaggaggtgaac**atgagaactctgactctaaatgaattagattctg tttctggtggtGGGGACCCACTCGCAGATCCTAACAGTCAGATCGTTCGCCAGATCATGTCCAATG CGGCCTGGGGGGCCGCCTTTGGTGCCAGAGGCGGTTTAGGGGGCATGGCGGTTGGTGCGGC TGGGGGCGTGACTCAAACAGTATTGCAGGGTGCTGCGGCGCATATGCCGGTCAACGTGCCG ATTCCTAAGGTGCCGATGGGTCCTAGCTGGAACGGGTCCAAAGGCTAA**tgctaaggaggtgcta*AT GAGCTTTTTGAACTTTGCCTTTAGCCCCGTGTTTTTTTCCATCATGGCCTGCTATTTCATCGTATG GCGTAATAAACGCAATGAATTTGTTTGCAACCGCCTGCTGTCGATTATCATCATCTCATTTTTAAT CTGCTTCATTTACCCATGGCTTAACTATAAAATTGAAGTGAAATACTACATCTTTGAACAGTTTTA CTTGTTCTGCTTCCTGTCGAGCCTGGTTGCGGTAGTCATTAACCTGATCGTATACTTCATTCTGTA CCGTCGCTGTATTTGA*ttgacgggcccatgagcctaggtcagtgcggccgctacggacatgtccgta*gctagcgaaa aaaaaacccgcccctgacagggcgggttttttttttaatttaacccccc*tacgattaattaaactgaagctttccaccataatg cc
Lsp:EntA	atccggagctcttagcaggaggt**gaacatgagagaaataacgttaaatgaaatgaataatgtctctggtgct ACCACCC ATAGCGGTAAGTATTACGGAAATGGAGTTTACTGTACCAAAAATAAATGCACCGTTGATTGG GCTAAAGCGACAACTTGTATCGCTGGTATGTCTATCGGCGGGTTCTTAGGGGGTGCCATTCCA GGCAAATGCTAA**tgctaaggaggtgcta*ATGAAGAAAAACGCCAAACAGATTGTTCACGAGTTATAT AACGACATTAGTATCAGCAAAGACCCGAAGTATTCGGACATCCTTGAGGTACTGCAAAAAGTGT ATCTGAAACTGGAAAAACAGAAATATGAGTTAGATCCAGGACCATTGATCAATCGTCTGGTAA ACTACCTGTACTTCACCGCCTATACCAACAAGATTCGTTTCACCGAATATCAAGAAGAGTTAATC CGCAATCTGTCTGAGATTGGACGCACAGCAGGAATCAACGGACTGTACCGTGCCGACTATGGA GATAAAAGTCAGTTCTAA* ttgacgggccc atgagcctagg tcagtgcggccgc tacggacatgt ccgta*gctag cgaaaaaaaaacccgcccctgacagggcgggttttttttttaatttaacccccc*tacgattaattaagtcaa
Vsp:EntA (for pMPES)	gagaattcaaggaggaaacagctgtgagctcttagcaggaggtgaac**atgagaactctgactctaaatgaattagattctg tttctggtggtACCACTCATAGCGGTAAGTATTACGGAAATGGAGTTTACTGTACCAAAAATAAA TGCACCGTTGATTGGGCTAAAGCGACAACTTGTATCGCTGGTATGTCTATCGGCGGGTTCTTA GGGGGTGCCATTCCAGGCAAATGCTAA**tgctaaggaggtgcta*ATGAAGAAAAACGCCAAACAGAT TGTTCACGAGTTATATAACGACATTAGTATCAGCAAAGACCCGAAGTATTCGGACATCCTTGAG GTACTGCAAAAAGTGTATCTGAAACTGGAAAAACAGAAATATGAGTTAGATCCAGGACCATTG ATCAATCGTCTGGTAAACTACCTGTACTTCACCGCCTATACCAACAAGATTCGTTTCACCGAATA TCAAGAAGAGTTAATCCGCAATCTGTCTGAGATTGGACGCACAGCAGGAATCAACGGACTGTA CCGTGCCGACTATGGAGATAAAAGTCAGTTCTAA*ttgacgggcccatgagcctaggtcagtgcggccgctac ggacatgtccgta*gctagcgaaaaaaaaacccgcccctgacagggcgggttttttttttaatttaacccccc*tacgattaatt aaactgaagctttccaccataatgcc
EntA	gagaattcaaggaggaaacagctgtgagctcctgatcatgctcaaggaggagtcag**atgaagcatttgaagatcctgagca ttaaggagACCCAACTGATATACGGAGGTACCACTCATAGCGGTAAGTATTACGGAAATGGAG TTTACTGTACCAAAAATAAATGCACCGTTGATTGGGCTAAAGCGACAACTTGTATCGCTGGTA TGTCTATCGGCGGGTTCTTAGGGGGTGCCATTCCAGGCAAATGCTAA**tcgtaggaggacgtac*ATG AAGAAAAACGCCAAACAGATTGTTCACGAGTTATATAACGACATTAGTATCAGCAAAGACCCG AAGTATTCGGACATCCTTGAGGTACTGCAAAAAGTGTATCTGAAACTGGAAAAACAGAAATAT GAGTTAGATCCAGGACCATTGATCAATCGTCTGGTAAACTACCTGTACTTCACCGCCTATACCAA CAAGATTCGTTTCACCGAATATCAAGAAGAGTTAATCCGCAATCTGTCTGAGATTGGACGCACA GCAGGAATCAACGGACTGTACCGTGCCGACTATGGAGATAAAAGTCAGTTCTAA*ggtcgaagctca gaggatcgtacaggagctcccacagtagttacagagggcccctgcagctatagcgaccctaggcctagaacatatagcggc cgcccatcacactgtgagcacatgtggacacttcaccacgtatctgg*gctagcgaaaaaaaaacccgcccctgacagggc*

	*gggttttttttaatttaaccccc*tacgattaattaaactgaagctttccaccataatgcc
pMPES MCS	gagaattcaaggaggaaacagctgtgagctcccacagtagttacagagggcccctgcagctatagcgaccctaggcctaga acatatagcggccgcccatcacactgtgagcacatgtggacacttcaccacgtatctgg*gctagcgaaaaaaaaaacccgc ccctgacagggcgggttttttttttaatttaaccccc*tacgattaattaaactgaagctttccaccataatgcc
Vsp:EntA (for pMPESb)	aaggaggaaacagctgtgagctcctgatcatgctcaaggaggagtcag**atgagaactctgactctaaatgaattagattct gtttctggtggtACCACTCATAGCGGTAAGTATTACGGAAATGGAGTTTACTGTACCAAAAATAA ATGCACCGTTGATTGGGCTAAAGCGACAACTTGTATCGCTGGTATGTCTATCGGCGGGTTCTT AGGGGGTGCCATTCCAGGCAAATGCTAA**ggtcgaagctcagaggatcgtacaggagctcccacagtagttac agagggccc
Vsp:HirJM79	aaggaggaaacagctgtgagctcctgatcatgctcaaggaggagtcag**atgagaactctgactctaaatgaattagattct gtttctggtggtGCGACATACTATGGAAATGGATTGTATTGCAATAAGGAGAAGTGTTGGGTCGA TTGGAATCAAGCTAAAGGAGAGATCGGAAAGATAATAGTGAACGGTTGGGTGAATCATGGT CCTTGGGCTCCCAGACGCTAA**ggtcgaagctcagaggatcgtacaggagctcccacagtagttacagagggccc
Vsp:EntP	ctgatcatgctcaaggaggagtcag**atgagaactctgactctaaatgaattagattctgtttctggtggtGCAACTCGCT CATACGGGAATGGGGTGTATTGTAACAATTCTAAATGTTGGGTGAACTGGGGTGAAGCGAA AGAAAACATCGCTGGTATCGTCATCTCTGGCTGGGCCTCAGGACTTGCAGGAATGGGTCATT AA**ggtcgaagctcagaggatcgtacaggagctcccacagtagttacagagggccc
Vsp:EntB	aaggaggaaacagctgtgagctcctgatcatgctcaaggaggagtcag**atgagaactctgactctaaatgaattagattct gtttctggtggtGAAAACGACCACAGAATGCCCAACGAGTTGAATCGCCCTAACAATCTTAGCAA AGGGGGAGCCAAATGCGGCGCGGCGATTGCAGGTGGACTTTTCGGGATACCGAAAGGACCG CTGGCTTGGGCCGCTGGATTAGCGAATGTTTACTCAAAATGTAACTAA**ggtcgaagctcagaggatc gtacaggagctcccacagtagttacagagggccc
pMPES RBS	Gagctcttagcaggaggtgaac
pMPESb RBS	Gagctcctgatcatgctcaaggaggagtcag

[1]: The following key is used to label components of AMP DNA fragments. Ribosomal Binding Sites: lowercased, underlined; Signal peptide: lowercased, bold; Mature peptide: uppercased, bold; Immunity Gene: uppercased, italicized; Terminator: lowercased, italicized, underlined.

Activity Assays

Agar Diffusion Assays

Figures 3a, b and S2: 0.6 µL of indicator strain overnight culture was mixed with 100 µL of BHI medium and spread onto BHI agar plates. Plates were allowed to dry, then 3 µL of overnight producer culture were spotted onto the plate and allowed to dry completely. Dry plates were then covered and incubated overnight at 37 °C for imaging the following day.

Figures 3c and S1: Liquid BHI agar was inoculated with 0.1 µL of *E. coli or Salmonella* overnight culture per mL medium. The inoculated agar was then poured into a petri dish and allowed to solidify. A 0.5-µL overnight culture of each *E. coli* strain was then dropped onto the

plate with the appropriate indicator strain and allowed to dry completely. Dry plates were then covered and incubated overnight at 37 °C for imaging the following day.

Figure S3: Holes were cut into BHI agar plates then filled with liquid BHI agar containing 1000× dilution of Nissle overnight cultures and allowed to solidify, then sealed with 30 µL sterile BHI agar. Plates were incubated overnight at 37° C, then liquid BHI agar was inoculated with 0.5 µL/mL overnight indicator culture and poured over the producer plate. Dry plates were then covered and incubated overnight at 37 °C for imaging the following day.

Supernatant Activity Assays

Producer and indicator strains were grown from plates for 12 h in 3 mL LB. Supernatants were filtered using a 0.22-µm filter (EMD Millipore, Billerica, MA, USA). Overnight indicator strain cultures were diluted in the appropriate growth medium by 10^5 to give ~5×10^3 CFU/mL cells. Sixty-two-point-five microliters of the diluted cultures were then combined with 187.5 µL of the supernatant, which had been appropriately diluted with pMPES supernatant. The plate was then incubated for 20 h at 37 °C with fast orbital shaking in a Synergy H1 plate reader (BioTek, Winooski, VT, USA). One Bacteriocin Unit (BU) is defined as the reciprocal of the highest dilution of supernatant that resulted in an increase in the culture's Time To Rise (TTR) compared to growth in the absence of AMPs. TTR values were calculated as the hours required to rise to ¼ of the maximum OD_{600} for a given indicator strain. TTRs were first determined for a minimum of three growth curves of the indicator strain in 75% pMPES supernatant. We considered this to be the baseline growth in the absence inhibition. *p*-values were then obtained from a left-tailed two-sample *t*-test comparing the TTR values for each supernatant concentration against the pMPES supernatant TTRs. BUs for each biological replicate were then reported as the reciprocal of the highest dilution of supernatant that resulted in an increase in the culture's TTR compared to growth in the absence of AMPs with $p < 0.05$. Values reported tables are the averages and standard deviations of BUs of three biological replicates.

Note in table 5.4 and 5.5, one bacteriocin unit is defined to be the reciprocal of the lowest volumetric fraction of supernatant that results in 50% inhibition of the indicator strain. 50% inhibition is achieved when the OD_{600} of the indicator treated with supernatant containing bacteriocins is less than half the $OD6_{00}$ of the indicator treated with supernatant containing no bacteriocins. These OD_{600} values are obtained at the time when the indicator strain exits

exponential growth. This time of measurement was selected so as to be applicable for all indicator strains regardless of differences in growth rates. See Materials and Methods of chapter 6 for further detail.

Peptide Isolation Attempts

Several attempts were made using SDS-PAGE with and without supernatant concentration steps to visualize and estimate absolute AMP production and secretion. NuPAGE Novex 4-12% Bis-Tris Protein Gels were used for all SDS-PAGE attempts (Life Technologies Carlsbad, CA, USA). Up to 40 mL of culture were concentrated for SDS-PAGE samples. However, we were unable to see bands using Coomassie-stained SDS-PAGE for any of the producing strains. We continuously encountered a blurred region in the gels at the molecular weight expected for the peptides (3.5–10 kDa). We suspect that even with sufficient quantities of peptides that this region may make visualization difficult. We were unable to resolve this issue using minimal media or desalting columns. Additionally, reverse-phase HPLC was performed on supernatants using a Dionex UltiMate 3000 UHPLC (Dionex, Sunnyvale, CA, USA) with an XBridge Peptide BEH C18 column (Waters Corp., Milford, MA, USA) but no distinct peaks could be linked to AMP activity due to background noise. In the future, we will attempt additional AMP isolation techniques, such as size exclusion column chromatography and mass spectrometry. We will also test alternative types of gels based on previous work in the literature.

Chapter 6: Characterization of *E. faecium* Resistance Development to Class IIa Bacteriocins

6.1 Introduction

Vancomycin-resistant enteroccocal infections pose a severe threat in hospital environments. The dense colonization of VRE in the GI tracts of patients is a known starting point of bacteremia and is a primary cause of VRE outbreaks in hospitals.

Throughout this thesis we have demonstrated the potential of using AMPs delivered by probiotic bacteria for the reduction of VRE in the intestinal tract. Class IIa bacteriocins currently comprise one of the largest, most well-characterized class of AMPs with specific activity against enterococci. Many class IIa bacteriocins have been found to have potent activity against VRE and therefore may offer a potential treatment or prevention option for these difficult-to-treat infections [1–3].

Nevertheless, like resistance to traditional antibiotics, resistance to these antimicrobial peptides (AMPs) is a concern. An understanding of the mechanism of resistance and impact of resistance development on the pathogen is essential in evaluating the application of these AMPs for treatment of VRE infections and the overall reduction of VRE in the gastrointestinal (GI) tract.

Numerous studies exploring class IIa bacteriocin resistance have been conducted for other pathogens, including *L. monocytogenes* and *E. faecalis* [4–7]. Evidence from these studies indicates that a mannose phosphotransferase system (ManPTS) is involved in resistance to this class of AMPs and it has been hypothesized that the transporter may act as an external target for the peptides [7–9]. It has also been hypothesized that a primary route to resistance is the down-regulation of this ManPTS [5,10]. ManPTS knock-out experiments in both *L. monocytogenes* and *E. faecalis* have supported this theory. Additionally, numerous studies using these pathogens have shown that resistant mutants often display altered metabolic activity compared to the wild type strains, which could significantly impact their ability to colonize a host [6,11].

While these studies may provide useful insight on the general mechanism of resistance to class IIa bacteriocins, similar work has yet to be done for *E. faecium*. The particular causes and effects of resistance to class IIa bacteriocins in *E. faecium* could play an important role in the future application and development of these AMPs against this pathogen.

In this study we explore the mechanism and fitness effects of *E. faecium* resistance development to class IIa bacteriocins. In previous studies we engineered *Lactococcus lactis* as an AMP delivery organism to produce the three class IIa bacteriocins- Enterocin A, Enterocin P, and Hiracin JM97 [1,3].

We first quantify the frequencies of resistance of four clinical isolates of antibiotic-resistant *E. faecium* to this cocktail of AMPs. We then evaluate the fitness and levels of resistance of 29 mutants of a particular clinical isolate, *E. faecium* 6E6. We then sought to identify putative genes involved in *E. faecium*'s resistance to the class IIa bacteriocins by comparing whole genome sequences of the wild type 6E6 and two resistant mutants.

These studies revealed mutations in both a σ54 transcription factor and in a σ54 factor activator protein, both putatively involved in the expression of a particular ManPTS operon. qRT-PCR revealed that this ManPTS was down-regulated in all five mutants compared to the wild type 6E6. Lastly, we demonstrate this ManPTS's direct involvement in peptide susceptibility by showing that heterologous expression of the ManPTS operon renders *L. lactis* susceptible to the bacteriocins.

Collectively, these studies provide strong evidence implicating a particular ManPTS in the resistance of *E. faecium* to class IIa bacteriocins and show that the down regulation of this PTS is a common feature of resistant mutants in *E. faecium* 6E6. The information provided here can help guide future considerations for the application and design of systems using class IIa bacteriocins for the treatment of vancomycin-resistant *E. faecium* infections. For example, we now have evidence of a wide-spread fitness defect among *E. faecium* resistant mutants which could impact the conditions under which they can colonize the GI tract. Additionally, we now have a proposed target protein, knowledge of which can aid in the future selection and utilization of AMPs with orthogonal mechanisms of action. These design considerations are further discussed herein.

6.2 Results

6.2.1 *E. faecium* Resistance Frequency to Class IIa bacteriocins

The frequency of resistance (FOR) is an important parameter in evaluating the ease with which resistance to an antimicrobial agent is developed. We tested the FOR of four antibiotic-

resistant clinical isolates of *E. faecium* to a cocktail of three class IIa bacteriocins (Enterocin A, Enterocin P, and Hiracin JM79). A combination of peptides was used because this has previously been shown to be more effective than treatments with the single peptides [1]. The results of the FOR tests are shown in Table 6.1. These values were obtained by plating dilutions of overnight cultures of the specified strain on gM17+ agar to obtain total colony forming units (CFUs)/mL and then on gM17+20% *L. lactis* supernatant (SN) containing Enterocin A, Enterocin P, and Hiracin JM79 to obtain the number of resistant mutants in the culture. Frequency of resistance was then calculated as the ratio of CFUs on the selective plate over CFUs on the non-selective plate.

Table 6.1. Class IIa Bacteriocin Resistance Frequencies and Activities of Four *E. faecium* Clinical Isolates

***E. faecium* Strain**	**Supernatant Activity (BUs)**	**Frequency of Resistance (Resistant CFU/Total CFU)**
6E6	55.8 ± 5.9	2.6E-05 ± 1.7E-06
7A	12.3 ± 1.6	5.8E-04 ± 9.6E-05
8E9	95.1 ± 7.9	3.1E-05± 6.1E-06
9B	47.3 ± 4.2	2.0E-05± 4.0E-06

FOR was determined in biological triplicate for each strain. One Bacteriocin Unit (BU) is defined as the reciprocal of the lowest v/v fraction of supernatant required to reduce the growth of the indicator strain by 50%; error represents the standard deviation of three biological replicates. The standard deviation represents the deviation in FOR and activity across triplicates.

Table 6.1 also reports the activity of the *L. lactis* pNZCA3 supernatant against the four strains in liquid culture. Throughout this work, inhibitory activity of the supernatants is reported in terms of Bacteriocin Units (BUs). One bacteriocin unit is defined to be the reciprocal of the lowest volumetric fraction of supernatant that results in 50% inhibition of the indicator strain. A more detailed description of activity calculations are provided in the Materials and Methods section of this chapter. These values allow a comparison of the sensitivity of the different strains tested.

Clinical isolates *E. faecium* 6E6, 8E9, and 9B all showed resistance frequencies on the order of 10^{-5} while *E. faecium* 7A exhibited an appreciably higher frequency on the order of 10^{-4}. Interestingly, the colonies of *E. faecium* 7A that arose on supernatant plates showed

significantly slower growth compared to those on gM17 alone, or compared to the 6E6, 8E9, and 9B mutants. This indicates that while 7A exhibited more spontaneous resistant mutants, these mutants still exhibited a noticeable level of susceptibility to the peptides.

The effect of SN concentration on FOR was also tested (data not shown). It was found that above ~10% SN, the FOR remained approximately the same. At 5% SN however, gM17 and selective plates produced similar numbers of colonies but colonies on plates containing SN were significantly smaller. This indicates that under these conditions, the minimum inhibitory concentration to stop growth of the wild type 6E6 is between 5% and 10% SN.

6.2.2 Level of Resistance

Two groups of class IIa bacteriocin resistant mutants were derived from *E. faecium* 6E6 and used in both the resistance level and growth studies. Group A mutants were selected by plating an overnight culture of *E. faecium* 6E6 on gM17+10% SN and collecting the resulting colonies. In contrast, Group B mutants were created by inoculating liquid medium containing 10% SN with wild type *E. faecium* 6E6 culture and allowing the culture to grow overnight. By using this method, bacteria were exposed to a continuous pressure for resistance selection. The overnight culture was then plated on gM17+10% SN and the resulting colonies were collected for further study. Figure 6.1 depicts this selection process.

Once both a Group A and a Group B population had been isolated, we aimed to examine the level and heterogeneity of their resistances. This was done both as a means of assessing the potential severity of resistance for the two classes of mutants as well as to gain information that could help elucidate the mechanism of resistance. To semi-quantitatively test the level of resistance, we performed stab-on-agar (SOA) tests for 15 Group A mutants and 14 Group B mutants. For these tests, individual plates were inoculated with the different mutants, allowed to solidify, then stabbed with a colony of *L. lactis* PNZCA3 producing Enterocin A, Enterocin P, and Hiracin JM79. Susceptibilities of the pathogens to the AMPs were then determined based on the diameter of the zone of inhibition, i.e. halo diameter. Halo diameter was ultimately selected as the measure of resistance because inhibition could be observed over a broader range of mutants compared to supernatant dilution tests.

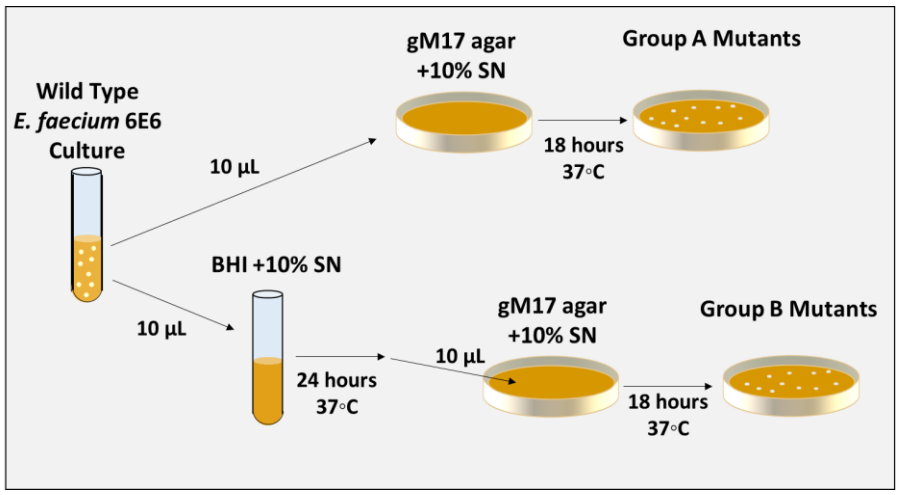

Figure 6.1. Creation of Group A and Group B Mutants: Two methods of resistance development were used to create the mutants in this study resulting in Group A and Group B mutants. All mutants were ultimately selected for by plating on gM17+SN which contained Enterocin A, Enterocin P, and Hiracin JM79. Group B mutants were first evolved overnight in a liquid culture containing the AMPs.

In the SOA tests, several of the mutants exhibited halos indicating that they remained susceptible to the AMPs, though at a reduced level compared to wild type 6E6. Table 6.2 shows mean halo sizes formed on the mutants exhibiting halos in both the A and B groups compared to the wild type 6E6 along with the percentage of mutants in each group exhibiting no visible halo. Figure 6.2 shows representative images of SOA tests of the wild type 6E6, a low level resistance mutant, and a high level resistance mutant.

Table 6.2. AMP Susceptibility Test Results of *E. faecium* 6E6 Resistant Mutants

Group	Mean Halo Diameter (mm)	Percent Showing No Halo
Wild Type 6E6	11.5 ± 0.0	0 %
Group A Mutants	6.4 ± 1.7	33 %
Group B Mutants	5.8 ± 2.0	64 %

Individual mutants (15 of Group A, 14 of Group B) were tested in biological triplicate to obtain individual mean halo diameters. Individual halo diameters were then used to calculate the group's mean halo diameter. Standard deviation of halo diameters across mutant averages are shown.

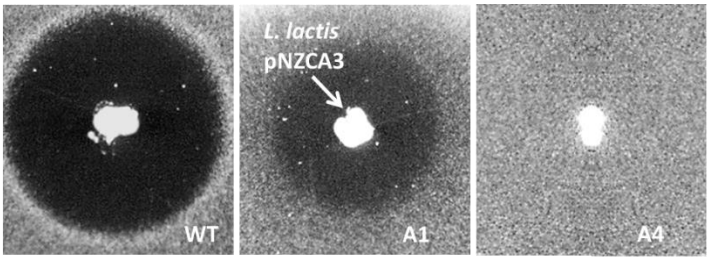

Figure 6.2. Representative Stab-on-agar Examples of *E. faecium* 6E6 Mutants: *E. faecium* 6E6 wild type or the indicated resistant mutants were used as the indicator strain. *L. lactis* pNZCA3 which produces Enterocin A, Enterocin P, and Hiracin JM79 was stabbed onto plates containing the indicator strains specified in the lower right of the image. Mutants were considered highly resistant if no halo was visible and were considered moderately resistant if a halo was visible. Numbers indicate the number of Group A and Group B mutants in each resistance class.

Figure 6.3 shows individual mutant halo sizes as well as the growth rates of the individual mutants on mannose which will be discussed later. For these tests, serial dilutions of SN were also spotted on the surface of the plate and the highest dilution resulting in inhibition was recorded. Isolates inhibited by undiluted supernatant are indicated with stars. WT *E. faecium* 6E6 was inhibited at the 64x dilution of supernatant. Thus the sensitivities of starred isolates are within 64 times that of the wild type 6E6. Note, the inhibitory dilution was the same across biological triplicates for all isolates.

On average, the Group B mutants exhibited smaller and more diffuse halos than the Group A mutants. Additionally, the fraction of Group B mutants considered to be highly resistant (exhibited no halo) was nearly double that observed in the Group A mutants (64 % versus 33 %). We acknowledge that some of the isolates from both the A and B cultures were likely siblings. Regardless of this fact, this data implies that for the two cultures as a whole, overnight growth in the presence of SN appears to have resulted in some selection for increased resistance. Despite this selection however, a significant fraction of all of the mutants remains partially susceptible to the AMPs.

	Growth rate on Mannose (mOD_{600}/min)	Halo Diameter (mm)
WT	5.3 ± 0.1	11.5 ± 0.0
A1*	3.3 ± 0.0	8.7 ± 0.6
A8*	1.4 ± 0.0	7.8 ± 2.4
A11*	3.3 ± 0.2	7.5 ± 0.5
A2*	1.3 ± 0.0	7.2 ± 0.6
A13*	3.3 ± 0.1	7.0 ± 2.9
A9*	0.9 ± 0.3	6.8 ± 0.4
A6	0.7 ± 0.0	6.7 ± 0.6
A7	0.4 ± 0.1	5.0 ± 2.6
A10	0.8 ± 0.0	4.7 ± 0.3
A5	0.7 ± 0.0	3.0 ± 0.0
A3	0.7 ± 0.0	0.0 ± 0.0
A4	0.7 ± 0.0	0.0 ± 0.0
A12	0.9 ± 0.0	0.0 ± 0.0
A14	0.8 ± 0.0	0.0 ± 0.0
A15	0.8 ± 0.0	0.0 ± 0.0
B2	1.1 ± 0.2	7.8 ± 1.0
B3	1.2 ± 0.2	7.5 ± 0.9
B1	1.0 ± 0.3	6.3 ± 0.6
B11	0.8 ± 0.1	3.8 ± 1.0
B9	1.0 ± 0.1	3.5 ± 0.5
B4	0.9 ± 0.1	0.0 ± 0.0
B5	1.0 ± 0.4	0.0 ± 0.0
B6	0.9 ± 0.2	0.0 ± 0.0
B7	0.9 ± 0.3	0.0 ± 0.0
B8	0.7 ± 0.0	0.0 ± 0.0
B10	0.8 ± 0.1	0.0 ± 0.0
B12	0.9 ± 0.1	0.0 ± 0.0
B13	0.8 ± 0.0	0.0 ± 0.0
B14	0.8 ± 0.1	0.0 ± 0.0

Figure 6.3. Individual Mutant AMP Susceptibility and Growth Rate on Mannose: Individual mutants were tested to obtain individual mean halo diameters and growth rates on mannose. Error represents the standard deviation of three biological triplicates. Stars indicate that mutant sensitivity was within 64 times that of the wild type 6E6. Color gradient: High values shown in green to low values shown in red.

6.2.3 Mutant Fitness Tests

Previous studies in *E. faecalis, L. monocytogenes,* and *L. lactis* showed that class IIa bacteriocin resistant mutants frequently exhibit changes in their metabolic and growth behavior which has been linked to the mechanism of resistance and may impact their ability to colonize a host [6,11]. We therefore tested the growth of all Group A mutants and Group B mutants in complex growth medium (BHI) as well as modified M9 medium containing a variety of different carbon sources (glucose, mannose, lactose, cellobiose, fructose, galactose, maltose) to gain insight on growth behavior.

Figure 6.4 shows the average exponential phase growth rate across all 15 Group A mutants and all 14 Group B mutants as well as the wild type *E. faecium* 6E6. Starred values

indicate the average growth rate of that group to be statistically different from the wild type 6E6 with $p < 0.05$ based on a two-tailed Student's t-test. Population averages are the averages of the growth rates of all mutants in the specified group. Each mutant was grown in triplicate and the individual mutant growth rates were calculated by averaging the biological triplicates. Individual growth rates are shown in Figure 6.5.

As previously seen in the resistance level studies, the Group A mutants showed more heterogeneous growth overall compared to the Group B mutants. Additionally, the Group B mutants showed significantly higher growth rates compared to the wild type 6E6 on six out of the eight media tested. Interestingly, one trend was extremely consistent across all mutants in both populations. All 29 mutants tested showed reduced growth in mannose compared to the wild type 6E6.

Additionally, a positive correlation was found between halo sizes of individual mutants and the mutant's growth rate on mannose. The Spearman's rank correlation coefficient between halo size and mannose growth rate was calculated to be 0.76 for mutants with non-zero halo diameters. The correlation coefficient was 0.61 when mutants with halo diameters of 0 mm were included in the calculation. This different is likely due to the fact that differences in resistance level cannot be distinguished for mutants exhibiting no halo. These results indicate that a higher level of resistance is correlated with poorer mannose utilization. Halo sizes and mannose growth rates are shown for all individual mutants in Figure 6.3.

	BHI	Glucose	Mannose	Lactose	Cellobiose	Fructose	Galactose	Maltose
WT	2.7	4.6	5.3	2.2	1.5	3.6	2.5	1.6
A	4.0*	4.7	1.3*	1.5*	1.8	3.6	3.1*	1.7
B	6.1*	5.3*	0.9*	2.1	2.3*	5.1*	3.4*	2.2

Figure 6.4. Comparison of Mutant and Wild Type 6E6 Growth Rates on Different Carbon Sources: The reported growth rates are in mOD600/min and were calculated from the exponential phase of the growth curves. The values are the average growth rates across all mutants in the reported groups. Starred values indicate the average growth rate of that group to be statistically different from the wild type *E. faecium* 6E6 with $p < 0.05$ based on a two-tailed Student's t-test. Color gradient: High values shown in green to low values shown in red.

	BHI	Glucose	Mannose	Lactose	Cellobiose	Fructose	Galactose	Maltose
WT	2.7 ± 0.2	4.6 ± 0.1	5.3 ± 0.1	2.2 ± 0.2	1.5 ± 0.2	3.6 ± 0.1	2.5 ± 0.2	1.6 ± 0.5
A1	2.9 ± 0.3	4.9 ± 0.1	3.3 ± 0.0	2.2 ± 0.0	2.3 ± 0.0	4.3 ± 0.2	3.3 ± 0.3	2.2 ± 0.2
A2	6.1 ± 0.1	4.4 ± 0.0	1.3 ± 0.0	2.0 ± 0.0	1.7 ± 0.0	4.0 ± 0.2	3.4 ± 0.1	2.0 ± 0.1
A3	6.3 ± 0.1	4.5 ± 0.2	0.7 ± 0.0	1.4 ± 0.2	2.2 ± 0.1	4.4 ± 0.1	3.1 ± 0.2	1.7 ± 0.3
A4	6.3 ± 0.1	5.3 ± 0.2	0.7 ± 0.0	1.7 ± 0.2	2.4 ± 0.1	5.0 ± 0.1	3.5 ± 0.1	2.1 ± 0.2
A5	6.2 ± 0.1	5.1 ± 0.1	0.7 ± 0.0	1.8 ± 0.1	2.5 ± 0.0	4.9 ± 0.1	3.4 ± 0.0	2.2 ± 0.3
A6	2.7 ± 0.3	4.1 ± 0.3	0.7 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	2.4 ± 0.1	0.1 ± 0.1
A7	2.1 ± 0.3	3.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.3	2.5 ± 0.0	-0.1 ± 0.4
A8	3.5 ± 0.6	5.4 ± 0.1	1.4 ± 0.0	2.0 ± 0.1	2.2 ± 0.1	5.1 ± 0.1	3.8 ± 0.4	2.5 ± 0.1
A9	2.4 ± 0.0	3.8 ± 0.2	0.9 ± 0.3	0.6 ± 0.2	0.9 ± 0.2	0.4 ± 0.1	2.4 ± 0.4	1.0 ± 0.5
A10	3.2 ± 0.1	4.9 ± 0.2	0.8 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	1.4 ± 0.7	0.1 ± 0.0
A11	2.7 ± 0.5	5.4 ± 0.1	3.3 ± 0.2	2.5 ± 0.1	2.2 ± 0.1	5.0 ± 0.1	3.3 ± 0.3	2.3 ± 0.4
A12	3.9 ± 0.4	4.9 ± 0.1	0.9 ± 0.0	2.2 ± 0.3	2.0 ± 0.1	4.8 ± 0.1	3.6 ± 0.3	2.4 ± 0.3
A13	2.5 ± 0.2	4.9 ± 0.2	3.3 ± 0.1	2.5 ± 0.1	2.4 ± 0.0	4.9 ± 0.1	3.5 ± 0.1	2.6 ± 0.2
A14	3.9 ± 0.3	4.8 ± 0.0	0.8 ± 0.0	2.0 ± 0.1	2.5 ± 0.0	4.9 ± 0.0	3.3 ± 0.1	2.3 ± 0.4
A15	4.9 ± 0.2	4.9 ± 0.2	0.8 ± 0.0	1.9 ± 0.1	2.5 ± 0.1	5.0 ± 0.1	3.2 ± 0.2	2.5 ± 0.6
B1	6.6 ± 0.7	5.4 ± 0.1	1.0 ± 0.3	2.2 ± 0.3	2.4 ± 0.1	5.0 ± 0.1	3.4 ± 0.1	2.2 ± 0.3
B2	6.2 ± 0.5	5.2 ± 0.0	1.1 ± 0.2	2.1 ± 0.2	2.4 ± 0.1	5.0 ± 0.0	3.3 ± 0.1	2.3 ± 0.4
B3	5.4 ± 1.0	5.3 ± 0.1	1.2 ± 0.2	2.3 ± 0.0	2.3 ± 0.1	5.0 ± 0.1	3.6 ± 0.4	2.3 ± 0.3
B4	6.6 ± 0.1	5.3 ± 0.1	0.9 ± 0.1	1.9 ± 0.2	2.3 ± 0.0	5.1 ± 0.1	3.4 ± 0.1	2.0 ± 0.3
B5	6.1 ± 0.2	5.4 ± 0.1	1.0 ± 0.4	2.0 ± 0.1	2.4 ± 0.1	5.1 ± 0.0	3.2 ± 0.2	2.5 ± 0.1
B6	3.8 ± 0.5	5.2 ± 0.1	0.9 ± 0.2	2.3 ± 0.0	2.5 ± 0.1	5.1 ± 0.1	3.8 ± 0.2	2.9 ± 0.5
B7	6.0 ± 0.5	5.3 ± 0.1	0.9 ± 0.3	2.2 ± 0.1	2.4 ± 0.0	5.0 ± 0.1	3.8 ± 0.4	2.8 ± 0.3
B8	6.6 ± 0.3	5.3 ± 0.1	0.7 ± 0.0	1.9 ± 0.2	2.2 ± 0.2	5.0 ± 0.1	3.1 ± 0.2	2.3 ± 0.2
B9	6.6 ± 0.3	5.4 ± 0.0	1.0 ± 0.1	2.5 ± 0.1	1.8 ± 0.0	5.0 ± 0.0	3.4 ± 0.1	2.0 ± 0.1
B10	6.2 ± 0.4	5.4 ± 0.1	0.8 ± 0.1	2.4 ± 0.2	2.4 ± 0.0	5.1 ± 0.0	3.5 ± 0.3	2.0 ± 0.2
B11	6.3 ± 0.3	5.3 ± 0.0	0.8 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	5.0 ± 0.0	3.5 ± 0.2	2.2 ± 0.3
B12	5.9 ± 0.4	5.3 ± 0.1	0.9 ± 0.1	2.0 ± 0.4	2.4 ± 0.1	5.0 ± 0.1	3.4 ± 0.1	1.8 ± 0.4
B13	6.9 ± 0.2	5.6 ± 0.0	0.8 ± 0.0	2.0 ± 0.0	2.4 ± 0.1	5.2 ± 0.1	3.0 ± 0.2	1.8 ± 0.2
B14	6.4 ± 0.3	5.5 ± 0.1	0.8 ± 0.1	2.1 ± 0.2	2.4 ± 0.0	5.2 ± 0.0	3.0 ± 0.2	1.6 ± 0.0

Figure 6.5. Individual Mutant Growth Rates on Different Carbon Sources: The reported growth rates are in mOD600/min and were calculated from the exponential phase of the growth curves. The values are the average growth rates across biological triplicates ± standard deviation. Color gradient: High values shown in green to low values shown in red.

6.2.4 Genome Sequencing of Resistant Mutants

Whole genome sequencing was done to enable unbiased identification of mutations potentially responsible for *E. faecium*'s resistance to class IIa bacteriocins. For this study, the wild type 6E6, one Group A mutant (A2) and one Group B mutant (B1) were selected for genome sequencing. The genomes were sequenced using PacBio sequencing then polished with Illumina.

The annotated wild type *E. faecium* 6E6 genome is reported on NCBI under genebank accession number GDSub30087. The total genome was found to be approximately 3.1 Mb and contained the core genome (2.97 Mb) along with a 382 kb plasmid and a 49 kb plasmid.

Comparison of the mutants with the wild type showed no major genomic rearrangements or epigenetic base modifications. Breseq alignment of the mutant Illumina reads against the wild type genome did however yield two mutations that resulted in changes to two proteins. These mutations were verified using Sanger sequencing. The Group A mutant exhibited a single point mutation in the ORF encoding a σ_{54} factor. This mutation resulted in the substitution of a valine for an aspartic acid at position 29. The Group B mutant did not show this same mutation; instead it had a short repeated fragment that introduced a frame shift in what appears to be a σ_{54} activator protein (referred to as ManR) upstream of a ManPTS operon. These mutations are depicted in Figure 6.6. No other mutations were definitively identified in either mutant.

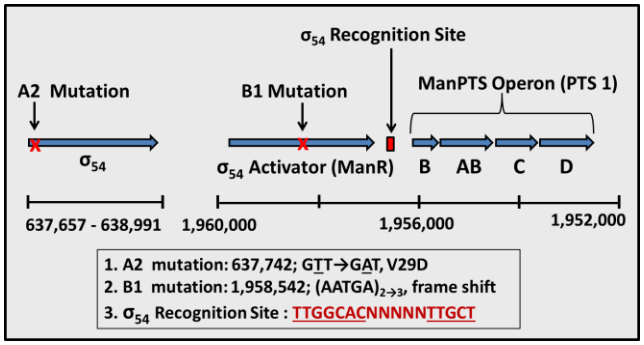

Figure 6.6. Mutations Identified in Spontaneous and Evolved Resistant Mutants: Mutations identified in the Group A mutant (A2) and Group B mutant (B1). The A2 mutation was found in the σ54 factor and B1 mutation was found in a σ54 activator protein upstream of a ManPTS. Numbers below genes indicate location in the genome. A σ54 recognition site was also found upstream of the ManPTS indicating its probable regulation by σ54 and the activator protein. Mutation results imply the involvement a σ54 controlled operon in bacteriocin resistance.

To identify other genes putatively under the control of σ_{54}, and therefore potentially responsible for resistance, the genome was searched for σ_{54} recognition sites containing the consensus sequence TTGGCACNNNNNTTGCT [7]. Five consensus sequences were found throughout the genome. Interestingly, all five of these regions were less than 150 base pairs upstream of operons encoding sugar transporter systems. One of these sugar transporter systems identified in the search was that depicted in Figure 5a which encodes for a ManPTS (PTS

1). The other putative σ_{54}-controlled PTS systems (PTS 2 - 5) are shown in Figure 6.7. As shown in the figures, three of the five recognition sites were found just downstream of σ_{54} activator proteins, providing further evidence for the control of these by the σ_{54} factor.

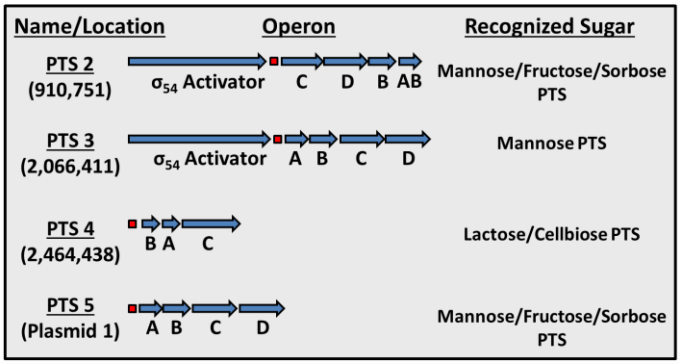

Figure 6.7. Other Coding Regions in *E. faecium* 6E6 Putatively Controlled by σ54: Other coding regions found within the *E. faecium* 6E6 genome immediately downstream of σ54 recognition site TTGGCACNNNNNTTGCT. Hypothesized sugar recognition and component labels (AB, A, B, C, D) are derived from homology to other annotated PTS systems reported in NCBI.

6.2.5 qRT-PCR of Sigma 54 PTS systems

We performed qRT-PCR on the genome-sequenced A2 as well as four additional resistant mutants (A4, A6, A11, A12) exhibiting a range of growth and resistance characteristics to determine if any of the σ_{54}-controlled genes were consistently down-regulated across the resistant mutants. For this study, only Group A mutants were used, in order to avoid introducing additional variables when comparing results across mutants. A4 was selected because it exhibited comparatively good growth in most media tested and a high level of resistance. A6 was selected because it exhibited a high level of resistance but generally poor growth. A11 was selected because it exhibited a low level of resistance and good growth on mannose compared to the other mutants. Lastly, A12 was selected because it exhibited moderate growth and a high level of resistance.

Figure 6.8 shows the $\log_2$(fold change) in gene expression of proximal gene of each of the five PTS systems described above compared to the wild type 6E6. Each point is the average of three biological replicates and error bars represent ±1 standard deviation across the biological triplicates. Values above zero indicate an increase in gene expression compared to the

wild type 6E6 while values below zero indicate a decrease in expression level. Starred values indicate a fold decrease < 1 with $p < 0.05$ based on a single-tailed Student's t-test. Note, the fold change in expression of PTS 4 component B for mutant 6 was found to be 52 ± 59. This value was truncated to improve visibility of the other data points.

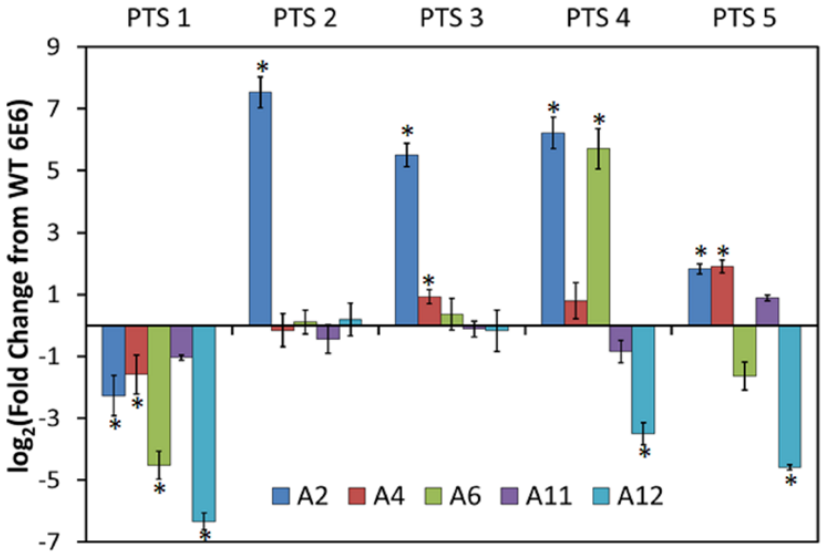

Figure 6.8. qRT-PCR of PTS Systems in Resistant Mutants Compared to the Wild Type 6E6: This graph reports the log2(fold change) in expression of five PTS systems putatively controlled by σ54 in mutants A2, A4, A6, A11, and A12 compared to the *E. faecium* 6E6 wild type. Values reported are the averages across three biological replicates. Error bars represent ±1 standard deviation across the biological triplicates. Values above zero indicate an upregulation of the gene compared to the wild type 6E6 while values below zero indicate downregulation of the gene. Starred values indicate a change in gene expression compared to 6E6wt with $p < 0.05$ based on a two-tailed Student's t-test.

The ManPTS (PTS 1) immediately downstream of ManR was down-regulated in four of the five resistant mutants with $p < 0.05$ and $p < 0.2$ for mutant A11 based on a Student's t-test. No other PTS systems were found to be consistently up or down-regulated across all mutants.

Expression of the three additional genes in PTS 1 (ManAB, ManC, and ManD) were also quantified and found to follow similar expression patterns to ManB across the mutants (Figure 6.9). These results support the statement that these four genes are in fact expressed as an operon. Expression of these genes was decreased in mutant A2, A6, and A12 with $p < 0.05$, $p < 0.1$ for mutant A4, and $p < 0.2$ for mutant A11 based on a Student's t-test. qRT-PCR was also

performed on the σ_{54} factor and ManR and showed the expression of these genes was essentially unchanged in the resistant mutants (Figure 6.10).

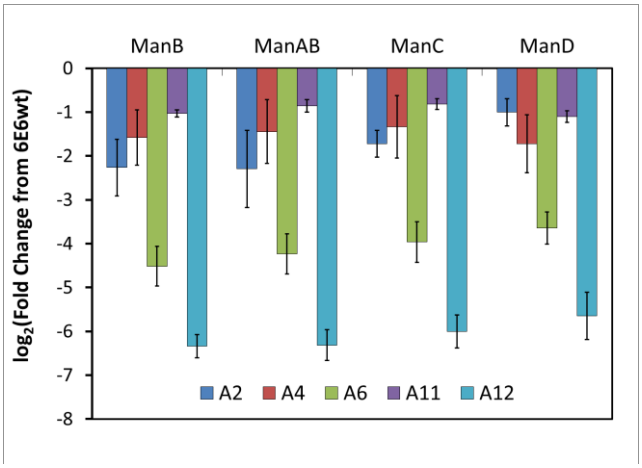

Figure 6.9. qRT-PCR of PTS 1 Operon Genes in Resistant Mutants Compared to the Wild Type 6E6: This graph reports the log2(fold change) in expression ManB, ManAB, ManC, and ManD of PTS 1 in mutants A2, A4, A6, A11, and A12 compared to the wild type *E. faecium* 6E6 (6E6wt). Values reported are the averages across three biological replicates. Error bars represent ±1 standard deviation across the biological triplicates. Values above zero indicate an upregulation of the gene compared to the wild type 6E6 while values below zero indicate downregulation of the gene. Starred values indicate a change in gene expression compared to 6E6wt with $p < 0.05$ based on a two-tailed Student's t-test.

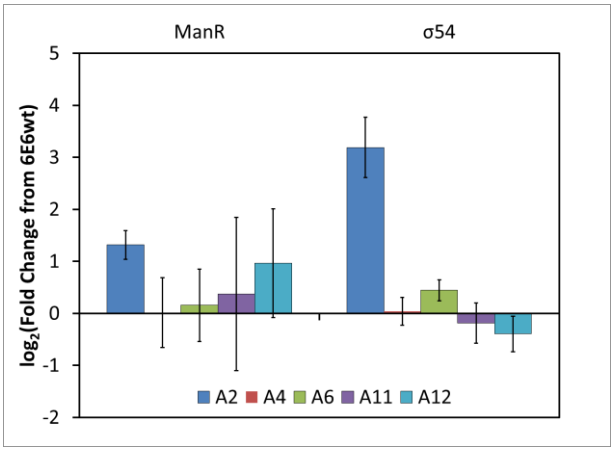

Figure 6.10. qRT-PCR of ManR and σ54 in Resistant Mutants Compared to the Wild Type 6E6: This graph reports the log2(fold change) in expression of the activator protein, ManR, and the σ54 transcription factor in mutants A2, A4, A6, A11, and A12 compared to the wild type *E. faecium* 6E6 (6E6wt). Values reported are the averages across three biological replicates. Error bars represent ±1 standard deviation across the biological triplicates. Values above zero indicate an upregulation of the gene compared to 6E6wt while values below zero indicate downregulation of the gene. Starred values indicate a change in gene expression compared to 6E6wt with $p < 0.05$ based on a two-tailed Student's t-test.

6.2.6 Sequencing of PTS 1 operon, activator protein, and σ_{54} of RT-PCR Mutants

Sanger sequencing was used to identify any mutations within the PTS 1 operon, activator protein, and σ_{54} of A2, A4, A6, A11, and A12. The mutations identified are reported in Table 6.3. This table also includes the $\log_2$(fold change) in PTS 1 expression, halo diameters and growth rates on mannose of the mutants for comparison.

Table 6.3. Mutations found in Group A mutants used in qRT-PCR Tests

Mutant	Mutations in σ_{54}, ManR, ManPTS	ManB Transcription Change from 6E6wt ($\log_2$(Fold Change))	Halo Diameter (mm)	Growth Rate on Mannose
A2	$\sigma_{54,}$ bp 86 substitution (V→D)	-2.27 ± 0.65	7.2 ± 0.6	1.3 ± 0.0
A4	ManC, bp 735 deletion	-1.58 ± 0.63	0.00 ± 0.00	0.73 ± 0.04
A6	None Identified	-4.52 ± 0.45	6.67 ± 0.58	0.71 ± 0.00
A11	ManR, transposon insertion	-1.03 ± 0.09	7.50 ± 0.50	3.30 ± 0.20
A12	$\sigma_{54,}$ bp 385 deletion	-6.34 ± 0.27	0.00 ± 0.00	0.87 ± 0.04

Mutations found by Sanger sequencing of σ54, ManR, and ManPTS (1) genes. RT-PCR results, halo diameters, and growth rates on mannose are provided for comparison. Error represents standard deviations across biological triplicates.

Mutant A4 was found to have a deletion at the end of the ManC gene in the PTS 1 operon. Mutant A11 was found to have a transposon insertion in the middle of the ManR activator protein. Mutant A12 was found to have a deletion in the σ_{54} factor. These results, particularly Mutant A4's direct mutation of ManC, again support the hypothesis of PTS 1's involvement in resistance.

No mutations were found in the $\sigma_{54,}$ ManR, or PTS 1 coding regions of A6 despite the clear decrease in PTS 1 gene expression. These results may be due to a mutation in the PTS 1 promoter of this mutant. Alternatively, there may be other essential regulatory proteins

involved in PTS 1 expression. For example, it has been shown that disruption of either ManR of the putative regulator Lin0142 in *Listeria* abolishes expression of the ManPTS and results in a resistant phenotype [12].

6.2.7 Effect of PTS 1 on Bacterial Susceptibility to Class IIa Bacteriocins

To verify PTS 1's role as a potential target of class IIa bacteriocins in *E. faecium*, we tested the ability of this PTS to confer peptide susceptibility to an otherwise unsusceptible Gram Positive bacteria, *L. lactis* NZ9000. Recall that the PTS 1 operon is comprised of four genes; ManB, ManAB, ManC, and ManD. For these studies, we amplified the following five fragments from the *E. faecium* 6E6 WT genome; ManB/ManAB, ManC/ManD, ManC only, ManD only, and ManB/ManAB/ManC/ManD (whole operon). These fragments were then inserted into a lactococcal expression vector (pNZC) under the control of a chloride-inducible promoter [3] and transformed into *L. lactis*. The activity of AMP-containing supernatant against *L. lactis* containing these different constructs was then compared to determine which, if any, of the fragments successfully conferred susceptibility. The results of these studies are shown in Table 6.4.

Table 6.4 shows that *L. lactis* expressing either ManC/ManD or the entire PTS 1 operon were highly susceptible to the cocktail of class IIa bacteriocins. These results indicate that expression of the PTS 1 operon does in fact result in bacterial susceptibility to the peptides. Additionally, these results show that the expression of both ManC and ManD is necessary to achieve the highest level of susceptibility. Interestingly, expression of ManC alone did result in some susceptibility but to less of an extent than the combination of ManC and D. We also note that in two of the three biological replicates, the supernatant activity against of ManD alone was found to be 2.5 BUs indicating that at higher concentrations of supernatant, ManD alone may also confer some level of susceptibility compared to *L. lactis* expressing no PTS 1 components.

Table 6.4 Class IIa Bacteriocin Susceptibility of *L. lactis* Expressing PTS 1 Components

L. lactis Construct	Supernatant Activity (BUs)
pNZC	< 2.5
pNZC:BA	< 2.5
pNZC:CD	161.2 ± 80.2
pNZC:C	2.9 ± 0.6
pNZC:D	< 2.5
pNZC:BACD	18.8 ± 6.5

L. lactis constructs listed were used to express the following PTS 1 genes *L. lactis*; pNZC, no PTS 1 genes; pNZC:BA, ManB/ManAB; pNZC:CD, ManC/ManD; pNZC:C, ManC; pNZC:D, ManD; pNZC:BACD, ManB/ManAB/ManC/ManD (whole operon). One Bacteriocin Unit (BU) is defined as the reciprocal of the lowest v/v fraction of supernatant required to reduce the growth of the indicator strain by 50%. Activity values are the average of three biological replicates and error represents standard deviation across replicates.

6.3 Discussion

Class IIa bacteriocins may offer a new option for the reduction of *E. faecium*, however, the resistance of this pathogen to these AMPs remains essentially uncharacterized. The goal of this study was to explore the risk and mechanism behind the development of *E. faecium*'s resistance to class IIa bacteriocins.

Herein, we have identified genes related to this *E. faecium* susceptibility to class IIa bacteriocins and provide evidence that their disruption is a common route to resistance in *E. faecium* 6E6. Additionally, we report on the frequency and level of resistance observed, the growth characteristics of the mutants, and some potential impacts of these characteristics in future applications.

The frequency of resistance (FOR) of a pathogen to a particular antibacterial agent is defined as the fraction of cells in a wild type 6E6 culture exhibiting spontaneous resistance to the antibacterial of interest (AMPs in this case). The FOR is a valuable parameter to consider for new antibacterial agents because it can provide insight on the ease with which resistance may

arise in clinical applications of the antibacterial. We evaluated the FORs of four *E. faecium* clinical isolates to a cocktail of three class IIa bacteriocins; Enterocin A, Enterocin P, and Hiracin JM79. Three of the four strains exhibited resistance frequencies on the order of 10-5 and the remaining strain was on the order of 10-4. The FORs observed here are comparable to those exhibited by both *E. faecalis* (10^{-4}–10^{-3}) and *L. monocytogenes* (10^{-6}-10^{-3}) to other class IIa bacteriocins [6,13].

In comparison, traditional antibiotics considered to have low instances of resistance exhibit FORs less than 10^{-9}-10^{-8} [14]. While these values are significantly lower than those observed for class II bacteriocins, it does not render these peptides obsolete. It should be noted that unlike resistance to traditional antibiotics, spontaneous resistance to class IIa bacteriocins appears to be relatively uncommon in nature based on a screen of *L. monocytogenes* susceptibilities to different class IIa bacteriocins [15]. This rarity could possibly result from a decline in the pathogen's fitness in its particular niche upon the development of resistance.

To identify genes involved in *E. faecium* resistance to class IIa bacteriocins, we selected one of the four clinical isolates, *E. faecium* 6E6, sequenced the genomes of two different resistant mutants and compared these to the wild type 6E6. These studies revealed the mutation of a σ_{54} transcription factor in one mutant and the disruption of a σ_{54} associated activator protein, ManR, in the other mutant.

Sigma factors are the components of the RNA polymerase holoenzyme responsible for recognition of a gene's promoter region. In other bacterial species, σ_{54} has been found to control numerous genes, often related to sugar transport and metabolism [16]. As stated previously, σ_{54} has been previously implicated in resistance to class IIa bacteriocins in both *E. faecalis* and *L. monocytogenes* [10,17]. In these studies, it is hypothesized that expression of the "target" ManPTS is controlled by σ_{54} and that resistance is conferred by disruption of σ54 and subsequent downregulation of expression of the ManPTS. Similarly, in our study we found a ManPTS operon was located downstream of ManR and a σ_{54} recognition sequence, TTGGCACNNNNNTTGCT, implying altered PTS expression could be controlled by these mutated proteins.

We use qRT-PCR to show that the ManPTS described above was in fact downregulated in the five resistant mutants tested. Additionally, we found a mutation in ManC of the ManPTS of one of the five mutants and mutations in ManR and σ_{54} in three other mutants indicating

these regions to be potential sources of resistance development. Interestingly, the disruption of ManR in A11 resulted in only a minor decrease in PTS 1 expression and partial resistance. This is in contrast to previous studies in *L. monocytogenes* in which ManR disruption decreased ManPTS expression to undetectable levels [8]. These results may indicate that an alternative σ_{54} associated activator protein exists in *E. faecium* that is capable of controlling PTS 1 expression. Alternatively, it is possible that the IM11 mutation results in a modified, but still semi-functional ManR.

It was also seen that PTS 1 was slightly down-regulated in mutant A4 despite no detectable disruption to its regulatory regions or genes. It has been previously shown in L. innocua that in the absence of glucose, ManAB down-regulates its own PTS expression by phosphorylating and consequently inactivating ManR [18]. It may be possible that the disruption of PTS 1's ability to transport sugar leads to a similar negative feedback response.

In order to prove a direct relationship between the *E. feacium* ManPTS and bacteriocin susceptibility, we expressed the ManPTS genes in *L. lactis* which is naturally not susceptible to the peptides used in this study (data not shown). We found that two genes in the ManPTS operon, ManC and ManD, were sufficient to confer peptide susceptibility in *L. lactis* indicating that they were in fact a target for class IIa bacteriocins. Additionally, we demonstrate that the expression of ManC alone is capable of directing peptide susceptibility though to a lesser extent.

Similar studies have been done in which heterologous expression of the *L. monocytogenes* ManPTS in *L. lactis* was shown to confer susceptibility to Leucocin A, Pediocin A, and Enterocin A [9]. These studies also found that ManC alone was necessary to obtain susceptibility. In another study, point mutations were introduced into ManC of *L. monocytogenes* and shown to alter peptide activity and specificity against the host implying that ManC may act as the target and recognition site of class IIa bacteriocins [19]. The additional activity we observed with the co-expression of ManC and ManD may be due to stabilizing effects between the two proteins as ManC and ManD are known to form a complex in the cell membrane [20].

Collectively, the results described herein indicate that *E. faecium* resistance development can result from either the direct mutation of the target ManPTS or from mutation of genes and regions involved in its expression. Unfortunately, due to difficulties transforming *E. faecium* 6E6, we were unable to perform ManPTS knock-out or complementation studies in *E.*

faecium. Nevertheless, we believe the results presented here to be strong evidence that this ManPTS is involved in peptide susceptibility.

It is important to note that the mechanism of resistance described here may be only one of many potential factors influencing overall level of resistance. For example, alterations in surface composition have been suspected to impact *E. faecium*'s susceptibility to mundticin KS, a class IIa bacteriocin [21]. Additionally, other genes may be involved in ManPTS expression. For example, two other regulators are known to impact ManPTS expression in *L. monocytogenes* [22]. Additionally, this study has used a cocktail of three class IIa bacteriocins. One must therefore consider how the use of individual peptides might impact the observed modes of resistance. Further studies will be required to explore these possibilities.

In addition to determining a mechanism of *E. faecium* resistance to class IIa bacteriocins, we also aimed to characterize the levels of resistance and fitness effects as these qualities play an important role in resistance development. We compared the levels of resistance of 29 *E. faecium* 6E6 mutants based on the diameters of their zones of inhibition. In these studies, 15 of the mutants were isolated by plating an overnight wild-type culture on plates containing supernatant (Group A mutants). The remaining 14 mutants (Group B mutants) were first grown overnight in supernatant then isolated the following morning.

Interestingly, resistance levels across the mutants were highly heterogeneous and a large fraction of both Group A and Group B mutants remained at least partially susceptible to the AMPs. This heterogeneity may be due to the diversity in mutations leading to resistance described above. As a population average, it does appear that the Group B mutants exhibited higher resistance levels compared to the Group A mutants. These results imply that the overnight incubation in SN likely resulted in some selection for higher levels of resistance. This hints that like traditional antibiotics, AMPs should be used sparingly to avoid selection of high-level resistance phenotypes.

As stated above, fitness of the resistant mutants is another important factor impacting both the rise of resistant phenotypes and their stability in a hospital environment. If a resistant mutant is a particularly poor grower, it may be able to dominate in the presence of the antibacterial, but once the drug is removed, it will be outcompeted by other bacteria. Previous work in *E. faecalis*, *L. monocytogenes*, and *L. lactis* shows that the development of class IIa bacteriocin resistance can result in significant changes in both the growth and metabolism of the

pathogen [6,11]. We thus sought to evaluate the fitness of the resistant mutants to the wild type 6E6 by comparing their growth in BHI as well as in minimal media containing a variety of different carbon sources.

While the resistant mutants did not show stunted growth in most media tested, it was found that both Group A and Group B mutants grew significantly slower and to a lower final density than the wild type 6E6 when mannose was provided as the primary carbon source. This result is in strong agreement with the previous hypothesis that the down-regulation of a mannose transporter is common trait across resistant mutants [5]. Additionally, when halo sizes and growth rates on mannose were compared for individual mutants, it was found that halo size was positively correlated with the growth rate on mannose. Based on this information, it would appear that the resistance phenotype is linked to disruption of the cell's ability to utilize mannose.

This proposed mechanism of resistance and this apparent reduction in mutant fitness may impact on *E. faecium*'s ability to colonize a host. In a previous study, it was found that by knocking out another ManPTS (PTS 2 in this study), *E. faecium*'s ability to colonize the GI tracts of mice was significantly impaired [23]. It was hypothesized that knocking out the ManPTS hindered the pathogen's ability to compete for resources. The involvement of PTS 1 in *E. faecium* colonization could be one reason spontaneous resistance to class IIa bacteriocins is rarely observed in nature; that the development of resistance ultimately disables the bacteria from taking hold in its niche. This hypothesis is further supported by the fact that ManPTS genes are most commonly found in bacteria derived from animal hosts indicating that these transporters could play an important role in colonization [24]. If this is the case, it would be of great interest to test the colonization of the resistant mutants and/or PTS 1 knock-outs compared to the wild type 6E6 in an in vivo model.

Analogous arguments could be made for some Gram-negative bacteriocins which are thought to target iron receptors [25]. Numerous studies have shown iron acquisition to be important for gram-negative bacterial infections, meaning the down-regulation of the iron receptor could impair bacterial colonization [26]. If this argument holds, the use of bacteriocins could offer a new treatment option with reduced occurrence of resistance. This is of course speculation and extensive studies would be required to support such a statement. Nevertheless, we find the concept intriguing.

It is important to note that this study only delves into the resistance characteristics of a single strain of *E. faecium*. In the future, it would be valuable to know if these same characteristics of reduced growth on mannose and down-regulation of this ManPTS are common across all *E. faecium* resistant mutants of class IIa bacteriocins. Based on the fact that this mechanism also appears to be present in *L. monocytogenes* and *E. faecalis*, and that the genes for this ManPTS are found in all 13 fully-sequenced *E. faecium* genomes available in NCBI, one might hypothesize that this mechanism is in fact a general one.

In summary, this chapter provides one of the first studies of class IIa bacteriocin resistance in *E. faecium*. We have characterized the frequency of resistance of multiple clinical isolates and showed that mutants are capable of evolving both higher levels of resistance and improved growth rates. Additionally, we show that all resistant mutants of *E. faecium* 6E6 exhibit stunted growth on mannose which could have important effects on the mutated pathogen's ability to colonize a host. Lastly, we provide evidence to suggest the identification of a particular ManPTS involved in *E. faecium*'s resistant phenotype. Ultimately, we have added another piece to the expanding puzzle of class IIa bacteriocin resistance and have begun to build a foundation of knowledge necessary for the application of these peptides against vancomycin-resistant *E. faecium*.

6.4 Materials and Methods

Bacterial Strains and Growth Conditions

Table 6.5. Bacteria Used in this Study

Bacteria Strain	Description	Source
Lactococcus lactis NZ9000	plasmid-free strain, derivative of MG1363; pepN::nisRK, nonbacteriocin producer	Mobitec
L. lactis pNZC	*L. lactis* NZ9000 containing a chloride-inducible expression vector	[3]
L. lactis pNZCA3	*L. lactis* NZ9000 producing Enterocin A, Enterocin P, and Hiracin JM79 under a chloride-inducible promoter	[3]

E. faecium 6E6	Ampicillin/Vancomycin/Linezolid resistant	University Of MN
E. faecium 7A	Ampicillin /Linezolid resistant	University Of MN
E. faecium 8E9	Ampicillin/Vancomycin /Linezolid resistant	University Of MN
E. faecium 9B	Ampicillin/Vancomycin /Linezolid resistant	University Of MN

All *E. faecium* strains are clinical isolates provided to us by Dr. Gary Dunny and Dr. Patricia Ferrieri from the University of Minnesota Medical School. Identification of these isolates was achieved using a combination of biochemical assays and automated identification on the Vitek2 instrument (BioMérieux). All *E. faecium* were grown overnight in BHI medium (ResearchProducts Intl, Mt. Prospect, IL) in a stationary flask or tube from a single colony at 37°C.

L. lactis strains were grown at 30 o C in M17 medium (Oxoid Ltd., Basingstoke, U.K.) supplemented with 0.5% (w/v) glucose (gM17). For production of Enterocin A, Enterocin P, and Hiracin JM79, *L. lactis* pNZCA3 was grown overnight (17-18 hours) at 30 o C in gM17. The following morning, the culture was centrifuged at 7,000 x g for 7 minutes. Supernatant containing the peptides was then filter-sterilized through a 0.22 µm filter (Merk Millipore Ltd., Cork, Ireland) and stored at 4 oC until use.

All strains will be provided upon request.

Frequency of Resistance

Overnight cultures of *E. faecium* 6E6, 7A, 8E9, and 9B were serially diluted in sterile PBS. 10 µL of each dilution was then plated on both gM17+agar plates and gM17+agar plates containing 20% *L. lactis* supernatant (SN). Plates were then incubated overnight at 37 ^{o}C and bacterial colonies were counted at the relevant dilution for all samples. Frequency of resistance was reported as colony forming units (CFU) on SN plate/CFU on gM17. Frequency of resistance was then averaged for the biological triplicates for each strain. The reported error is the standard deviation of the frequency of resistance of the averaged triplicates.

Minimum Inhibitory Concentration Assays

Overnight indicator strain cultures were diluted in the appropriate growth medium by 10^4 to give ~$5x10^4$ CFU/mL cells. 180 µL of the diluted cultures were then combined with 120 µL of the supernatant which had been diluted with *L. lactis* pNZC supernatant to give seven 2x dilutions resulting in concentrations of 40 - 0.625 v/v % for *L. lactis* and *E. faecium* 7A, or 10 - 0.156 v/v % for *E. faecium* 6E6, 8E9, and 9B. The plate was then incubated for 20 hours at 37°C for *E. faecium* or 32°C for *L. lactis* within a Synergy H1 plate reader (BioTek Instruments, Inc., Winooski, VT). Inhibitory activity of the *L. lactis* pNZCA3 supernatant is reported in terms of bacteriocin units (BUs). One bacteriocin unit is defined to be the reciprocal of the lowest volumetric fraction of supernatant that results in 50% inhibition of the indicator strain.

50% inhibition is achieved when the OD_{600} of the indicator treated with supernatant containing bacteriocins is less than half the OD_{600} of the indicator treated with supernatant containing no bacteriocins. These OD_{600} values are obtained at the time when the indicator strain exits exponential growth. This time of measurement was selected so as to be applicable for all indicator strains regardless of differences in growth rates. A summary of the calculations used to calculate supernatant activity in terms of BUs is shown below.

1. Graphically identify time ($t_{measure}$) at which indicator exits exponential growth.
2. Calculate inhibition (I) at $t_{measure}$ for all supernatant fractions tested against a given indicator strain.

$$I = 1 - \frac{OD_{600}(+\text{bacteriocins})}{OD_{600}(-\text{bacteriocins})}$$

3. Determine lowest supernatant fraction resulting in I=0.5.
4. Calculate supernatant fraction for I=0.5 by linear interpolation of inhibition between the two fractions immediately larger and smaller than 0.5. Supernatant activity (BUs) is the reciprocal of this calculated fraction.
5. Repeat for three biological triplicates then calculate averages and standard deviations across triplicates.

Isolation of Resistant Mutants

A schematic of the isolation process is shown in Figure 6.1.

Group A Mutants

A 2 mL culture of *E. faecium* 6E6 was grown overnight. The following morning, 10 µL of the overnight culture was plated on a gM17+ agar plate containing 10% SN by volume. The plate was then incubated overnight at 37 ^{o}C. The following morning, 15 colonies on the selective plate were collected and stored for future use.

Group B Mutants

A 2 mL culture of *E. faecium* 6E6 was grown overnight. The following morning, 2 mL of BHI medium +10% supernatant was inoculated with 10 µL of the overnight culture and incubated for 24 hours at 37 ^{o}C. The following morning, 10 µL of the culture was plated on a gM17 agar plate containing 10 % AMP supernatant by volume. The plate was incubated overnight and the following morning 14 individual colonies were collected and stored for use.

Stab-on Agar Diffusion Assays

Mutants and wild type 6E6 were grown overnight in triplicate. To produce individual plates, 10 mL of unsolidified BHI + agar was transferred to a 15 mL conical tube, inoculated with 5 µL of an overnight culture, and inverted 6 times to ensure proper mixing. The inoculated agar was then poured into a petri dish, allowed to solidify, then stabbed with *L. lactis* pNZCA3. Additionally, 5 µL of eight 2x dilutions of SN were pipetted on all plates and allowed to dry to provide a comparison of strain halo size to MICs from supernatant dilutions. Plates were then incubated overnight at 37 ^{o}C. Following incubation, halo diameters were measured and the highest dilution showing inhibition was recorded.

Minimal Media Growth Tests

Modified M9 medium was produced by supplementing a 500 mL solution of M9 salts with 1.5 g yeast extract (Becton, Dickinson, and Company, Le Pont de Claix, France), 5 g casamino acids (BD and C), 250 µL of 2 M MgSO4, and 25 µL of 2 M CaCl2. The indicated sugar (glucose, fructose, mannose, cellobiose, lactose, or maltose) were then added to a final concentration of 0.4% (wt/v). Three individual cultures of each mutant were grown overnight in 350 µL of BHI at 37°C in a 96 well plate without agitation. The following morning, 30 µL of the

overnight culture was used to inoculate 270 µL of the specified minimal media and growth was monitored on a Synergy H1 Multi Mode plate reader at 37°C without agitation (BioTek Instruments, Inc., Winooski, VT). Average growth rate (mOD600/min) for all mutants was calculated by taking the slope of the linear regions of the growth curves and averaging the rates across biological triplicates. Averages reported in Figure 4 represent the averages across all Group A mutants (15 mutants) and Group B mutants (14 mutants) tested.

Genome Sequencing and Mutation Analysis

Genomic DNA of the wild type 6E6, A2, and B1 were isolated using a DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol with an additional 30 minute initial cell lysis step. Genomic DNA was sequenced using a PacBio RS II sequencer (commissioned to Mayo Clinic Bioinformatics Core, Rochester, MN). Genomes were assembled de novo using the HGAP 3 protocol from the SMRT Analysis v. 2.3 software package [27]. The resulting contigs were trimmed and circularized based on sequence overlap analysis using the Gepard dot plot generator [28]. Assembled genomes were then polished using the PacBio RS Resequencing 1 protocol [27]. DNA modifications were identified using the PacBio RS Modification and Motif Analysis protocol and Gepard was used to search for genomic rearrangements between the wild type 6E6 and mutants [29].

Illumina sequencing was also obtained for the wild type 6E6, A2, and B1 to further polish the genomes and for use in identifying point mutations. Illumina reads were mapped to the consensus genome sequences of their respective genomes using Bowtie2 and then analyzed with Pilon (30) to generate the final polished genome sequences [30,31]. Breseq was then used to identify mutations between the wild type 6E6 and resistant mutants [32]. Sanger sequencing was used to verify the mutations identified.

The assembled genome of the wild type 6E6 is published under NCBI accession number GDSub30087. The raw PacBio reads of the wild type 6E6, A2 mutant, and B1 mutant are published under accession numbers SRR3104404, SRR3104405, and SRR3104409 respectively. The raw Illumina data is also published for all three strains under accession numbers SRR3105311 (WT), SRR3105312 (A2), and SRR3105313 (B1).

qRT-PCR

E. faecium was grown from a single colony in 2 mL of BHI at 37°C until the culture reached an OD600 of ~0.35-0.5 at which point the culture was in mid-log growth phase. At this point, 1.5 mL of the culture was removed and treated with 3 mL of RNAprotect® Bacteria Reagent (Qiagen, Inc., Valencia, CA). Cells were pelleted via centrifugation and the pellet was stored at -80 ^{o}C until further processing. Biological triplicates were collected for the wild type 6E6 and mutants.

RNA was isolated from cell pellets using a protocol similar to that previously described (32) but with a 10 minute-longer incubation period. Briefly, cells were lysed by re-suspending the pellet in ~200 µL of lysis buffer (180 µL 30 mg/ml lysozyme in water, 4 µL 5000 U/ml mutanolysin in water, 0.4 µL 1mM EDTA, 2 µL 10 mM Tris, pH=8.0) and incubated at 37°C for 20 minutes. 700 µL of RTL buffer from the Quiagen RNeasy kit supplemented with 7 µL β-mercaptoethanol was then added to the lysed cells and the solution was vortexed. 500 µL of 100% ethanol was then added, mixed by pipetting, then the solution was applied to the RNeasy column and remaining purification was accomplished according to the Quiagen RNeasy Handbook.

Purified RNA was processed using the TURBO DNA-free kit (Ambion, Austin, TX) to eliminate any contaminating genomic DNA. For each sample, 400 ng of pure RNA was then reverse transcribed using the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative PCR was then performed on a Bio-Rad CFX touch thermocycler (Bio-Rad Laboratories, Hercules, CA) using iTaq™ Universal SYBR® Green Supermix (Bio-Rad) with ~ 20 ng cDNA per 20 µL qPCR reaction.

Fold changes were found by calculating the ΔCq of each biological replicate from the average Cq values of three technical replicates. The ΔCqs were then averaged across wild type 6E6 biological triplicates and used to calculate ΔΔCq for each biological triplicate of the mutants compared to the wild type 6E6. ΔΔCq's were then averaged and fold change was calculated as $2^{-\Delta\Delta Cq}$ based on the approximation of ~100% primer efficiency which was verified by kinetic analysis of the qPCR curves using LinRegPCR [33,34]. Changes in gene expression were considered to be significant if the mean of the mutant ΔCq's was found to different from the mean of the wild type 6E6 ΔCq's with $p < 0.05$ based on a two-tailed Student's t-test.

Assembly of pNZC:PTS1 Constructs

The sequences of all primers discussed herein are provided in Table 6.6. The entire PTS 1 region was first PCR amplified from the *E. faecium* WT 6E6 genome using primers ManB_F and ManD_R. All PCRs were done using Q5 DNA polymerase (New England Biolabs, Ipswich, MA). The ManB/ManAB fragment was then amplified using ManB_assembly_F and ManAB_assembly_R. The ManC/CD fragment was amplified using ManC_assembly_F and ManD_assembly_R. The ManC fragment was amplified using ManC_assembly_F and ManC_assembly_R. The ManD fragment was amplified using ManD_assembly_F and ManD_assembly_R. pNZC, a chloride-inducible *Lactococcus* expression vector was digested with restriction enzymes NcoI and SphI (NEB). PCRs and digested backbone were purified using QIAquick PCR purification kits (Quiagen) then assembled using NEBuilder Hifi DNA Assembly (NEB). For the creation of pNZC:BACD, a new ManB/ManAB fragment was amplified using ManB_assembly_F and ManAB2_assembly_R. pNZC:CD was digested with NcoI, then the PCR and digested backbone were purified using QIAquick PCR purification kits (Quiagen) then assembled using NEBuilder Hifi DNA Assembly (NEB). All assemblies were first transformed into electrocompetent *E. coli* MC1061 F' (Lucigen, Middleton, WI). Successful transformants were identified by colony PCR with pNZ8048F/R then verified by Sanger sequencing. Constructs were then transformed into electrocompetent *L. lactis* NZ9000 as previously described with 0.5 w/v % glycine in the growth medium [35].

Table 6.6. Primers Used for the Construction of pNZC:PTS 1 Vectors

Primer Name	Primer Sequence (5'-…-3')
ManB_F	GGAGGTGGTTGTTCACACAG
ManD_R	GATTTGCCCGTAATCTGTCAG
ManB_assembly_F	CAATCATAAATATAAGGAGGTATGCCATGGATGGATATTCGATTGGCACGTATAG
ManAB_assembly_R	CTCTAGAACTAGTGGTACCGCATGCTTACGCTTTTGCTAATTCAGCTTTTG

ManC_assembly_F	CAATCATAAATATAAGGAGGTATGCCATGGCATGTCTATTATTTCAATAATTTTAGTCGT
ManD_assembly_R	CTCTAGAACTAGTGGTACCGCATGCTTACGCTTTTGCTAATTCAGCTTTTG
ManC_assembly_R	CTCTAGAACTAGTGGTACCGCATGCTTAATAGTCATTCAAAATGTCGCC
ManD_assembly_F	CAATCATAAATATAAGGAGGTATGCCATGGATGGCAGAAGAAAAAATCAAATTATCAA
pNZ8048_F	GCCCCGTTAGTTGAAGAAGG
pNZ8048_R	CAATTGAACGTTTCAAGCCTTGG
ManAB2_assembly_R	CAACTAATACGACTAAAATTATTGAAATAATAGACATG

Chapter 7: Summary and Concluding Remarks

Each year over 2 million antibiotic resistant infections are reported in the United States, over 23,000 of which results in death [1]. While the prevalence of antibiotic resistant infections has been on the rise for multiple decades, only recently have we recognized the severity of the threat. There is now a strong, global incentive to both reduce the overuse of antibiotics as well as to develop new drugs targeting pathogens for which few treatments remain.

Antimicrobial peptides (AMPs) offer a vast reservoir of unexplored antimicrobial activity but remain largely unusable for internal infections because of high production costs and poor bioavailability. The gastrointestinal tract (GI) is a common origin of bacterial infections but remains unreachable for most AMPs via oral or intravenous administration. We propose to overcome this AMP delivery challenge by engineering probiotic bacteria that can survive passage though the GI tract and produce AMPs targeting the pathogen of interest at the site of infection in the intestines.

In this thesis, we focus on the development of AMP-based probiotics for the elimination of vancomycin-resistant *Enterococcus* (VRE) from the GI tract. VRE is an ubiquitous pathogen in hospital environments where it is found in approximately one in ten patient GI tracts [2]. When a patient is treated with broad-spectrum antibiotics, their native microbiota are depleted and VRE proliferate to fill the newly-available niches. Dense VRE colonization of patients vastly increases the patient's risk of bacteremia and is a known source of pathogen dissemination throughout the hospital environment. Based on this mode of infection, we propose that the use of AMP-based probiotics with specific activity against VRE could offer a means of reducing VRE colonization and expansion. Throughout this thesis, we focus on the development of probiotics targeting the two species responsible for the majority of VRE infections, *E. faecium* and *E. faecalis* [1].

Herein, we have developed two probiotic platforms for the reduction of VRE in the GI tract. In chapter 3, we describe our first probiotic platform which uses the species *Lactococcus lactis*. *L. lactis* is a Gram-positive species of lactic acid bacteria commonly used in the production of fermented dairy products. Due to its long term use in the food industry, *L. lactis* is considered

to be safe for consumption and has now been used in numerous studies (including phase I clinical trials) for the delivery of therapeutic proteins to the intestinal mucosa.

In chapter 3 we present a chloride-inducible expression vector for the delivery of AMPs targeting VRE from *L. lactis*. We demonstrate that the chloride-inducible promoter is highly active at typical chloride concentrations found within the GI tract. We then show the efficacy of this expression system in producing three class IIa bacteriocins (Enterocin A, Enterocin P, and Hiracin JM79) to inhibit several strains of *E. faecium* and *E. faecalis* in laboratory cultures. This study provided promising evidence for efficacy of using a chloride-inducible *L. lactis* AMP expression system for the reduction of VRE in vitro. This study was published in the peer-reviewed journal, Applied and Environmental Microbiology [3].

In chapter 4 we develop two VRE colonization models in mice with the aim of using these models to test the efficacy of our probiotics in reducing VRE in a GI tract environment. In the first model, mice are colonized with *E. faecium* JL282 or JL277 in the absence of antibiotic treatment. This model is intended to preserve the native microbiome. In the second model, mice are colonized with a vancomycin-resistant clinical isolate, *E. faecium* 8E9. In this model, *E. faecium* colonization was assisted by the concurrent administration of vancomycin during the infection period and was intended to more closely mimic conditions typical of VRE expansion in humans.

We then use the two models to test the ability of the *L. lactis* system from chapter 3 to reduce VRE fecal carriage in mice. In four of the five trials, *Enterococcus* carriage was decreased in mice treated with *L. lactis* producing enterocins compared to the untreated group. These studies provided an initial proof of concept for *L. lactis* as an AMP delivery organism, however, future improvements will be necessary to improve the extent and rate of VRE reduction. We are considering numerous strategies, including evolving more pronounced bile resistance in lactis, engineering stronger expression and secretion constructs, and testing polymeric coatings of lactis that will enhance its viability in the gut.

Chapter 5 introduces the second probiotic platform, which uses the probiotic species *E. coli* Nissle 1917 (EcN). EcN was selected for our application because it has a long history of use as a commercially-available probiotic and is compatible with the myriad of genetic tools and

techniques developed for lab strains of *E. coli*. In this chapter, we describe the challenge of secreting heterologous AMPs from *E. coli* and propose the use of the Microcin V secretion machinery as a general AMP transporter.

We then test the versatility of the Microcin V transporter using an AMP expression vector that includes the Microcin V transporter as well as a powerful synthetic promoter (ProTeOn+) to drive AMP expression. We refer to this vector as pMPES for "Modular Peptide Expression System." We then demonstrate pMPES's ability to produce and secrete seven different class II bacteriocins derived from a variety of producer species. The results from these studies are published in the peer reviewed journal Pharmaceuticals [4]. These initial studies showed promise for the use of the Microcin V transporter for the secretion of AMPs from EcN, however, production needed to be improved to yield and effective therapeutic.

In the second part of chapter 5 we introduce several improvements to pMPES to increase peptide production and to eliminate unnecessary plasmid components. These changes ultimately result in a new modular expression vector, pMPES2. We then show that the alterations made to create pMPES2 yielded over a 20x improvement in EcN activity against *E. faecium* in laboratory cultures. pMPES2 was then used to simultaneously express the three anti-enterococcal peptides, Enterocin B, Hiracin JM79, and Enterocin A to further improve EcN activity over the single peptide systems.

Using the antibiotic-assisted colonization model in chapter 4, we show that VRE carriage in mice treated with EcN expressing the three enterocins is significantly lower than VRE carriage in both untreated mice ($p = 0.011$) and mice treated with EcN expressing no AMPs ($p = 0.098$). Though these trials must be repeated, these results are extremely promising and demonstrate the potential for using EcN as an AMP delivery organism.

The development of bacterial resistance is a key concern in the development of any new antimicrobial technology. Class IIa bacteriocins are among the largest class of AMPs with specific activity against VRE and were used in both the *L. lactis* and EcN probiotic platforms. Thus, in the final study of this thesis, we examine the development of *E. faecium* 6E6 resistance to a cocktail of three class IIa bacteriocins; Enterocin A, Enterocin P, and Hiracin JM79.

In this chapter, we discuss whole genome-sequencing on two resistant isolates and identify mutations in a σ_{54} transcription factor and a σ_{54}-associated activator protein, ManR. We then identify what appears to be a σ_{54}-controlled mannose phosphotransferase system (ManPTS) which we show to be down-regulated in all five mutants tested suggesting a role of this ManPTS in resistance development. Further sequencing revealed that four of the five mutants had mutations in either σ_{54}, ManR, or ManC of the ManPTS. The ManPTS's link to bacteriocin activity was confirmed by heterologously expressing this transporter in *L. lactis* and showing that it resulted in susceptibility of the otherwise unaffected strain. This work is currently under review for publication in the peer-reviewed journal Antimicrobial Agents and Chemotherapy.

In combination with previous literature on *E. faecalis* and *Listeria* resistance to class IIa bacteriocins, the results our study described inchapter 4 imply this the ManPTS may act as an external receptor of class IIa bacteriocins and that resistance is frequently achieved via downregulation or direct mutation of this protein target. The bacterial target and mechanism of resistance elucidated herein may prove useful in the rational design and selection of future AMPs. Additionally, this mechanism may have major implications regarding pathogen fitness in the host GI tract as it was found that all resistant mutants exhibited impaired growth on mannose. However, further studies would need to be done to evaluate the effects of these mutations on colonization and pathogenicity.

Collectively this thesis provides a strong foundation for the development of AMP-based probiotic bacteria for the reduction of VRE in the GI tract. Importantly, the tools created herein can be applied to the development of systems targeting numerous different pathogens. We do however recognize the very important questions of potential risks and health hazards from GMO consumption. To further this technology, we must address the concerns regarding risks to patients, health care providers and the environment.

In the future, we will consider numerous biocontainment strategies to address the concern of microbial and genetic transfer to the environment. One promising approach is to use the auxotroph transgene containment method and replace an essential gene involved in the survival of the probiotics. A comprehensive approach for tighter control and to minimize probiotic

survival in the environment as well as DNA transfer risk is the GeneGuard strategy. In this approach plasmids are engineered with conditional origins of replication, rich-media compatible auxotrophies, and toxin–antitoxin pairs [5]. In future experiments we may also perform additional studies to evaluate peptide toxicity in mammalian guts, probiotic strain survival and release in feces, and the transfer of DNA to other microbes.

Over the past decade, we have experienced an increasing number of untreatable bacterial infections raising the fear that we may soon enter what some refer to as a "post-antibiotic era." There is now a pressing need for new antibiotic options for the treatment of these and future pathogens. Though much work remains to be done, the technology explored in this thesis offers a promising alternative antibiotic therapy for the treatment of GI tract-derived diseases. With further improvements to efficacy and control, we hope that one day AMP-based probiotics may open an entirely new avenue of antibacterial treatment options.

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