Bacteriocin Prevalence and Resistance in a Clinical *E. faecium* Cohort: Rise of the Class IIa Bacteriocin Bac43

by

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Dedication

This dissertation is dedicated to my family and all the people who have supported me on this journey.

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Abstract

Vancomycin resistant enterococcus (VRE) is a healthcare associated pathogen that presents a serious public health risk. To control transmission, we must first understand the mechanisms by which VRE comes to colonize and dominate the gastrointestinal tract. Bacteriocins are antimicrobial toxins that can be utilized in bacterial competition. Enterococci compete extensively in the gut and bacteriocins can be used as a weapon in this competition to free up space and resources for the producing cell to proliferate. In this work, we explore bacteriocin presence and resistance in a clinical cohort of *E. faecium* isolates collected over six years from a single hospital system.

In Chapter 2, we utilize computational and phenotypic methods to identify bacteriocin genes and assess functionality. We identified two bacteriocins, enterocin A and bac43, with high prevalence. While the structural gene for enterocin A was found in 98% of isolates, the rest of the operon required for production was subject to numerous variations resulting in only six phenotypically functional isolates out of 2428. Bac43 was highly associated with emerging MLSTs 117 and 80 and increased from 8% to 91% presence over the six-year collection period. The genes encoding bac43 were also highly conserved and conferred phenotypic functionality. These results implicate bac43 as a potentially relevant factor in *E. faecium* transmission.

In Chapter 3, we explore bac43 resistance *in vivo* using our *E. faecium* clinical cohort. Bac43 targets the mannose phosphotransferase system (manPTS) for insertion and pore formation in the cell membrane, ultimately resulting in cell death. While mutations to the manPTS can confer

resistance, manPTS has also been implicated as an important factor in *E. faecium* colonization. We applied three methods including a genomic analysis of the entire cohort, a phenotypic screen of a representative subsample of isolates, and an inquiry of isolates with potentially disruptive mutations in the manPTS. These investigations revealed manPTS is largely conserved and isolates with high-level bac43 resistance are rarely identified, suggesting these isolates are selected against *in vivo*. However, we did identify a small subset of *E. lactis* (formerly known as Clade B *E. faecium*) with four times greater resistance to bac43 than sensitive isolates. These data support the theory that mutation of the manPTS resulting in resistance is unfavorable *in vivo*, but strains can evolve to have an intermediate level of resistance without a detrimental impact fitness.

In Chapter 4, we generate *E. faecium* fluorescent reporter strains carrying the genes for bac43 production and sought to design a system for studying competition between bacteriocin-producing and non-producing strains using the model organism *C. elegans*. While strain movement could be tracked though the *C. elegans* gut, strains rapidly transversed through the gut making this method unfavorable for analyzing the influence of bacteriocin production. However, our generation of *E. faecium* fluorescent reporters with and without functional bac43 genes will serve as valuable resources to visualize competitive interactions in alternative models.

Here we document the rapid rise of bac43 in a cohort of 2428 clinical *E. faecium* isolates collected over six years from a single hospital system. These works support bac43 as a possible contributing factor to *E. faecium* gut colonization and is an important area for future work. Additionally, we advance our current knowledge on bacteriocin resistance and provide useful tools to continue investigating the role of bacteriocins in clinical settings.

Chapter 1 Introduction

1.1 *Enterococcus faecium*

Enterococcus faecium is a Gram-positive opportunistic pathogen.[1] While *E. faecium* and other enterococci are common members of the human gastrointestinal tract, *E. faecium* has the potential to cause serious infections particularly in healthcare environments.[1,2] *E. faecium* is an intrinsically resilient and hardy organism with a highly plastic genome prone to the acquisition various genetic elements that can confer resistance to antibiotics.[3,4] In the 1980s, strains began to emerge carrying resistance to the antibiotic vancomycin.[5] Vancomycin resistant enterococcus (VRE), which refers mainly to *E. faecium* and *E. faecalis*, spread globally and is currently classified as a serious public health threat by the Centers for Disease Control, resulting in an estimated 54,000 infections and 539 million dollars in health care costs annually.[6,7]

Enterococcal infections tend to originate with intestinal colonization from which the bacteria can then spread to other parts of the body including the blood, heart, and urinary tract.[8,9] In healthcare environments there are several factors increasing susceptibility to intestinal colonization by VRE including prolonged treatment with antibiotics, receiving an organ transplant, presence of an indwelling device, and admittance to intensive care units.[9–13] These factors can increase exposure to VRE as well as cause perturbations to the protective gut microbiota, allowing VRE an opportunity to expand and dominate the gastrointestinal tract.[14–17] Studies have shown colonization of the gastrointestinal tract puts patients at a higher risk for developing blood stream infections.[12,13,17,18] Colonized patients also represent a major route of exposure for other patients and healthcare workers, and for environmental contamination.[19]

Preventing colonization and controlling transmission of VRE are important areas of intervention to reduce the spread of VRE. This has included measures such as hand hygiene, contact precautions, surveillance screening, and antibiotic stewardship.[9,20] However, VRE is already endemic in many healthcare settings and expanding resistance profiles make VRE infections difficult to treat. VRE remains an ongoing threat that requires vigilant control measures.

1.1.1 Species classification

E. faecium is a diverse species found widely in nature, from being present in different foods for fermentation as well as a commensal member of the mammalian microbiome.[21] With the rise of *E. faecium* as a nosocomial pathogen, it was important to characterize the population that is implicated in human disease.[22] Historically, the *E. faecium* population has been divided into two main clades: clade A, hospital-associated, and clade B, community-associated.[23] The hospital associated clade A is further split into clade A1 comprising mostly strains responsible for infections in hospitals, and clade A2 representing isolates more associated with sporadic infections in animals and humans in the community. Clade B refers primarily to non-clinical, commensal isolates found from humans in the community, but these isolates have also been implicated in human disease. Clade A strains differ from clade B by their tendency to carry various virulence factors as well as high-level resistance to aminoglycosides, ampicillin and vancomycin.[24,25]

Enterococcus lactis was initially isolated from raw milk cheese and, while closely related to *E. faecium*, was characterized as novel species based primarily on differences in 16S rRNA sequences.[23,26,27] However, advancements in whole genome sequencing have allowed for a re-evaluation of this classification.[28] Belloso Daza et al. identified genomic clustering of *E. lactis* and clade B *E. faecium* isolates that indicated they were the same species.[23] As a result, clade B

community associated *E. faecium* strains have recently begun to be reclassified as *E. lactis*. Clinical microbiology labs, however, rarely make this distinction between *E. lactis* and *E. faecium*.

1.2 Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides that when released extracellularly by producer cells can inhibit or kill sensitive cells in the surrounding environment.[29] As a result, bacteriocins can play a role in bacterial competition in polymicrobial environments and influence population structures.[30,31] Enterococci, many of which produce bacteriocins,[32] compete extensively in the gut environment.[33,34] Bacteriocin production has been hypothesized to play a role in pathogen transmission and intestinal domination, but this is almost entirely unexplored in a clinical environment. We therefore sought to study the influence of bacteriocin production in clinical *E. faecium* isolates.

1.2.1 Ecological theory of bacteriocin production

Bacteria live in dense, diverse communities where they must compete for resources. Bacteria have therefore developed phenotypic weapons to provide an advantage in microbial competition, one of these weapons being bacteriocin production.[31,35,36] Bacteriocins can provide a significant strategic advantage by eliminating competitors in the surrounding environment allowing the producer access to space and resources for proliferation.[31] However, this advantage is not without a cost as resources and energy must be diverted for bacteriocin production and immunity against its own toxin.[31] In some contexts, cells that have developed resistance to the bacteriocin represent a cheater phenotype where they incur the, theoretically, lesser cost of resistance while profiting off the greater benefits of nearby bacteriocin producers.[37,38]

Chao and Levin demonstrated in an *Escherichia coli* model of colin E3 producer and sensitive strains that producers will have the advantage only in spatially structured environments, or if they are sufficiently common in well-mixed environments.[39] When producers can locally utilize the resources made available by killing their sensitive counter parts, the benefit of production outweighs the cost. In a well-mixed environment like a flask, however, producers have a greater barrier to overcome as the resources and space made available by killing competitors would be widely dispersed rather than locally accessible.[38,39] Producers must therefore be present in sufficiently high initial density to overtake the sensitive cells that are not incurring the costs of bacteriocin production.

Besides producers and sensitives, there is also a third player to consider of resistant mutants. Resistant cells could result through mutations, or by encoding a gene for bacteriocin immunity without the associated gene for production. Theoretically, these players could reap the benefits of the producers without the same costs.[31,37] We can begin to model the interactions and fitness between bacteriocin producer, sensitive, and resistant players as a rock-paper-scissors dynamic. Resistant cells can have an intermediate cost, being greater than sensitive cells but less than producers. In this model, sensitive cells will outgrow resistant cells, resistant cells will outcompete producers, and producers will kill the sensitives.[38] These interactions have been demonstrated in *E. coli in vitro*[40] and *in vivo.*[41] In the well-mixed environment of a flask, resistant cells quickly dominate the culture.[38] However, when interactions are localized in a spatially structured environment, populations of all three players were able remain.[38] This *E. coli* experimental model supports the idea that resistant mutants help maintain diversity while the population is in an equilibrium point, preventing the surge of a single player.

1.2.2 Experimental validation of bacteriocins used for competition

The strongest support for the role of bacteriocin production, in the gut specifically, comes from Kommenni et al. In a mouse model of enterococcal gut colonization, it was shown that an *E. faecalis* strain carrying a plasmid encoded bacteriocin, bacteriocin 21 (bac-21), was able to outcompete *E. faecalis* strains lacking the bacteriocin.[42] These bac-21 producing strains were able to directly kill their competitors, and the plasmid was able to maintain its own survival through conjugation within the gut.[42] Through killing and conjugation, bacteriocin producers were able to establish and maintain long-term gut colonization. This study supports how bacteriocins can be used to displace competitors within the gut environment.

1.2.3 Bacteriocins used for competition in natural systems

A few studies have implicated bacteriocins as factors conferring a competitive advantage in natural environments. Within the nasal microbiome benign *Dolosigranulum pigrum* was found to be present when pathogenic species like *Staphylococcus aureus* are absent.[43] *D.pigrum* was shown inhibit *S. aureus in vitro* potentially as a result of encoded bacteriocin genes, implicating bacteriocins as an influencing factor in *D. pigrum*'s interspecies role shaping the nasal microbiome.[43] Bacteriocins encoded by *Streptococcus pneumoniae* have also been implicated in mediating competition within the nasal microbiome.[44] Woodworth et al. studied the use of fecal microbiota transplant (FMT) to protect against intestinal colonization by multi-drug resistant organisms.[45] They found FMT facilitates intra-species competition and the bacteriocins produced by the FMT *E. coli* strains could have aided in the replacement of the non-bacteriocin producing baseline strains.[45] Finally, Wagner et al. demonstrated hospital associated clade A1 and A2 *E. faecium* isolates encoded a significantly higher number of bacteriocins compared to commensal

E. lactis isolates.[46] These human clinical isolates were found to more often inhibit human commensals, and bacteriocin production could be a means by which nosocomial strains displace commensals.[46] Together, these studies demonstrate bacteriocins are commonly present in natural systems and could contribute to a competitive advantage.

1.2.4 Bacteriocin classification

This work focuses mainly on class IIa bacteriocins. While the typing scheme for gram-positive bacteriocins has been debated, at least two classes are widely agreed upon.[47,48] Class I includes posttranslationally modified lantibiotics (lanthionine containing bacteriocins), and class II comprises small (<10 kDa) nonmodified heat-stable bacteriocins. Class II can be further subdivided into IIa (antilisterial pediocin-like bacteriocins), IIb (two-peptide bacteriocins), and IIc (other non-pediocin-like one-peptide bacteriocins). Class IIa bacteriocins are the largest collection of bacteriocins in class II and have been used in food preservation given their targeting of the food-borne pathogen *Listeria monocytogenes*.[29] Class IIa bacteriocins are characterized by their hydrophilic N-terminal region harboring the conserved "pediocin box" sequence YGNGV (Tyr-Gly-Asn-Gly-Val), a variable hydrophilic and/or amphiphilic C-terminal, and a conserved disulfide bond which stabilizes the N-terminal β-sheet-like domain and is crucial for antibacterial activity.[49]

1.2.5 Class IIa bacteriocins: mechanism of action and resistance

Class IIa bacteriocins attack their targets by creating pores within the target cell membranes which leads to dissipation of the proton-motive force and leakage of cellular components, ultimately leading to cell death.[49] Class IIa bacteriocins' N-terminal β-sheet-like domain mediates interaction with the mannose phosphotransferase system in the membrane of target cells while the C-terminal hairpin-like domain causes a conformational change leading to

pore formation (Figure 1.1A).[49,50] Bacteria producing class IIa bacteriocins also produce cognate immunity proteins which protect them from cell death by their own bacteriocin. Data suggests these immunity proteins provide protection through interaction with the intracellular side of the cell membrane and blockage of the pore created by the bacteriocin(Figure 1.1B).[49–53] The exact mechanism of interaction responsible for immunity specificity between different class IIa bacteriocins requires further study.

Class IIa bacteriocins recognize and target the mannose phosphotransferase system (manPTS) on sensitive cells.[54–57] Phosphotransferase systems (PTS) are primarily responsible for mediating the uptake and phosphorylation of carbohydrates but have also been found to be the receptor for some bacteriocins and bacteriophages.[58,59] PTS compromise two cytoplasmic proteins (enzymes I) and a variable number of sugar specific enzyme II complexes.[59] Enzymes II are classified into five phylogenetically distinct superfamilies; the mannose/sorbose/fructose superfamily is referred to as manPTS.[58] ManPTS is a complex of four different protein subunits (EIIA, EIIB, EIIC, and EIID). Subunits EIIA and EIIB are located in the cytoplasm while EIIC and EIID form a membrane bound complex. The EIIC/EIID complex is targeted by class IIa bacteriocins for bacteriocin insertion and pore formation in the bacterial membrane.[54,55,60,61]

Resistance to class IIa bacteriocins like mesentericin Y105,[56,57] lactococcin A,[53,55] and pediocin PA-1[62] has been studied in multiple different species including *Listeria monocytogenes*,[55,56] *Lactococcus lactis*,[53,55] *Enterococcus faecalis*,[57,62] and *Enterococcus faecium*[63]. Through these works, resistance has been primarily tied to mutations in the manPTS subunits themselves and regulation of manPTS by the sigma-54 transcription activator and the sigma-54 associated activator protein (ManR). Resistant mutants have been shown to arise readily *in vitro* under laboratory conditions and has been attributed to downregulation of the

manPTS stemming from alterations to the sigma-54 transcription activator or to the sigma-54 transcription activator, ManR.[54–57,63,64] Resistance has also been associated with changes in the composition of unsaturated fatty acids and phospholipids in the bacterial membrane.[65] Genetic mainputation of the EIIC and EIID subunits confirmed their necessity for bacteriocin sensitivity as mutation of either gene resulted in resistance[61]. While resistance has been extensively studied *in vitro*, the occurance of resistance *in vivo* has yet to be fully understood.

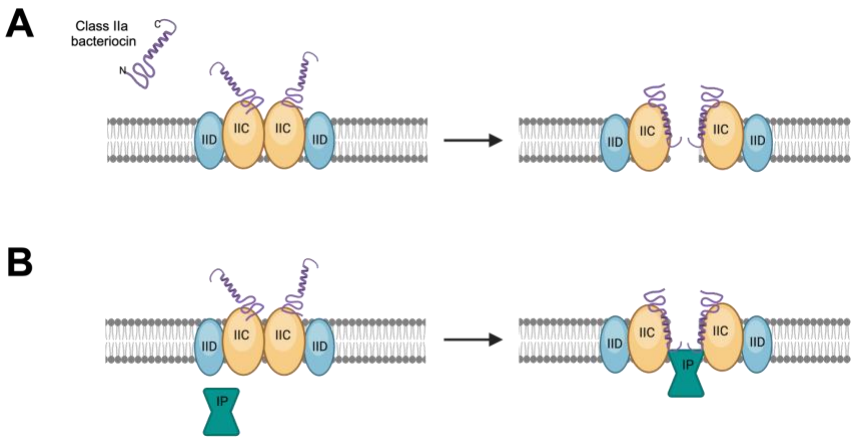


Figure 1.1: Mechanism of class IIa bacteriocin action and immunity. (A) The N-terminal region of the bacteriocin interacts with the manPTS EIIC subunit causing a conformational change in the EIIC/EIID membrane complex creating a pore in the membrane which leads to cell death. (B) The immunity protein (IP) of immune cells blocks the pore created by the bacteriocin by binding to the manPTS. Figure adapted from Kjos et al.[55] and created using BioRender.

1.2.6 Enterocin A and bacteriocin 43 (bac43)

Enterococci produce a wide variety of bacteriocins, known as enterocins.[34,66] Here we focus primarily on two class IIa bacteriocins enterocin A and bacteriocin 43 (bac43). Enterocin A was first identified from a strain of *E. faecium* and is one of the most commonly identified of the class IIa enterocins.[34,66,67] The locus responsible for enterocin A production is encoded on an 9kb region of the chromosome and encodes seven genes.[68] This includes genes encoding structural and immunity proteins (*entA* and *entI*, respectively), an inducer peptide (*entF*), a

histidine kinase and response regulator (*entK* and *entR*, respectively), and an ABC-transporter and accessory protein (*entT* and *entD*, respectively).[68] Enterocin A production is regulated through quorum sensing by a three-component regulatory system consisting of the inducer peptide, histidine kinase, and response reglator.[68,69] The inducer peptide (which induces its own synthesis) is first exported through the bacteriocin ABC transporter. Once the externalized inducer peptide reaches a sufficient concentration, the transmembrane histidine kinase is activated and phosphorylates the response regulator, ultimately triggering transcription of the enterocin A locus.[51,68,69] This process also triggers a positive feedback loop as the response regulator activates the genes encoding the three-component regulatory system.[51]

The class IIa bacteriocin of primary interest in this work is bac43. One identification of bac43 was from a clinical cohort of vancomycin resistant *E. faecium* isolates from the Michigan Medicine hospital system collected between 1994 and 1999.[70] This bacteriocin was found to be active against multiple bacterial species including *E. faecium, E. faecalis, L. monocytogenes, C. botulinum*, and *S. aureus.*[70,71] Bac43 (also known as hiracin JM79[71] and bacteriocin T8[72]) was found to be encoded on a 6.1kb mobilizable plasmid pDT1. This plasmid carries the bac43 structural and immunity genes (*bacA* and *bacB*, respectively) as well as genes for plasmid mobilization and replication (Figure 1.2). Unlike the regulation and export described for enterocin A, the N-terminal of bac43's structural gene contains a leader sequence that directs bac43 to be externalized through the *sec*-dependent pathway.[71] No genes associated with bacteriocin regulation have been identified on this plasmid.

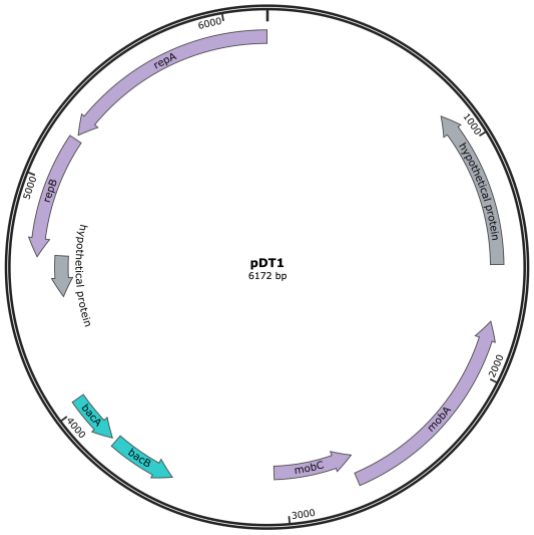


Figure 1.2: Map of bac43 encoding plasmid pDT1. pDT1[70] is a 6.2kb plasmid that encodes genes for bac43 structure and immunity (teal, *bacA* and *bacB*, respectively), plasmid mobilization (lavender, *mobC* and *mobA*), plasmid replication (lavender, *repA* and *repB*), and two hypothetical proteins (gray). Figure created by SnapGene.

1.3 Clinical cohort of *E. faecium* isolates

Between 2016 and 2021, routine surveillance for VRE was performed on nine high risk adult units as part of Michigan Medicine's infection control practice. All patients were screened with rectal swabs on admission and weekly thereafter. Samples were also collected for any patients with *E. faecium* blood stream infections. The cohort utilized throughout this work includes the first VRE faecium positive rectal swab and/or the first positive *E. faecium* blood culture of 2300 patients between 2016 and 2021. This comprises a total of 2428 isolates where 2150 are from perirectal swabs and 278 are from blood cultures. Genomes for each of these isolates were sequenced and assembled. This valuable cohort allows for thorough genotypic and phenotypic analyses of clinical *E. faecium* samples and temporal trends assessments. Of note, clinical microbiology labs rarely distinguish between *E. faecium* and *E. lactis* resulting in some isolates later identified to be *E. lactis* within this cohort.

1.4 Summary

Here we investigate bacteriocin production in a clinical cohort of 2428 *E. faecium* isolates collected over a six-year time period. In Chapter 2, we used genomic methods to identify bacteriocin genes within this cohort, phenotypic assays to test for functionality, and assess trends of bacteriocin presence over time. After identifying the bacteriocin producing population, in Chapter 3 we explore resistance to the most prominent bacteriocin in our cohort bac43. Finally, in Chapter 4 we present a protocol for the generation of fluorescently labeled *E. faecium* bac43 producer, sensitive, and immune strains for use in competition assays.

Chapter 2 Increasing Prevalence of Bacteriocin Carriage in a Six-Year Hospital Cohort of *E. faecium*

This chapter is published work:

Garretto, A, Dawid S, Woods, R. (2024). Increasing prevalence of bacteriocin carriage in a 6-year hospital cohort of *E. faecium.* J Bacteriol. 206(12):e0029424.

2.1 Abstract

Vancomycin-resistant enterococci (VRE) are important pathogens in hospitalized patients; however, the factors involved in VRE colonization of hospitalized patients are not well characterized. Bacteriocins provide a competitive advantage to enterococci in experimental models of colonization, but little is known about bacteriocin content in samples derived from humans and even less is known about their dynamics in the clinical setting. To identify bacteriocins which may be relevant in the transmission of VRE, we present a systematic analysis of bacteriocin content in the genomes of 2,248 patient-derived *E. faecium* isolates collected over a 6-year period from a single hospital system. We used computational methods to broadly search for bacteriocin structural genes and a functional assay to look for phenotypes consistent with bacteriocin expression. We identified homology to 15 different bacteriocins, with 2 having a high presence in this clinical cohort. Bacteriocin 43 (bac43) was found in a total of 58% of isolates, increasing from 8% to 91% presence over the 6-year collection period. There was little genetic variation in the bac43 structural or immunity genes across isolates. The enterocin A structural gene was found in 98% of isolates, but only 0.3% of isolates had an intact enterocin A

gene cluster and displayed a bacteriocin-producing phenotype. This study presents a wide survey of bacteriocins from hospital isolates and identified bac43 as highly conserved, increasing in prevalence, and phenotypically functional. This makes bac43 an interesting target for future investigation for a potential role in *E. faecium* transmission.

2.2 Introduction

Vancomycin-resistant enterococci (VRE) are healthcare-associated pathogens that are transmitted on surfaces and between patients and healthcare workers, resulting in an estimated 54,500 infections and 5,400 deaths each year.[6] Most clinical infection with VRE are caused by either *E. faecalis* of *E. faecium*, with vancomycin-resistant *E. faecium* being distinguished from other common nosocomial pathogens by its increased propensity to transmit in hospital settings.[73] VRE is endemic in many health care settings resulting in opportunities for patients to acquire VRE during their hospital stay.[11,74] When VRE transmits to previously uncolonized patients, particularly to those with perturbations to the gut microbiota by antibiotics, it can dominate in the gastrointestinal community before causing infections.[14,75] The mechanisms that promote this colonization and gut domination by VRE, however, are unclear prompting a need to better understand the underlying biology.

Bacteriocins are ribosomally synthesized antimicrobial proteins and are commonly used by bacteria to provide a competitive advantage in polymicrobial environments.[30,38] Bacteriocins are produced by many lactic acid bacteria, including enterococci,[32] and have been proposed to play a role in shaping and maintaining the human microbiome and resisting colonization by pathogens.[30,76] Their production has also been suggested to play a role transmission and intestinal domination, but this role is almost entirely unexplored in the

clinical setting. Within the gut, enterococci compete extensively among themselves and with other species.[33] In a mouse model of enterococcal gut colonization, it was demonstrated that *E. faecalis* harboring bacteriocin 21 (bac-21) was able to outcompete *E. faecalis* strains lacking this bacteriocin.[42] The bacteriocin-producing *E. faecalis* were also able to maintain long-term gut colonization in contrast to the non-bacteriocin producing strain. Gut colonization is a necessary first step in gut dominance,[14,75] and patients colonized with high densities of VRE have a greater amount of shedding into the environment leading to more opportunities for transmission.[77] Therefore, bacteriocin production has the potential to increase gut dominance and lead to greater transmission yet an understanding of the specific bacteriocins that could play this role is lacking.

Here, we present genomic analysis of a cohort of 2,428 *E. faecium* blood and perirectal isolates collected over a six-year period from a single hospital system. This sizeable cohort allows for an expansive search for a variety of potential bacteriocin loci, an analysis of how bacteriocin carriage changes over time, and the ability to shed light on how these bacteriocins could promote *E. faecium* transmission in this important setting. We identified many previously described bacteriocins in the patient derived isolates, most of which were found in limited numbers or did not have consistent phenotypic evidence of activity. However, bac43, a plasmid encoded bacteriocin, was found in 58% of isolates and became more prevalent over the study period. Isolates encoding bac43 had phenotypic evidence of bacterial inhibition and were found throughout the phylogenetic tree with evidence of both horizontal and vertical acquisition events.

2.3 Methods

2.3.1 Ethics

This study was approved by the University of Michigan Institutional Review Board (ID no. HUM00102282), which determined that informed consent was not required as all data utilized were collected for patient treatment purposes.

2.3.2 Sample Collection

E. faecium samples were collected from the Michigan Medicine hospital system between 2016 and 2021. Perirectal (PR) swabs were collected as part of the VRE surveillance screening protocol. Patients were screened upon admission to any of the hospital's nine high risk units and then weekly during their stay in those units. Blood (BL) samples were collected from all patients with *E. faecium* bloodstream infection.

Per hospital protocol, perirectal (PR) swabs were streaked on VRE select plates (Bio-Rad) by the clinical microbiology laboratory. Each positive VRE select plate was transferred to our research laboratory where an isolated pink colony was selected at random, re-streaked twice on Brain Heart Infusion (BHI) agar (Becton, Dickinson and Company, 211065), and stored in BHI broth (Becton, Dickinson and Company, 237500) supplemented with 20% glycerol at -80ºC.

Blood (BL) samples were obtained from the clinal microbiology laboratory having been streaked on either BBL TSA II 5% SB or BBL chocolate II agar plates. Random isolated colonies were selected and re-streaked twice on BHI plates with overnight growth at 35ºC. If there were multiple clearly distinct morphologies, one of each was selected. Freezer stocks were created from a single colony stored in BHI broth supplemented with 20% glycerol at -80ºC.

2.3.3 Sequencing and assembly

DNA extraction from clones was performed using the Omega Bio-tek Mag-BIND Bacterial DNA 96 Kit (M2350). Illumina library preparation was performed using Collibri PCR-free ES DNA Library Prep Kit for Illumina - Invitrogen (CAT# A39123196) and sequencing was performed using Illumina Nova Seq or HiSeq platform. Reads were processed using a snakmake pipeline for read trimming using Trimmomatic[78](v0.39), *de novo* assembly using Unicycler[79](v0.4.8), and annotation using Prokka (v1.14.6).[80] Multilocus sequence types were identified using the software mlst[81](v2.22.0).

2.3.4 Hybrid assemblies

Select genomes were assembled using a combination of long and short read data. Long read sequencing was performed using a MinION with a Flongle flow cell from Oxford Nanopore Technologies. Libraries were prepared using the Rapid Barcoding Sequencing Kit (SQK-RBK004) and the Flongle flow cells were primed using the Custom Kit (CUST-KIT). Sequencing data were obtained using the MinKNOW software from Oxford Nanopore Technologies. Adapters were trimmed from long-read sequences using Porechop[82](v0.2.3), *de novo* assemblies were generated using Unicycler[79](v0.4.8), and assemblies were annotated using Prokka[80](v1.14.6).

2.3.5 Identification of bacteriocins in genome assemblies

A list of bacteriocin protein sequences was obtained from BAGEL4,[83] including bacteriocin from classes 1-3. Sequences identical at the amino acid level were removed using the sRNAtoolbox.[84] Bacteriocin sequences within our assembled genomes were identified by creating custom BLAST databases of each assembly.[85] tblastn was then used to query bacteriocin sequences against each genome assembly database. Output files were filtered

using R (v4.3.1). If multiple hits were made to overlapping regions of a genome, the hit with the greatest bitscore was kept. Hits with an e-value greater than 1e-08 and a percent identity less than 60% were removed. Hits from all assemblies were clustered using CD-HIT[86] based on 90% sequence identity and 75% minimal alignment coverage for the longer sequence.

Searches were also performed using nucleotide sequences of other genes involved in bacteriocin production and immunity for enterocin A, bac43, enterocin NKR-5-3B (Appendix Table A.1). These genes were used as the query against each of the custom BLAST databases for each genome assembly.

2.3.6 Variant calling and phylogenetic analysis

Variants were identified using a custom snakemake pipeline. Trimmed reads were mapped to an internal reference strain *E. faecium* BL00198-1 and variants were called using snippy.[87] A core genome was produced using snippy-core; samples with less than 250,000 base pairs aligned were excluded. The core alignment was then cleaned using snippy-clean_full_aln, and a phylogenetic tree was produced using Gubbins.[88] Gubbins was run for six iterations where the first tree built using 'rapidnj' and 'JC' model. The remaining five iterations were built using 'raxmlng' and the 'GTR' model. Samples with less than 75% homology to the reference strain were excluded from tree generation to reduce errors in tree construction, but all samples were retained for subsequent analyses. The tree figure was rendered and annotated using ggtree in R (v4.3.1). For visualization purposes, MLSTs with less than 20 isolates associated with them are classified as "Other". Sequences with less than five isolates associated with them are classified as "Other".

2.3.7 Variation analysis of enterocin A gene cluster

Variants in the enterocin A gene cluster and the bacteriocin 43 encoded plasmid were identified using an internal snakemake pipeline. For enterocin A, trimmed reads were mapped to internal reference with the entire enterocin A gene cluster (BL02040-1). Variants were called using FreeBayes[89] with the following parameters: minimum base quality greater than or equal to 10 and minimum alternate fraction less than or equal to 0.5. Variants were annotated using SnpEff.[90] Insertion elements were identified and annotated using panISa[91] and ISFinder.[92] Areas of low read coverage indicating gene deletions were identified using SAMtools[93] depth and filtering to positions with at least 10% of the average coverage across the whole gene locus. Any variants or insertion elements that were assigned to regions identified as deletions were considered artifacts and were excluded using custom R scripts (v4.3.1). Read pileups were visualized using igv-reports.[94] Variants and low coverage regions were manually inspected using these read maps; mapped regions were identified in isolate genome assemblies to confirm presence or absence of variants and low coverage regions.

2.3.8 Variation analysis of bac43 encoding plasmid pDT1

Trimmed reads were mapped to an internal reference isolate, PR46485-1-C1, which encodes a plasmid identical to the previously published pDT1 sequence.[95] Variant calling, annotation, identification of deletions, and insertion element identification was repeated for pDT1 with the same methodology as for enterocin A. Additional analyses for pDT1 included coverage calculations across the plasmid and bac43 specific genes using SAMtools[93] coverage. Isolates with less than 45% mean depth or less than 55% coverage across the plasmid were excluded from further analysis. Variant positions were accepted if the RO (reference observations) to DP (total reads mapped) ratio was less than 10%, and if the RO

was less than 30 reads. Percent identity was then calculated as the number of non-variant positions divided by the total number of bases called. Isolates were finally filtered by those with at least 99.5% identity to the aligned portions of the pDT1 plasmid or if the BLAST analysis identified the full structural gene of bac43.

2.3.9 Visualizing genetic associations among variants

Genetic associations between variants in the enterocin A gene cluster and pDT1 were visualized using PHYLOViZ[96](v2.0) using the goeBURST Full MST algorithm (goeBURST distance). Subcluster annotations were made based on shared variants and/or variations within the same genomic regions. Gene homology maps were created using Easyfig[97](v2.2.2) with a minimum identity threshold of 60% was utilized.

2.3.10 Bacteriocin activity assay

Putative bacteriocin activity was assessed using a modified soft-agar assay for bacteriocin production and immunity.[95] Isolates were streaked from freezer stock onto BHI agar plates and incubated at 35ºC overnight. Single colonies were resuspended in 50ul saline and poked into a soft agar lawn containing a bacteriocin sensitive strain. If results were discordant with functional predictions, a higher inoculum was tested by using a toothpick to poke a single colony directly into the lawn. After incubation at 35ºC overnight, putative killing effect was determined by the presence of a halo of bacterial clearing around the poke into the bacteriocin sensitive lawn. Immunity to enterocin A was determined poking an enterocin A producer (BL02040-1) into a soft agar bacterial lawn of the strain being tested and looking for the absence of a halo of bacterial clearing.

Immunity to bac43 was determined by the ability to grow in the presence of supernatant derived from a bac43 producing strain (PR46485-1-C1). Bac43 supernatant was

obtained by incubation of PR46485-1-C1 in 45mL BHI at 35ºC, 130rpm, for 24 hours. The culture was centrifuged for 15 minutes at 3220rcf and the supernatant was filter sterilized twice (0.22um filter). Colony resuspensions were plated on BHI agar supplemented with 10% bac43 supernatant. Plates were incubated at 35ºC overnight. Immunity to bac43 was determined by bacterial growth on BHI plates supplemented with 10% bac43 lysate.

Isolates were chosen for the bacteriocin production and resistance assays as follows. When sequences were predicted to be nonfunctional (i.e. deletions or insertion elements present in bacteriocin genes) only one isolate with that mutation was tested. If the isolate with the presumed non-functional bacteriocin was found to be negative on the bacteriocin production and immunity assays, other isolates carrying the mutation were not tested. For sequence isolates from multiple MLSTs, one isolate was tested from each of the MLSTs present in at least 20 of all isolates.

2.3.11 Data availability

All scripts and pipelines are available on GitHub (https://github.com/woodslab/bacteriocin_search). The whole genome sequencing project for our reference *E. faecium* isolates have been deposited at DDBJ/ENA/GenBank: BL00198-1 (accession JBEFKN000000000), BL02040-1 (accession JBEFKM000000000), PR46485-1-C1 (JBEFKL000000000). The versions described in this paper are JBEFKN010000000, JBEFKM010000000, and JBEFKL010000000, respectively.

2.4 Results

2.4.1 Samples included

The cohort for this study included the first *E. faecium* isolate from each patient with a VRE *faecium* positive rectal surveillance swab between 2016 and 2021. This consisted of 2300 patients (Table 2.1). Additionally, 278 patients were found to have *E. faecium* growth in blood cultures. The first isolate from surveillance and blood culture per patient was sequenced. One-hundred and twenty-eight patients had both a perirectal (PR) swab and a blood (BL) sample taken during their stay in the hospital resulting in a total of 2428 *E. faecium* isolates.

Table 2.1: Sample summary.

Number of patients	2300
Number of isolates	2428
Number of perirectal (PR) isolates	2150
Number of blood (BL) isolates	278
Number of patients with both a PR and BL isolate	128

2.4.2 Bacteriocins identified in clinical cohort

BLAST hits to bacteriocin structural genes in our cohort were clustered based on sequence identity. There were 17 bacteriocin clusters identified with homology 15 different bacteriocins (Figure 2.1A, Appendix Table A.3). Most bacteriocin sequence clusters were rare, being found in less than 3% of isolates. The majority of these low frequency clusters have previously been characterized from *E. faecium*,[98–104] but enterocin SE-K4[105] and bac31[106] were characterized from *E. faecalis* and pneumolancidin[107] was characterized from *S. pneumoniae*. Many isolates also encoded multiple bacteriocin sequences (Figure 2.1B). We chose the four most common cluster hits to investigate further as their higher prevalence suggested a potentially relevant role in hospital transmission. This includes the enterocin A cluster (2395 or 98.6% of

isolates), two cluster hits to bac43 (1402 or 57.7% of isolates and 988 or 40.7% of isolates, respectively), and enterocin NKR-5-3B cluster (466 or 19.2% of isolates)(Figure 2.2A-D).

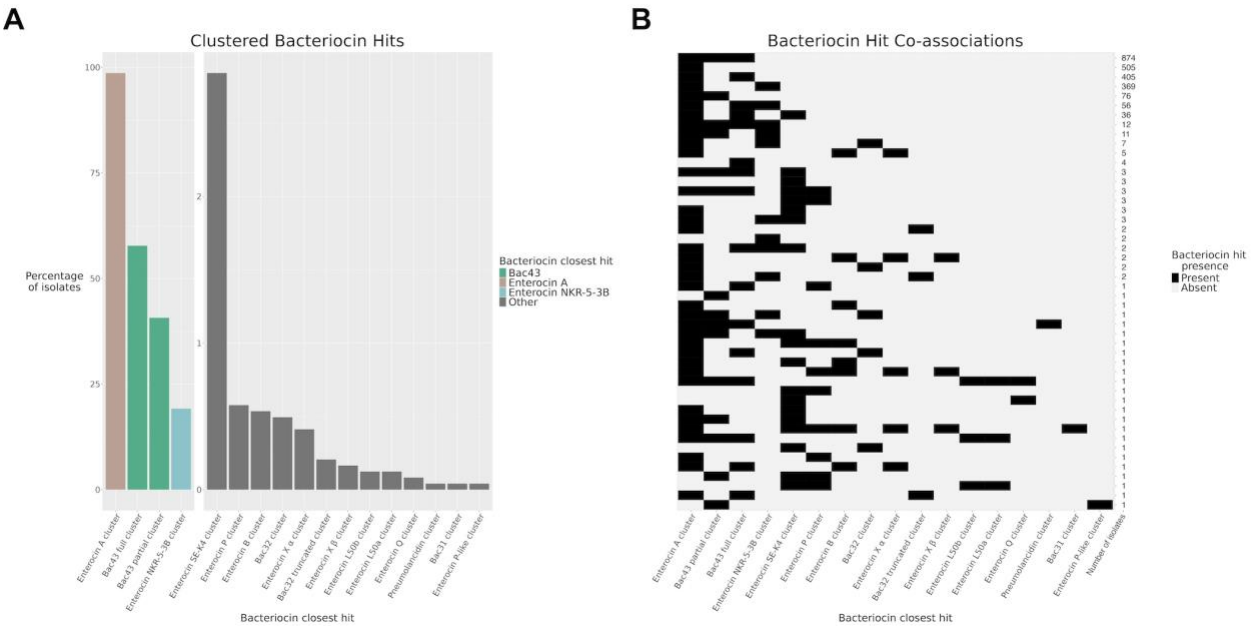


Figure 2.1: Frequency of bacteriocin gene sequence hits. Bacteriocin sequences were classified into 17 different clusters with homology to 15 different bacteriocins. (A) Four bacteriocin clusters were identified as present in over 19% of isolates: enterocin A cluster, bac43 full cluster, bac43 partial cluster, and enterocin NKR-5-3B cluster. (B) Patterns of hits to multiple bacteriocin sequences followed by the number of isolates found to have each pattern.

The enterocin NKR-5-3B cluster shares 87.3% amino acid homology to the structural gene *enkB* of enterocin NKR-5-3B and was identified in 19.2% of isolates. This class IIc circular bacteriocin was previously characterized on a 9kb chromosomal region of *E. faecium.*[108] The previously described functional operon includes five genes associated with enterocin NKR-5-3B: *enkB* the bacteriocin structural prepeptide, *enkB1* and *enkB3* for secretion and self-immunity, *enkB2* for maturation, and *enkB4* for self-immunity. None of the clinical isolates contained the entire previously described 9kb region. Rather we found homology to *enkB* and approximately 200 bp of *enkB1* (Figure 2.2D). Given the lack of predicted function, this cluster was not further investigated.

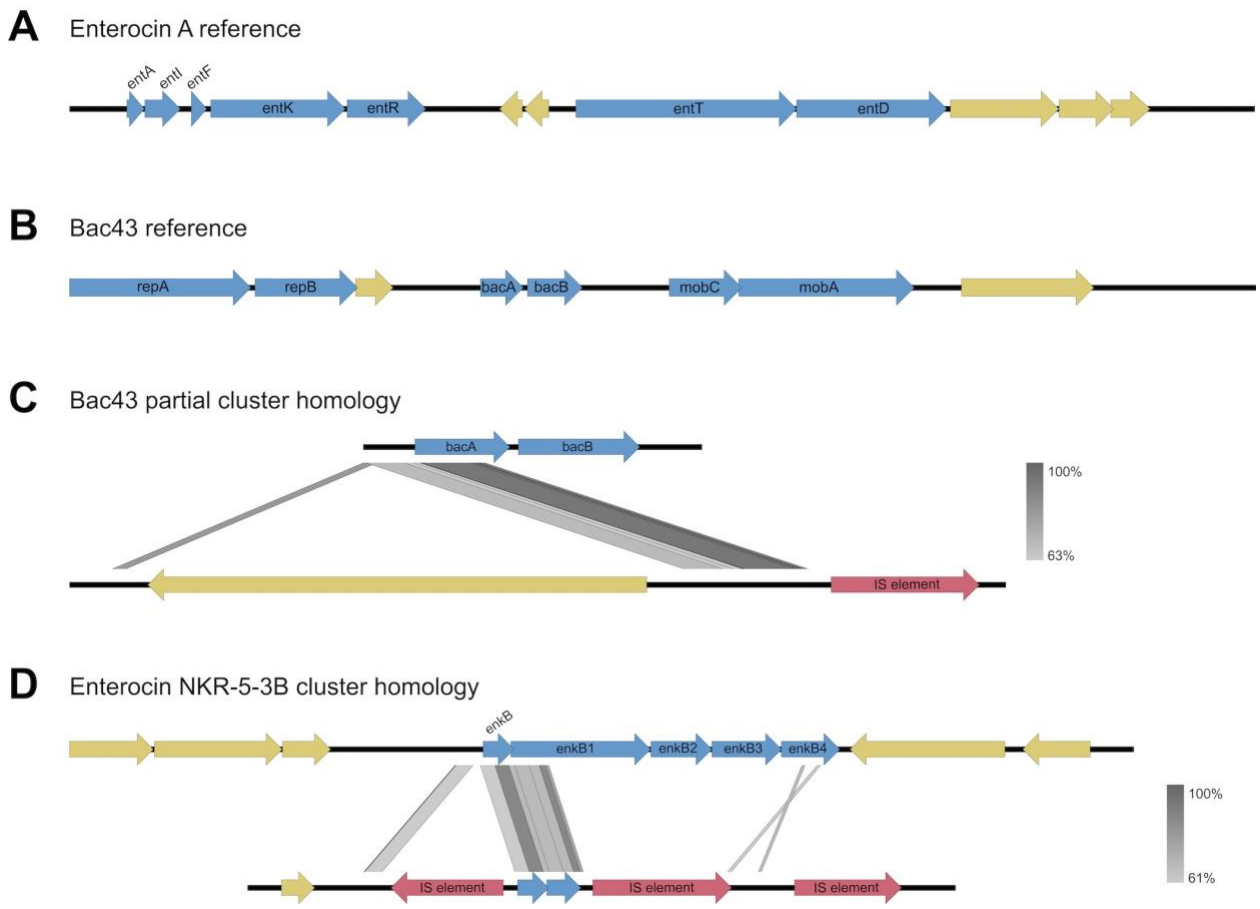


Figure 2.2: Gene maps and homology to previously identified bacteriocins. Genes related to bacteriocin production are blue, hypothetical proteins are yellow, and insertion elements are red. (A) Internal reference of the enterocin A gene cluster. (B) Internal reference of the bacteriocin 43 encoding plasmid pDT1. (C) Homology of the bacteriocin 43 genes to the bac43 partial cluster BLAST hit. (D) Homology of the enterocin NKR-5-3B genes to the enterocin NKR-5-3B cluster BLAST hit.

2.4.3 Analysis of the enterocin A gene cluster

The enterocin A structural gene, *entA*, was identified in 98.6% of isolates.[67,68] Enterocin A is a class IIa bacteriocin previously characterized in *E. faecium* strain CTC492. In that strain, the enterocin A gene cluster is encoded on a 10.5kb region of the bacterial chromosome[68] (Figure 2.2A), and the locus includes genes for structural protein (*entA*), self-immunity (*entI*), the peptide pheromone (*entF*), the two-component regulatory system (*entK* and *entR*), and the peptide transporter (*entT* and *entD*).

We identified variant positions, insertion elements, and deletions by comparing isolates to an internal reference. This reference sequence has a missense variant in the *entI* gene (position 246717 in BL02040-1, G to A) compared to the previously published sequence.[68] We identified 87 unique sequences of the enterocin A gene cluster in our collection, 61 of which were found in only a single isolate. These sequences were assigned to five subclusters labeled A through E (Figure 2.3A). Subcluster A is centered around sequence 1, which is found in 84% of all isolates. Sequence 1 isolates have a large deletion spanning *entFKRTD*. Of the 41 other sequences in subcluster A, 29 share this deletion (Figure 2.3B). Four sequences in subcluster A (4, 6, 9, and 12) have all the genes required for enterocin A function intact. In subcluster B, four sequences (3, 49, 73, and 85) also have all the enterocin A genes. The other sequence in subcluster B (35) has an insertion element in histidine protein kinase *entK* likely interrupting function. Subcluster C sequences have an insertion element that is likely inactivating the regulator gene *entR* (Figure 2.3B). Subcluster D shares the insertion element in *entR* with subcluster C plus an additional insertion element the immunity gene *entI* also likely interrupting function (Figure 2.3B). Subcluster E has three sequences which all have the complete enterocin A gene cluster but with approximately 30 synonymous and missense mutations.

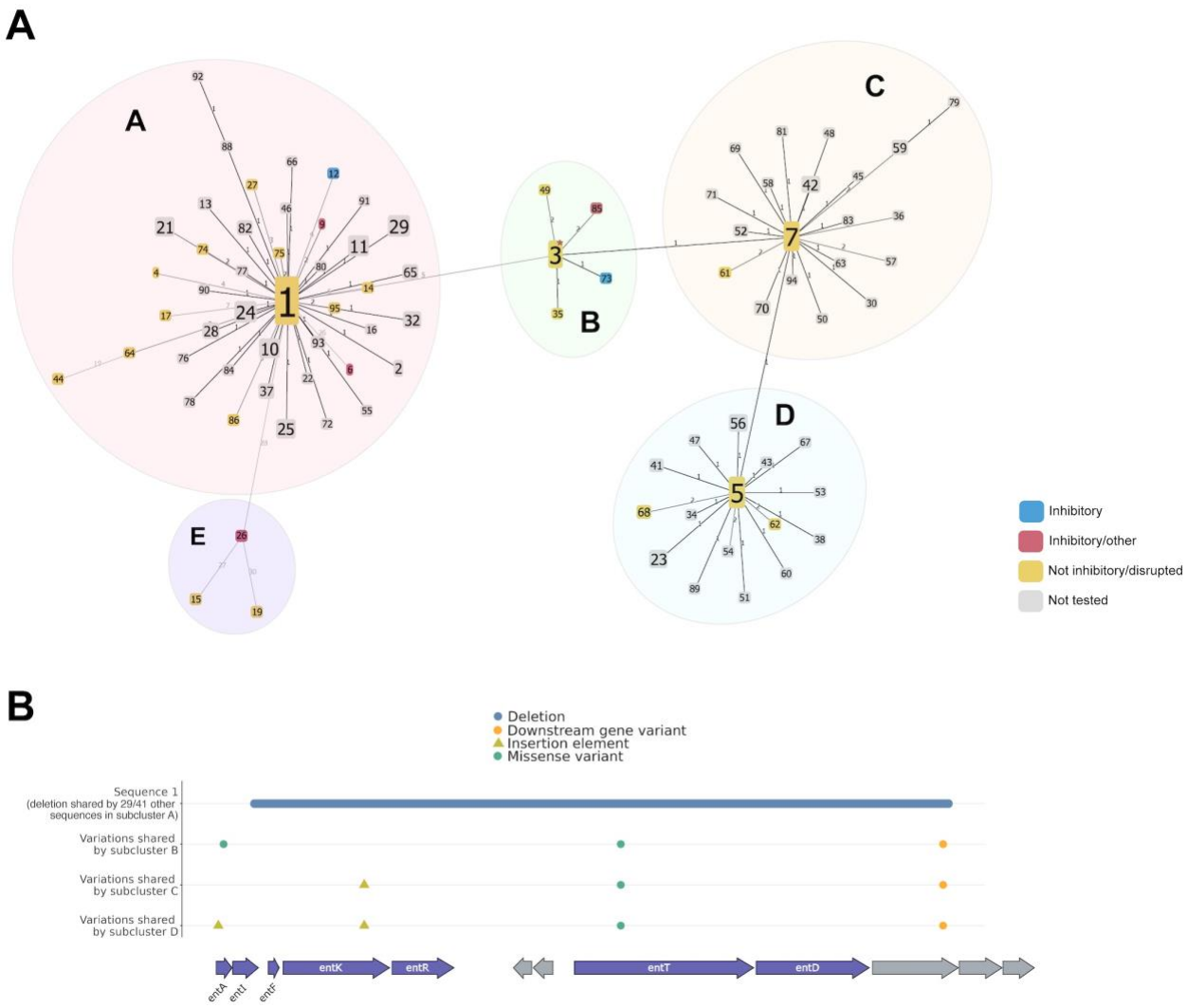


Figure 2.3: Enterocin A sequence clusters. Isolates were assessed for SNPs, insertion elements, and gene deletions within the enterocin A gene locus and are clustered based on matching patterns of variations (sequences). (A) Each node is a unique sequence of the enterocin A locus and branch numbers represent the number of variations between the sequences. Node size increases based on the number of isolates belonging to that sequence. The results of phenotypic assays on tested strains are noted on each sequence node. Inhibitory sequences (blue and red) have both the full enterocin A locus and can kill a bacteriocin sensitive strain, inhibitory/other sequences (red) also encode other bacteriocins. Not inhibitory/disrupted sequences (yellow) either lacked inhibition in phenotypic assays and/or had disruptions in the enterocin A locus that would predict nonfunction. Sequence 3 isolates are marked as non-inhibitory due to mixed phenotypic results. (B) Gene maps aligned with the enterocin A reference locus detailing shared variations among the subclusters.

2.4.4 Analysis of bac43 gene clusters

Bacteriocin 43 (bac43) is a class IIa *sec*-dependent bacteriocin previously isolated from clinical vancomycin resistant *E. faecium* isolates.[95] The 6.1kb plasmid pDT1 encodes the bac43 structural gene *bacA*, the gene for self-immunity *bacB*, plasmid mobilization genes *mobC* and *mobA*, and plasmid replication genes *repA* and *repB*[95](Figure 2.2 B).

Bac43 was identified as the closest bacteriocin hit in two common clusters. One of these, referred to as 'bac43 partial' cluster, was found in 40.7% of isolates and aligned only to part of the *bacA* coding region, has no homology to the immunity gene, and the start codon found on *bacA* was absent. Thus, we do not expect it to be functional. The aligned bac43 partial sequence displayed only 87.5% homology to previously published *bacA*, and were not found on contigs with other pDT1 genes (Figure 2.2C).

The second cluster, 'bac43 full,' was identified in 57.7% of isolates and displayed 99.5–100% amino acid homology to the published *bacA*. Sequences falling into the bac43 full cluster also have homology to the immunity gene *bacB* and are often found on contigs consistent with the pDT1 plasmid.

Variants in the pDT1 plasmid were identified by comparing isolates to an internal reference genome carrying a plasmid identical to the one previously published.[95] Four isolates discordant with phenotypic predictions were assayed using PCR for the presence of the *bacA* (Appendix Table A.2). PCR revealed an absence of this gene, and these isolates were removed from further analysis. We identified 106 unique sequences of the bac43 encoding plasmid pDT1, only six of which included variation in the bacteriocin or immunity genes. Thus, based primarily on variation in the plasmid backbone, these sequences are organized into nine subclusters (Figure 2.4). Subcluster A is centered around sequence 3

which is found in 39.7% of isolates and is identical to the published pDT1 sequence.[95] Most of the variation within this subcluster is outside of *bacA* and *bacB*, but four sequences had a mutation in one of these genes: a synonymous variant in *bacA* (sequence 82), an insertion element in *bacB* (sequence 14), and two with insertion elements in different positions in *bacA* (sequences 57 and 84). Additionally, sequence 65 (subcluster H) has a missense variant in *bacB*. Sequences 9 and 73 (subcluster D) have lost the bac43 genes but retained portions of the pDT1 plasmid. The remainder of the sequences are characterized by insertion elements and/or alterations to the plasmid backbone.

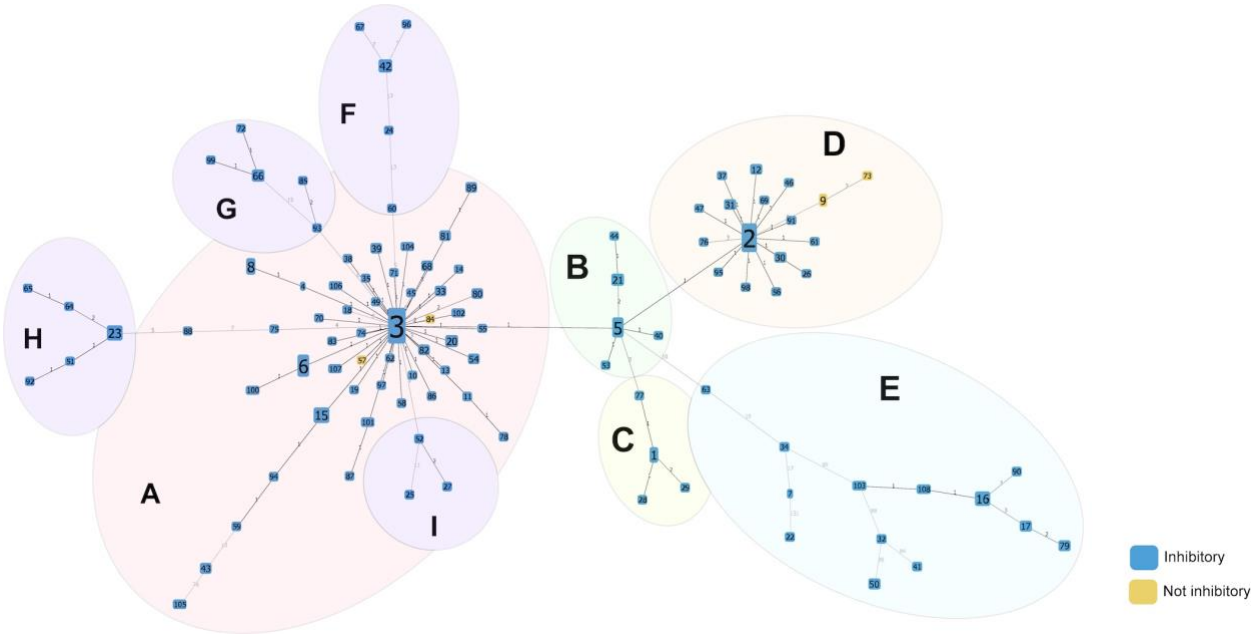


Figure 2.4: Clustered variations within the bac43 encoding plasmid pDT1. Isolates were assessed for variants, insertion elements, and gene deletions within the bac43 encoding plasmid pDT1 and are clustered based on matching patterns of variations (sequences). Each node is a unique sequence of pDT1 and branch numbers represent the number of variations between the sequences. Node color represents phenotypic testing results. Node size is determined by number of isolates belonging to that sequence. Inhibitory sequences (blue) can kill a bacteriocin sensitive strain.

2.4.5 Phenotypic evaluation of bacteriocin containing hits

Phenotypic assays were performed to determine whether strains produce a phenotype of (1) killing of a strain with no bacteriocin sequences and (2) immunity to killing by strains that encode a known bacteriocin producer (for enterocin A) or a crude extract (for bac43).

For strains encoding the enterocin NKR-5-3B-like sequence, we tested seven isolates from different MLST backgrounds. As expected, none of the isolates demonstrated inhibition against a bacteriocin sensitive strain.

For enterocin A, eleven sequences were classified as potentially functional based on the absence of deletions or insertion elements into the gene cluster. Isolates encoding six of these sequences showed both consistent killing of the sensitive strain and resistance to killing by an enterocin A producer consistent with a fully functional enterocin A locus (sequences 6, 9, 12, 26, 73, and 85)(Figure 2.5A); four of these isolates (sequences 6, 9, 26, and 85), however, also encode other bacteriocins such as enterocin B, enterocin P, enterocin Xα, and enterocin SE-K4 which could not be ruled out as the source of inhibition. The five sequence 3 isolates that were tested gave mixed results. Four of the five tested isolates encoding sequence 3 demonstrated an inhibitory and resistant phenotype that could be consistent with a functional enterocin A locus, but the contribution of other bacteriocins could not be excluded. The only sequence 3 isolate that encodes no other bacteriocins had no inhibitory activity but did demonstrate resistance to killing by enterocin A exhibiting that the sequence 3 enterocin A locus is in fact nonfunctional. This resistant noninhibitory pattern was also demonstrated by sequence 49. As neither of these isolates encode other bacteriocins the enterocin A resistance could stem from *entI* or chromosomal mutations. The remaining three sequences did not have an inhibitory phenotype. The sequence 4 isolate showed resistance

without inhibition but unlike the preceding isolates, the tested isolate also encodes other bacteriocins. Sequence 15 and 19 lack both inhibitory activity and enterocin A resistance; both have numerous mutations that could have resulted in non-function (Figure 2.5B).

Consistent with the proposed contribution of other bacteriocin loci to both inhibition and immunity, of the 16 sequences that were predicted to be non-functional due to deletions of or insertions into key bacteriocin genes, seven had both inhibitory activity and enterocin A immunity. These isolates all were predicted to encode other bacteriocins.

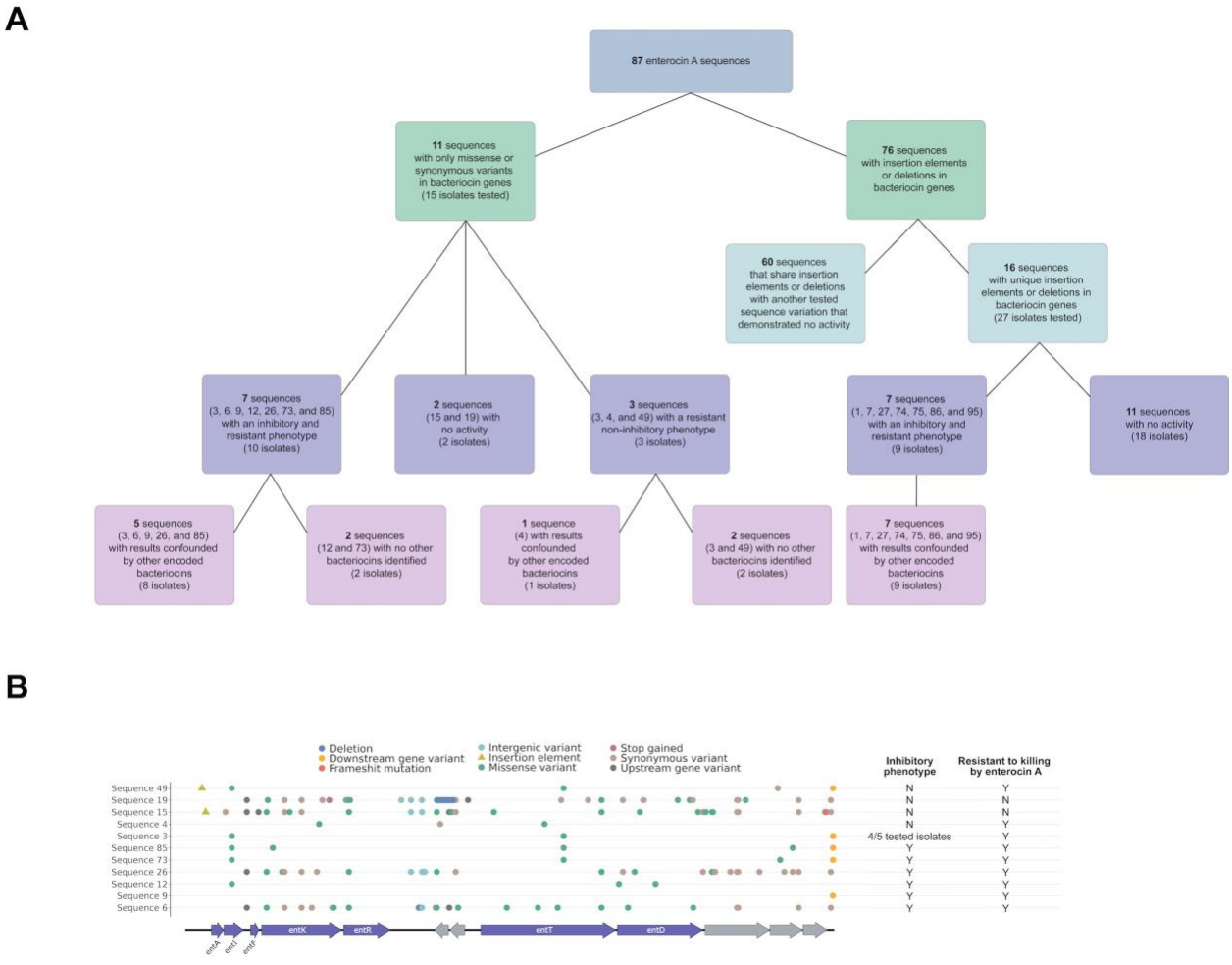


Figure 2.5: Enterocin A phenotypic analysis.(A) Overview of enterocin A isolates tested, functional expectations, and experimental results. (B) Gene maps aligned with the enterocin A reference locus for sequences that were expected to be functional (i.e. no deletions or insertion elements within the bacteriocin genes).

Phenotypic testing was performed on 132 isolates representing 106 sequences with pDT1 variants (Figure 2.6A). Ninety-nine of these had 100% conserved sequences of *bacA* and *bacB* but other mutations in the pDT1 plasmid; all 124 isolates tested with these 99 sequences were able to kill the bac43 sensitive strain and were resistant to killing by a bac43 producer. Among the five sequences with mutations in the bac43 genes, three retained both an inhibitory phenotype and bac43 resistance: sequence 65 (missense variant in *bacB*), 82 (synonymous variant in *bacA*), and 14 (insertion element in *bacB*). However, no inhibition or resistance to bac43 were observed by isolates with an insertion element in *bacA* (sequences 84 and 57).

Because the variant analysis aligned sequences to the full pDT1 plasmid, two sequences (9 and 73) were identified that lost the bac43 genes but retained portions of the plasmid (Figure 2.6B); these isolates did not demonstrate inhibition or resistance to bac43.

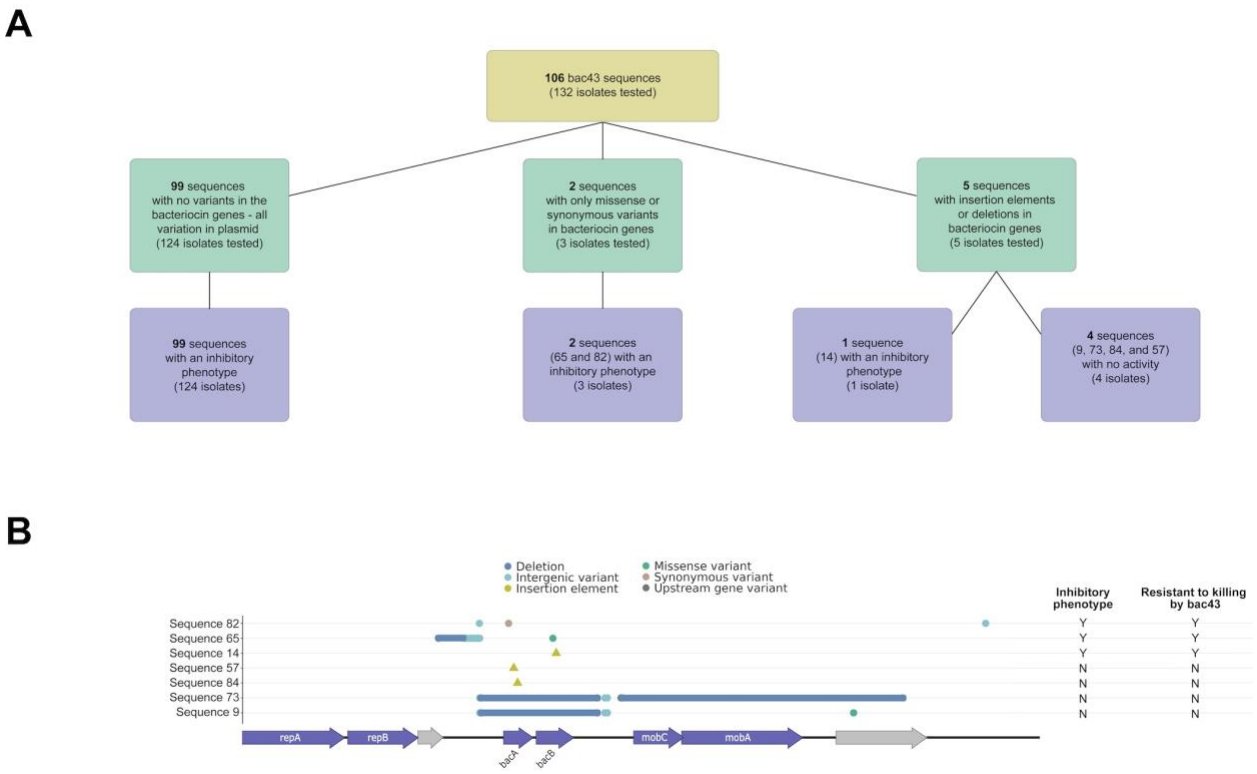


Figure 2.6: Bac43 phenotypic analysis. (A) Overview of bac43 isolates tested, functional expectations, and experimental results. (B) Gene maps aligned with the bac43 encoding plasmid pDT1 for sequences with variants in the bac43 genes *bacA* and *bacB.*

2.4.6 Phylogenetic analysis

A phylogenetic tree of the isolates was created to examine the evolutionary relationships between the bac43 and enterocin A sequences. The enterocin A sequences cluster together supporting a predominantly vertical mode of transmission (Figure 2.7). Sequences are not restricted to MLSTs: for example, sequence 1 with a deletion of *entFKRTD* is found in multiple MLST backgrounds suggesting this wide-spread deletion may have occurred before MLST differentiation. Five of the seven phenotypically functional isolates encoding the full enterocin A locus (six sequence variations plus the enterocin A reference) passed the homology thresholds for inclusion on the tree. Four of these isolates cluster together while one is found in another cluster surrounded by nonfunctional sequence 1 isolates consistent with horizontal gene transfer of the intact gene locus.

For bac43, similar sequences tend to cluster together consistent with prominence of vertical transmission (Figure 2.7). Sequence 3 is mostly found within MLST 117 and sequence 2 is associated with MLST 80. However, occasionally the same sequence can be found in distant clades and MLSTs supporting horizontal transmission events. For example, sequence 3 is also found in MLST 80 and sequence 2 in found in MLST 117. Functional bac43 is found in 18 of the 59 MLSTs (seven of nine MLSTs found in more than 20 isolates).

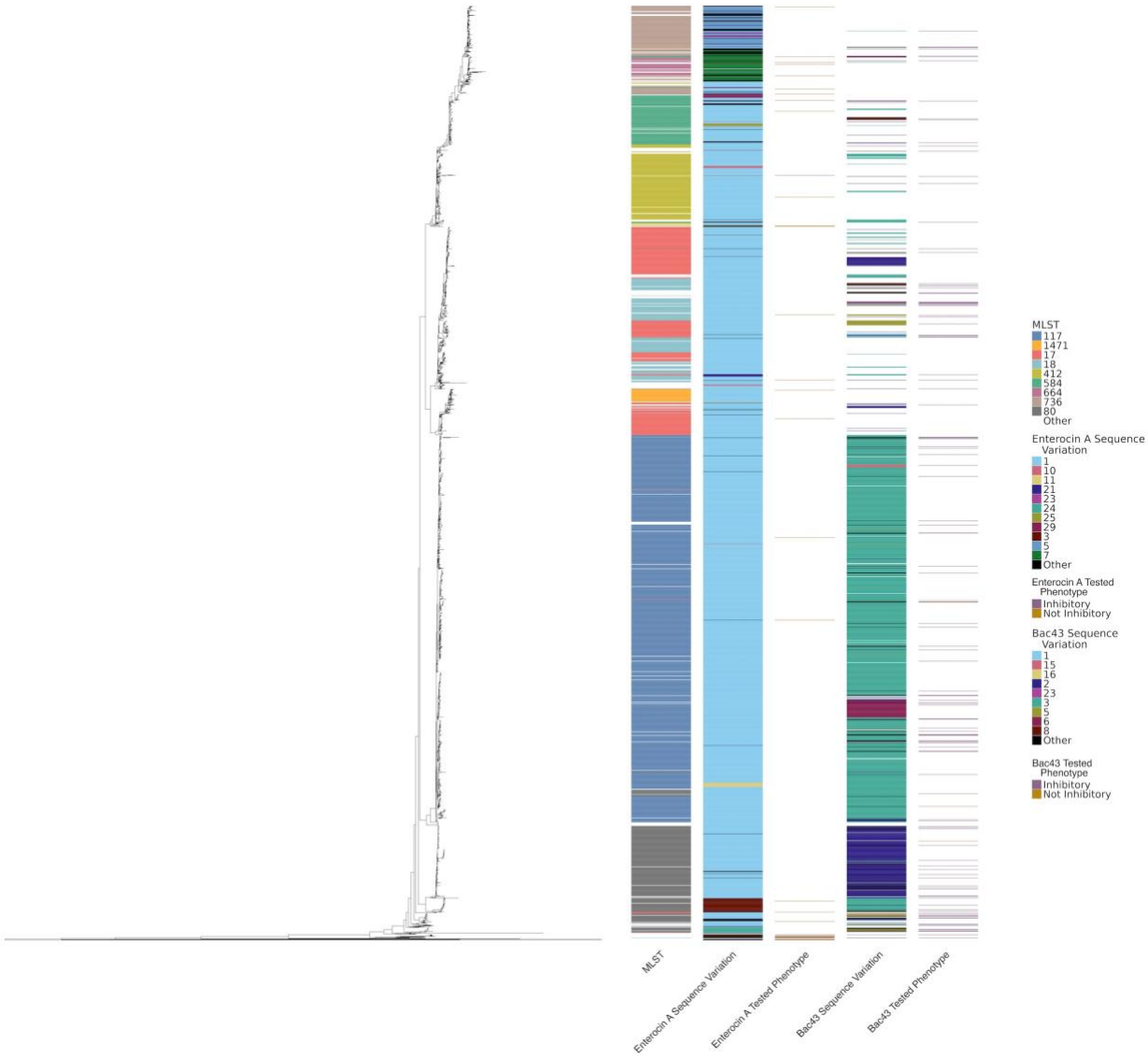


Figure 2.7: Phylogenetic mapping of bacteriocin genotype and phenotype. Core genome phylogeny with recombination removed by Gubbins. From left to right heatmaps are as follows: MLST, enterocin A sequence variations, phenotype of tested enterocin A isolates, bac43 sequence variations, phenotype of tested bac43 isolates. MLSTs with less than 20 isolates are classified as "Other."

2.4.7 Temporal trends

The frequency of bac43 increased over the study period, rising from 8% in the first quarter of 2016 to 91% presence by the end of 2021 (Figure 2.8A). At the beginning of the collection period, no single MLST was present in more than 20% of isolates. However, MLST 117 subsequently rose to become the dominant sequence type with MLST 80 steadily increasing (Figure 2.8B). Isolates encoding bac43 are highly associated with MLSTs 117 and 80 accounting

for 66.6% and 18.5% of bac43 isolates, respectively. By contrast, the six isolates that demonstrated functional enterocin A activity were dispersed over only the first five years of the study and had no clear association with MLSTs.

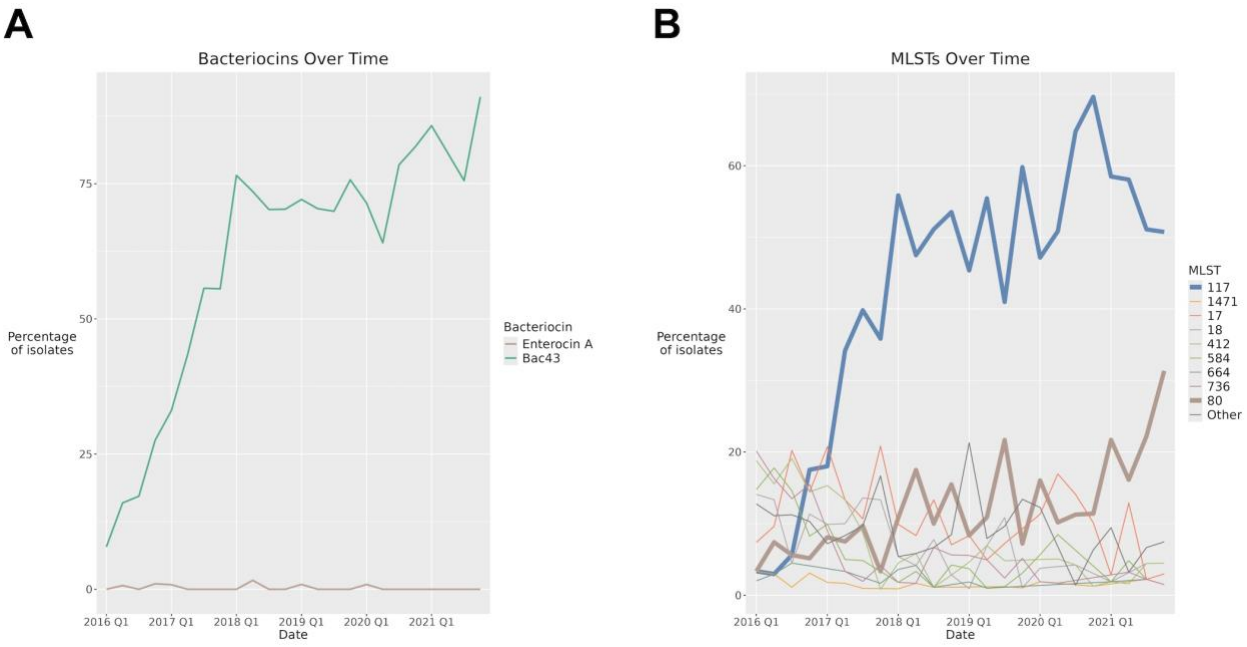


Figure 2.8: Temporal trends in bacteriocin variants and MLST. Presence of (A) functional bac43 and enterocin A sequences by quarter year and (B) MLSTs over the six-year study period, over the study period by quarter year. MLSTs with less than 20 isolates are classified as "Other." Data includes first isolate per patient only.

2.4.8 Prevalence by isolate source

Our collection protocol identified vancomycin resistant perirectal (PR) isolates, while blood (BL) isolates include both vancomycin resistant and vancomycin sensitive isolates. When we restrict our cohort to only those with vancomycin resistance, we have 2142 PR isolates and 167 BL isolates. Phenotypically functional enterocin A was found in 0.09% of PR isolates and 0% of blood (BL) isolates (p-value 1), while phenotypically functional bac43 was found in 59.1% of PR isolates and 56.9% of BL isolates (p-value 0.62)(Table 2.2). We find no significant differences in bacteriocin presence between PR and BL isolates. As an aside, when included all isolates (VRE and VSE) we found bac43 was present is significantly more PR isolates

suggesting a potential association between bac43 and vancomycin resistance (Appendix Table A.4).

Table 2.2: Bacteriocin presence between vancomycin resistant perirectal (VRE PR) and vancomycin resistant blood (VRE BL) isolates. Significance values: p-value < 0.05 *, p-value < 0.001 **

Bacteriocin	Percentage of VRE PR isolates (n=2142)	Percentage of VRE BL isolates (n=167)	p-value	Odds ratio
Enterocin A	0.09%	0%	1	Infinity
Bac43	59.1%	56.9%	0.62	1.09

2.5 Discussion

Enteroccocci occupy the intestinal tract of a broad array of host species and compete intensively with other inhabitants of this niche[75]. Bacteriocins are commonly found in enterococci and believed to be effective weapons in this competition,[30] but little is known about the role they play in clinical settings. Here we investigate the epidemiology and evolution of bacteriocins in *E. faecium* in a clinical setting by analyzing bacteriocin genetic content and phenotype of patient-derived isolates collected over a six-year period. Bacteriocin 43 (bac43) stands out as being the most common functional bacteriocin and shows a dramatic increase in frequency over time.

Bac43 was first isolated from a collection of VRE isolates from this same hospital in the late 1990s[95]. From isolates collected over five years, bac43 was identified in 3.3%. From our current study, we find the bac43 genes and its encoding plasmid to be mostly conserved with 40.7% of our isolates encoding a plasmid identical to the initially described plasmid.[95] When considering both sets of data, we see that bac43 has been present within this hospital for over two decades with a recent rise in frequency. However, this observational genomic survey cannot identify a causal role for bac43 in this increase in frequency over time. It is tempting to suggest bac43 could impact colonization and transmission given the strong

phenotype it produces in the lab. Indeed, prior work using a mouse model of intestinal colonization showed that *E. faecalis* harboring a plasmid encoded bacteriocin bac-21 were able to eliminate bac-21 sensitive strains over the course of their experiment.[42] That study also demonstrated bac-21 plasmid was actively conjugated within the GI tract.[42] *In vitro* data also showed the bac43 plasmid pDT1 is capable of conjugation.[95] If pDT1 acts similarity in being advantageous to *E. faecium* for invasion and domination of the human gut, this could explain how bac43 has come to dominate our clinical cohort. However, there are alternative explanations. The strong association between bac43 and the MLSTs that have become dominant in our cohort (117 and 80), suggest these could be rising due to hitchhiking with some other features of these genomes. The rise of ST117 in hospital systems has been documented globally during the 2010s but the possible causes to the rise of ST117 in particular have yet to be identified.[109–112] It is also possible that bac43 could be acting as a selfish plasmid, mostly serving to maintain to own existence with little benefit to the host bacterium. Finally, we have focused on the intraspecific competition, but is it also possible that bac43 could impact interspecific competition, especially among closely related species.[95]

Prior studies have identified bac43 from clinical isolates of vancomycin resistant and sensitive enterococci[46,95,113] and demonstrated a correlation between bac43 and vancomycin resistant *E. faecium.*[113] Consistently, we also find that bac43 is associated with vancomycin resistance. After correcting for this association, we see a similar presence of bac43 in strains from both colonization and infection.

This study also adds clarity to the contribution of enterocin A through genomic and phenotypic analyses. While the structural gene for enterocin A is found in 98.6% of *E. faecium* isolates within this cohort, the vast majority of these appear to be non-functional.

Seven of the 11 sequences with a full enterocin A locus (0.3% of isolates) demonstrated an inhibitory phenotype, but four of these also encode other bacteriocins. The potential contribution of other bacteriocin loci is best demonstrated by sequence 3 isolates; four of the five tested isolates that encode other bacteriocins demonstrated an inhibitory and resistant phenotype, while the one isolate that lacked other bacteriocins only showed resistance. Similarly, several isolates that had only portions of the enterocin A locus and were not predicted to be functional had both inhibition and enterocin A immunity but all encoded other bacteriocins. We also identified three isolates with the full enterocin A locus that did not have an inhibitory phenotype but did appear to be resistant to killing by enterocin A. While these isolates still retain the enterocin A immunity gene, this resistance could also reflect mutations in the bacteriocin target site, mannose phosphotransferase system.[63]

This study represents one of the first systematic analyses of bacteriocins from a clinical cohort and identifies bacteriocin 43 (bac43) as being mostly conserved and increasing in prevalence in clinical *E. faecium* isolates over a six-year collection period. As bacteriocins have been shown to be effective weapons in bacterial competition, bac43 is an important target for future investigation of the role of bacteriocins in the transmission of vancomycin resistant *E. faecium*.

Chapter 3 Class IIa Bacteriocin Resistance and Genetic Variation in Mannose PTS Genes in a Clinical *E. faecium* Cohort Experiencing a Rise in Bacteriocin Prevalence

3.1 Abstract

Vancomycin resistant *Enterococcus faecium* are a serious public health threat. These bacteria are colonizing opportunistic pathogens that transmit silently among hosts, but the mechanisms favoring colonization are not well characterized. Bacteriocins have been suggested to confer a competitive advantage in this setting. Two recent studies have demonstrated a rise in carriage of bac43, a class IIa bacteriocin, in hospital associated *E. faecium*. This finding raises questions regarding the phenotypes of the non-bac43 producing *E. faecium* population. Resistance to bac43 emerges readily *in vitro* via disruptive mutations in the presumed target of bac43, the mannose phosphotransferase system (manPTS). However, the mutations identified *in vitro* have been suggested to carry a large fitness cost *in vivo*. Here we investigate whether bacteriocin resistance arises *in vivo*, and if so, if it involves the manPTS genes and is associated with impaired mannose utilization. We perform three analyses on a previously described cohort of 2428 *E. faecium* isolates where bac43 carriage was shown to increase over time. This includes (1) a genomic analysis of the entire cohort, (2) a phenotypic analysis of a representative subsample, and (3) an analysis of isolates with disruptive mutations in manPTS. While manPTS was overall highly conserved and we rarely identified high-level resistant mutants, a subset of isolates further characterized as *E. lactis* (formerly known as clade B *E. faecium*) was found to carry allelic variants of manPTS conferring 4-fold greater resistance to bac43 supernatant

without a defect in mannose utilization. This work may help to explain the emergence of bac43 producing strains given the selective pressure against resistance.

3.2 Introduction

Vancomycin-resistant enterococci (VRE) are opportunistic pathogens that have become endemic to healthcare environments.[114] Patients treated with antibiotics are particularly at risk for colonization by VRE as the resulting alterations in the gastrointestinal community leave it susceptible to domination by VRE.[14,75,115] However, the molecular mechanisms of intestinal colonization and domination by VRE are unclear. Bacteriocins are antimicrobial proteins that can have been proposed to confer an advantage in bacterial competition.[30,38,116] Bacteriocin production in enterococci is common,[66] and in mice bacteriocin production can assist in enterococcal domination of the intestine.[42,117] Thus, bacteriocins have the potential to influence the transmission of this serious pathogen.[117,118]

Resistance to bacteriocins is an important consideration when studying the ecological dynamics associated with bacteriocin production. Bacteriocin production can be advantageous in spatially structured environments where the producers can utilize the resources made available by killing their sensitive counterparts.[39] However, when a bacterium is added that confers resistance to the bacteriocin, but does not produce the bacteriocin, a three-way interaction can emerge that resembles that of a rock-paper-scissors game. In this situation, the bacteriocin producer outcompetes the bacteriocin sensitive strain, the producer can be outcompeted by the resistant non-producer, and the bacteriocin sensitive can outcompete the resistant non-producers.[40,41] This rock-paper-scissors dynamic has been explored through significant theoretical and *in vitro* experimentation and can potentially allow for the maintenance of

diversity, but only when interactions are localized and there is an equilibrium point preventing the surge of a single player.[40]

Despite the theoretical and experimental advances, very little is known about how such interactions unfold in naturally occurring populations. Recently, we described the prevalence of bacteriocin 43 (bac43) in cohort of 2,248 patient derived *E. faecium* isolates, in which the prevalence grew from 8% to 91% over six years in a single hospital.[119] The structural and immunity genes of bac43 were enocded on a plasmid that was genetically highly conserved, and was phenotypically functional. Similar to our work, Mills et al. showed that bac43 was enriched among globably emerging strains of vancomycin resistant *E. faecium.*[117]

Class IIa bacteriocins, including bac43, are charaterized by the presence of a unique motif known as the 'pediocin box.'[51] These bacteriocins attack sensitive cells through targeting of the mannose phosphotransferse system (manPTS).[54–57] While bacterial PTS systems are primarily responsible for the the uptake and phosyphorylation of carbohydrates, they may also be targeterd by bacteriocins. In *E. faecium*, manPTS is composed of four protein subunits (EIIB, EIIAB, EIIC, and EIID).[63] The membrane-bound complex formed by subunits EIIC and EIID is recognized by the N-terminus of class IIa bacteriocins for binding followed by insertion of the C-terminus creating a pore in the cytoplasmic membrane which ultimately results in cell death.[54,55,60] As a result, the EIIC/EIID subunit complex confers sensitivity to class IIa bacteriocins.[61] Resistance to class IIa bacteriocins arises readily *in vitro* under laboratory conditions.[120,121] This has been demonstrated to result from downregulation of the manPTS as a result of alterations to the sigma factor, sigma-54, or ManR, the sigma-54-associated activator protein,[54–57,63,64] but could also be attributed to changes in the composition of unsaturated fatty acids and phospholipids in the bacterial membrane.[65] Furthermore, mutants reistant to a cocktail

of bac43, enterocin A, and enterocin P were shown to have a growth defect *in vitro* when mannose was the primary carbon source provided.[63]

Currently, little is known about the the prevalence of class IIa bacteriocin resistant mutants in naturally occuring populations. This may be a result of the critical contributions of manPTS during colonization. ManPTS serves important roles in carbohydrate uptake but may also impact in stress reposonses and carbon catabolite repression.[62,122,123] Zhang et al. demonstrated a version of the manPTS gene cluster was highly enriched in *E. faecium* isolates from clinical infections (*E. faecium* clade A) compared to human commensals (*E. lactis*, formerly known as clade B *E. faecium*[23]).[124] This manPTS gene cluster was also found more often in MLSTs associated with clinical infections (e.g. MLSTs 117, 80, 17, and 18).[124] Additionally, Zhang et al. demonstrated that loss of the EIID subunit impaired *E. faecium* intestinal colonization of antibiotic treated mice. This result suggests that manPTS could be important in colonization and that resistance may not be readily identified *in vivo.*[124] The EIIC and EIID subunits were also found to be essential for *E. faecium* growth in human serum, and a EIIC transposon mutant presented a loss of virulence in a zebrafish model of infection.[125] These results suggest manPTS contributes plays an important role in colonization and pathogenesis in *E. faecium*.

Here, we perform additional analysis on the six-year clinical cohort of *E. faecium* where we previously described the rise of bac43, to better understand the potential role of bac43 resistance and the involvement of manPTS *in vivo*. This investigation reveals no general trends in resistance, and that resistance remains rare among strains that do no carry bac43. In the few cases where we do see bac43 resistance, is it not associatd with decrease mannose utilization like our *in vitro* selected resistant mutants.

3.3 Methods

3.3.1 Ethics

This study was approved by the University of Michigan Institutional Review Board (ID no. HUM00102282), which determined that informed consent was not required as all data utilized were collected for patient treatment purposes.

3.3.2 Sample collection

E. faecium samples were collected from the Michigan Medicine hospital system between 2016 and 2021 as previously described.[119] Perirectal (PR) swabs were collected as part of the VRE surveillance screening protocol. Patients were screened upon admission to any of the hospital's nine high risk units and then weekly during their stay in those units. Blood (BL) samples were collected from all patients with *E. faecium* bloodstream infection. Single colonies for each sample were isolated and stored in Brain Heart Infusion (BHI)(Becton, Dickinson and Company, 237500) broth supplemented with 20% glycerol at -80ºC.

3.3.3 Sequencing and assembly

DNA from clones was extracted and sequenced using Illumina Nova Seq or HiSeq platform as previously described.[119] *In vitro* selected resistant mutants were sequenced using Illumina Whole Genome Sequencing (200 Mbp) through SeqCenter in Pittsburgh, PA. Reads were trimmed using Trimmomatic[78](v0.39), *de novo* assembled using Unicycler[79](v0.4.8), and annotated using Prokka[80](v1.14.6). Multi-locus sequence types (MLST) were identified using the software mlst[81](v2.22.0).

3.3.4 Identification of manPTS genes in genome assemblies

ManPTS genes were identified in our genomes by BLAST using the manPTS genes (*manR*, *manX_1*, *manX_2*, *manY*, and *manZ)* from *E. faecium* strain 6E6 (Genbank accession number NZ_CP013994)[63] as a query (Appendix Table B.1). Custom BLAST databases of each assembly were created[85] and blastn was then used to query manPTS sequences against each genome assembly database.

3.3.5 Bacteriocin sequence identification

Bacteriocin sequences within this cohort were previously identified.[119] Briefly, custom BLAST databases of were generated for each isolate assembly.[85] tblastn was then used to query bacteriocin sequences from BAGEL4[83] against each genome assembly database.

3.3.6 Variant analysis

Variants were identified using a custom snakemake pipeline. Trimmed reads were mapped to *E. faecium* strain 6E6 as the manPTS targeted by class IIa bacteriocins in this sample was previously annotated.[63] Analysis of variants was included the entire manPTS and regulatory region (*manR*, *manX_1*, *manX_2*, *manY*, and *manZ,* positions 1953429 to 19559881). Variants were called using FreeBayes[89] with the following parameters: minimum base quality greater than or equal to 10 and minimum alternate fraction less than or equal to 0.5. Variants were annotated using SnpEff[90]. Insertion elements were identified and annotated using panISa[91] and ISFinder[92]. Areas of low read coverage indicating gene deletions were identified using SAMtools[93] depth and filtering to positions with at least 10% of the average coverage across the whole gene locus.

Any variants or insertion elements that were assigned to regions identified as deletions were considered artifacts and were excluded using custom R scripts (v4.3.1). Read pileups were visualized using igv-reports.[94]

Trimmed reads for *in vitro* selected resistant mutants were mapped to the parental sensitive strain BL02235-1. Variant calling and annotation, insertion element identification, and low coverage region identification was performed, as above, but applied to the entire genome.

3.3.7 Phylogenetic analysis

Fasta files were generated for each sample the entire manPTS and regulatory region using SAMtools[93] consensus on each samples bam file for the region of NZ_CP013994.1:1953429-1959881. Muscle5[126](v5.1) was used to generate a multiple sequence alignment. RAxML-NG[127](v1.2.2) was used to generate an alignment of non-duplicate sequences. A phylogenetic tree was produced using Gubbins[88] on non-duplicated manPTS alignment. Gubbins was run for five iterations where the first tree built using 'rapidnj' and 'JC' model. The remaining four iterations were built using 'raxmlng' and the 'GTR' model. Phandango[128] was used for visualization of recombination regions. Regions of homology were visualized using Easyfig.[129]

3.3.8 Sample typing

Variants were identified using snippy[87] and typing strains of *E. lactis* (strain LMG25958, Genbank accession JADZMB010000001.1) and *E. faecium* (strain NBRC100486, Genbank accession NZ_BCQD01000001.1). Percent identity to each reference was calculated as the number of bases aligned per isolate minus the number of variants called divided by the number of bases aligned. Species identity was designated by the species with the greatest percent identity. The genome assemblies of isolates with a greater percent identity to *E. lactis* were submitted to the Type (Strain) Genome Server[130] for confirmation.

3.3.9 Sample selection for temporal and MLST analysis

For our representative screen, we selected isolates from different MLSTs and collection dates. Seven MLSTs were present in at least 50 isolates over the collection period (ST 117, 17, 80, 412, 18, 736, and 584). Isolates previously identified to encode class IIa bacteriocins were excluded.[119] Up to three isolates from each of these major MLSTs were selected per year for a total of 113 isolates.

3.3.10 Sample selection for high impact variant analysis

Isolates with uncommon manPTS sequences were selected for more in-depth testing. This included isolates with unique sequences from clades 2 and 3 and isolates with high impact variants. High impact variants were identified start lost, stop gained, or frameshift mutations, insertion elements upstream of or within genes of interest, or deletions within the genes of interest.

3.3.11 Growth in mannose and glucose

Isolates were streaked from freezer stock onto Brain Heart Infusion (BHI) agar (Becton, Dickinson and Company, 211065) and incubated overnight at 35ºC. Colonies were resuspended in 0.85% saline to a McFarland standard between 0.4 and 0.6 McF. 20ul of each colony resuspension were added to 180ul filter sterilized (0.22um filter) chemically defined media (CDM, Appendix Table B.2) containing 1% wt/vol glucose or mannose. Samples were incubated at 35ºC, 500rpm, in the BioTek LogPhase 600 plate reader for 20hrs.

3.3.12 Growth in supernatant

Bac43 supernatant was made by incubating a bac43 producer (PR46485-1-C1) in 50mL BHI batches at 35ºC, 135rpm overnight. Cultures were centrifuged for 20min at 3220rcf and

filter sterilized (0.22um filter) as previously described.[119] To assess sensitivity to the bac43 supernatant, isolates were grown from freezer stock in 250ul BHI and incubated at 35ºC, 135rpm. Cultures were diluted in BHI to 10^{-5}. For isolates in our representative screen, 100ul diluted culture was added to either 100ul 0.85% saline or 100ul bac43 supernatant and were incubated at 35ºC, 500rpm, in the BioTek LogPhase 600 plate reader for 20hrs. For our quantitative testing of isolates from clades 2, and 3, and isolates with high-impact manPTS variant, we performed broth microdilution, whereby the bac43 supernatant was diluted 2-fold using 0.85% saline from down to 2^{-6}. 100ul diluted culture was added to 100ul of each dilution of supernatant (i.e. 1, 0.5, 0.25, 0.125, 0.0625, 0.0312 and 0.156).

3.3.13 Isolation of bac43 resistant mutants

Following an existing protocol[63] we selected for resistant mutants *in vitro* to use in comparison to any *in vivo* resistant mutants. Briefly, group A mutants were selected through plating 10ul of an overnight culture of BL02235-1 onto a BHI agar plate containing 10% bac43 supernatant (SN) by volume. After incubation colonies were re-streaked on BHI+10% SN and stored. Group B mutants were plating on BHI+10% SN after overnight growth in BHI+10% SN.

3.3.14 Analysis of phenotypic data

Growth rates were calculated using the R package growthcurver[131] using the function 'SummarizeGrowthByPlate.' If the difference in OD between time 0hr and time 20hr was less than 0.1 OD_{600}, growth rate was set to 0. EC50 values were estimated using the R package drc[132] using the log-logistic model 'LL.4.'

3.4 Results

3.4.1 Genomic analysis reveals a small population of isolates with high divergence in manPTS.

ManPTS consists of four subunits (EIIB, EIIAB, EIIC, and EIID, encoded by *manX_1*, *manX_2*, *manY*, and *manZ*, respectively) and an upstream sigma-54 transcriptional activator ManR encoded by *manR*. Isolates were analyzed for genomic relatedness by aligning them to the 6kb region of *E. faecium* 6E6 containing these five genes.[63] Among the 2428 clinical *E. faecium* isolates, there were 103 unique alignments to the region of *manR* to *manZ*. Three main phylogenetic clades were identified (Figure 3.1A). Clade 1 accounts for 2407 isolates (99.1%) with 99.3-100% nucleotide identity to the manPTS reference. This clade also shares a unique 61bp region in *manR* that was not detected in the other two clades. The eight isolates in clade 2 present differences from the reference with SNPs throughout the gene cluster, particularly within *manR*, suggestive of recombination events not detected by Gubbins[88] (Figure 3.1B). Clade 3 isolates all share a recombination event identified by Gubbins[88] spanning 399bp and involving subunits *manX_2* and *manY*. The 14 isolates in clade 3 further deviated from the other isolates with recombination events in *manX_2* and *manR*, and mutations resulting in a high level of divergence in *manZ*, *manY*, and *manX_2* (Figure 1B). Isolates were then assigned to *E. faecium* or *E. lactis*, as described in the methods. All clade 1 isolates were identified as *E. faecium* while clade 2 isolates were found to be *E. lactis* (previously classified as clade B *E. faecium*[23]). Of the 14 isolates in clade 3, 13 were also found to be *E. lactis* and one was *E. faecium* (MLST 59).

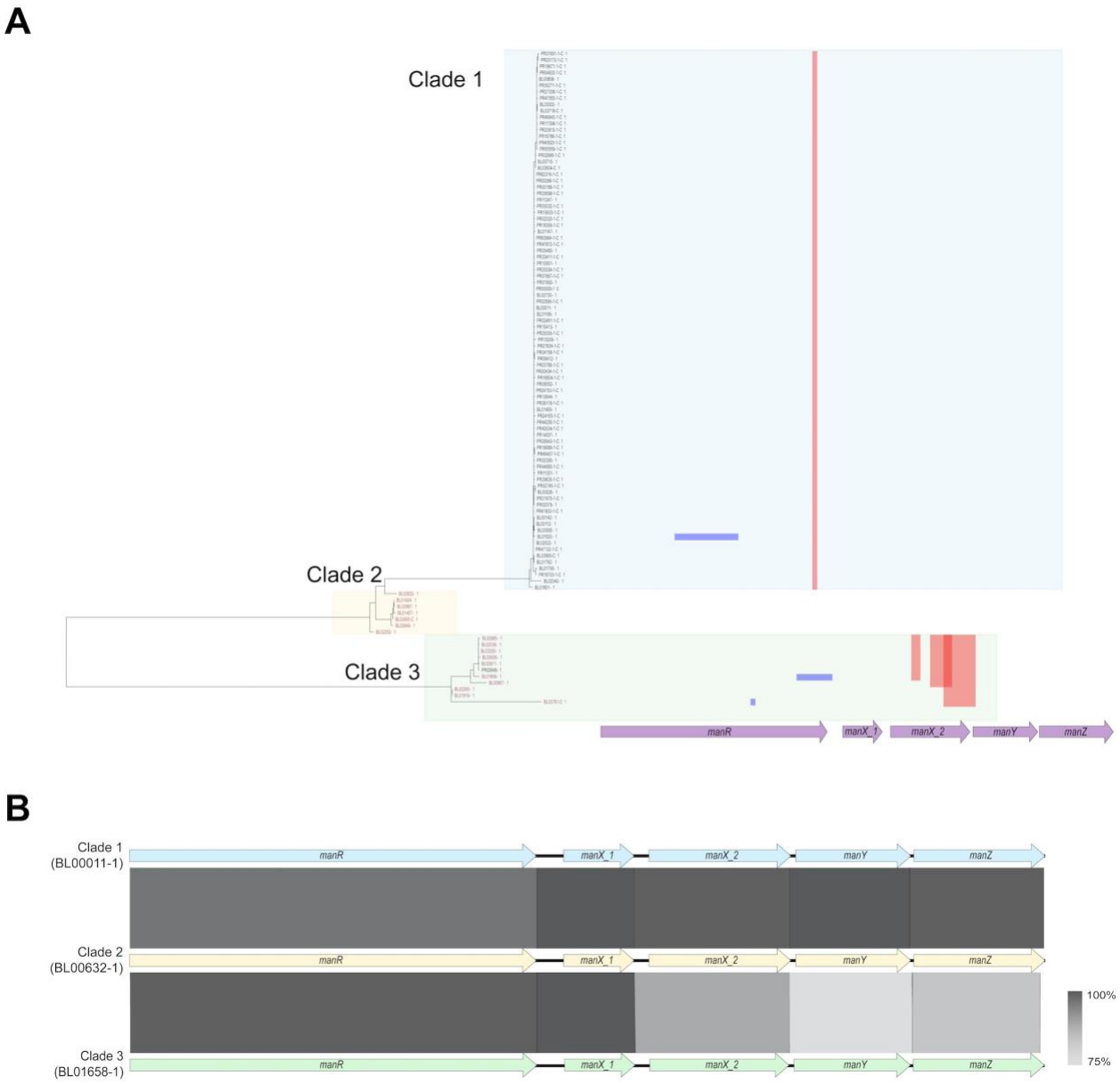


Figure 3.1:Phylogenetic and genomic analysis of the manPTS operon. (A) Phylogenetic tree with three clades created from a multiple sequence alignment of the manPTS operon with duplicates sequences removed. Isolates in black are *E. faecium* and isolates in red are *E. lactis*. Recombination events are identified by Gubbins and Phandango. Red bars indicate recombination occurring in the internal branches (shared by isolates with common ancestry), and blue bars indicate recombination occurring in terminal branches (unique to isolates). (B) Homology mapping between clade representative isolates. Clade 2 is mapped to clade 1, and clade 3 is mapped to clade 2.

3.4.2 **In vitro** ***isolated bac43 resistant mutants demonstrate high level resistance to a bac43 supernatant and a growth defect in mannose.***

To begin to explore the phenotypic traits of bac43 resistant mutants, we first selected resistant mutants *in vitro*. Briefly, we isolated mutants from clinical *E. faecium* sample BL02235-1. Group A mutants (A-1, A-2, and A-3) were isolated through a single selection with bac43 supernatant (SN), and group B mutants (B-1 and B-2) through two rounds of selections with bac43 SN (see Methods). All five resistant mutants expressed high level resistance when grown in bac43 SN compared with the parental sensitive strain BL02235-1(Figure 3.2A). To determine if our *in vitro* mutants had a growth defect in mannose uptake, strains were grown in a chemically defined media (CDM) where mannose (M) or glucose (G) was the primary carbon source, and a control with no additional sugar source added (NS). The parental sensitive strain BL02235-1 had similar growth in both mannose and glucose (Figure 3.2B). However, all five resistant mutants demonstrated decreased growth rates in mannose compared to growth in glucose, with the difference being statistically significant in four of the isolates. While the difference in growth rate for RM A-2 was not statistically significant (p-value 0.173) the impact on growth in mannose is still evident by the lowered OD after 20hrs. In the no sugar control, isolates showed little to no growth demonstrating these mutants are still able to utilize mannose yet to a lesser degree than the non-resistant counterpart. These results support impairment of the manPTS as the mechanism conferring bac43 resistance in *in vitro* selected resistant mutants.

To look for any genomic differences from the parental strain BL02235-1, whole genome sequencing was performed for the *in vitro* selected resistant mutants. The only variations identified were in the manPTS and/or its regulation (Figure 3.2C). RM A-1 and RM B-2 shared a frameshift mutation in *manZ*, RM A-2 had a missense mutation in *manY*, and RM A-3 had a

missense mutation in *manR*. RM B-1 had a 144bp in-frame deletion in *manR*. While all five isolates had similar growth in bac43 SN, the mutation in the gene encoding subunit EIIC from RM A-2 appears to have a lesser impact on mannose utilization compared to the isolates with mutations in the genes encoding ManR and EIID.

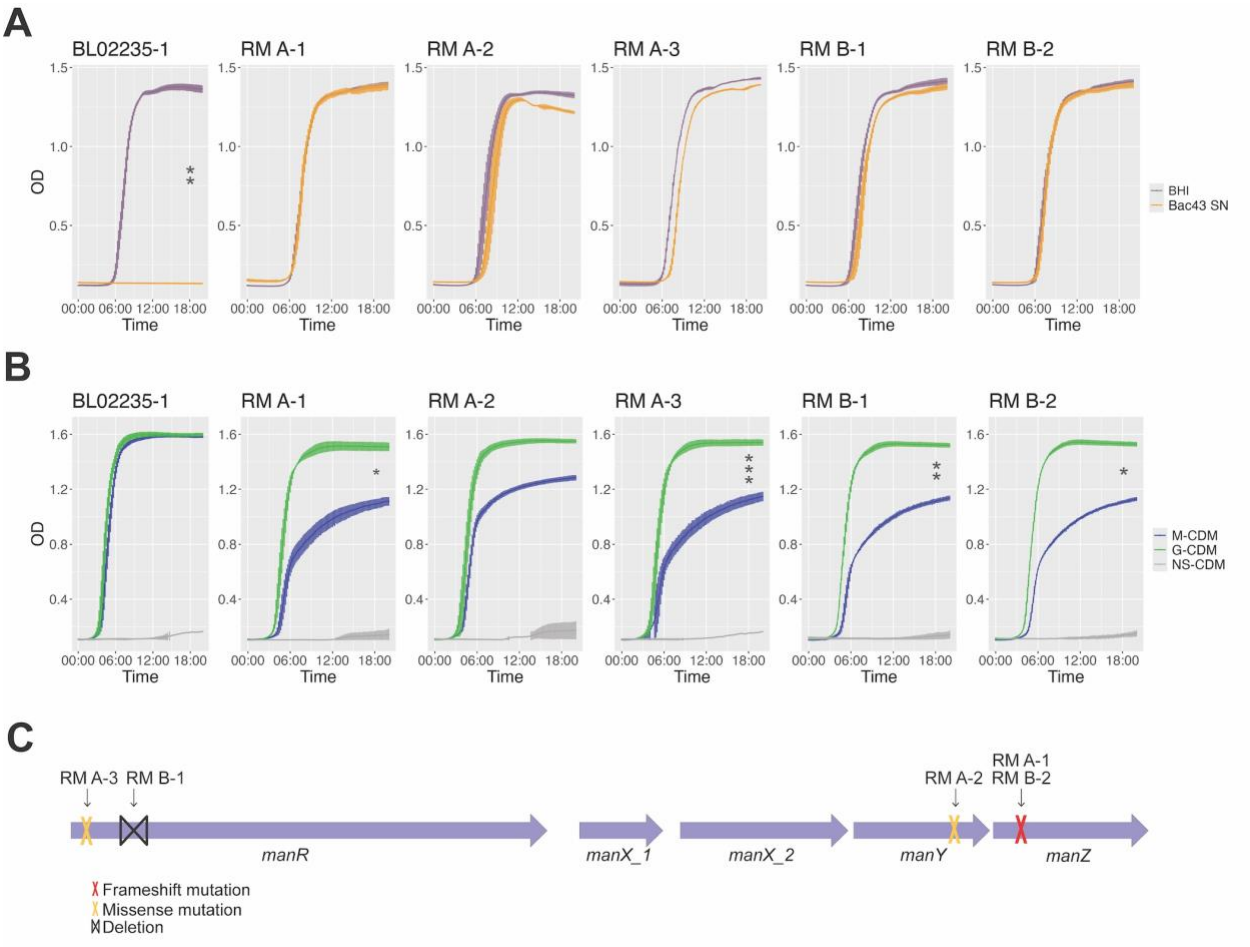


Figure 3.2: Growth curves of *in vitro* selected resistant mutants. Curves represent an average of three replicates and the standard deviation. Unpaired t-test with Bonferroni correction was used for determining a significant difference in the growth rates of the two curves. Significance values: p-value < 0.05 *, p-value < 0.01 **, p-value < 0.001 ***. (A) Growth curves of sensitive parental strain (BL02235-1) and resistant mutants (RMs) grown in nutrient rich media (BHI) and bac43 supernatant (SN). (B) Growth curves of sensitive parental strain (BL02235-1) and RMs grown in a chemically defined media with mannose (M-CDM), glucose (G-CDM), or no sugar (NS-CDM). (C) Location of manPTS variants present in resistant mutants when compared to the parental strains BL02235-1.

3.4.3 Representative screen of isolates reveals resistant mutants are rarely observed in vivo and there is no association with MLST or time.

We next used phenotypic assays to screen for resistance and phenotype/genotype correlations using a collection of clinical isolates differing by MLST and date of isolation. Up to three isolates per major MLST per year were randomly selected resulting in 113 *E. faecium* isolates. One isolate was removed from analysis as it did not reach stationary phase after 20hrs of growth in nutrient rich media for a total of 112 isolates. All isolates genomically fell into clade 1. Growth rate was calculated for all isolates grown in BHI (WT) and bac43 supernatant (SN) (Figure 3.3A).

Ninety-nine isolates failed to grow in SN in three separate replicates. Twelve isolates presented slight growth in SN but only in one or two of the three replicates. One isolate (PR26943-1-C1) presented consistent growth in SN, although the growth rate was lower than our *in vitro* selected resistant mutants. Given the overall growth in supernatant of 17 out of 336 cultures (~5.1%), the probability of finding growth in all three replicates of PR26943-1-C1 is unlikely ($p= 0.014$, binomial sampling, accounting for 112 comparisons), suggesting this isolate is resistant or mutates to resistance at a higher rate than the other isolates. We found neither sample collection date (two-way ANOVA p-value 0.484) nor MLST (p-value 0.645) were significant factors in explaining the variance in growth in SN.

To determine if there was a correlation between bac43 resistance and growth in mannose, isolates were tested in a minimal media with mannose or glucose as the primary carbon source. PR26943-1-C1, which grew in bac43 SN in all three replicates, also demonstrated decreased growth in mannose compared to growth in glucose (Figure 3.3B). Another isolate (PR27634-1-C1) similarly presented a decreased growth rate in mannose but showed no resistance to the SN.

Collection date was not predicted to be a significant factor in explaining the variance in the difference in growth rate between glucose and mannose (two-way ANOVA p-value 0.7834). While MLST was predicted to be statistically associated with the difference in growth rate between glucose and mannose (two-way ANOVA p-value 0.000987), MLST was also associated with estimating growth rate in glucose (one-way ANOVA p-value 0.000602) and growth rate in mannose (one-way ANOVA p-value 0.00662) independently. This suggests any MLST differences are related to overall growth rather than having an association with bac43 resistance.

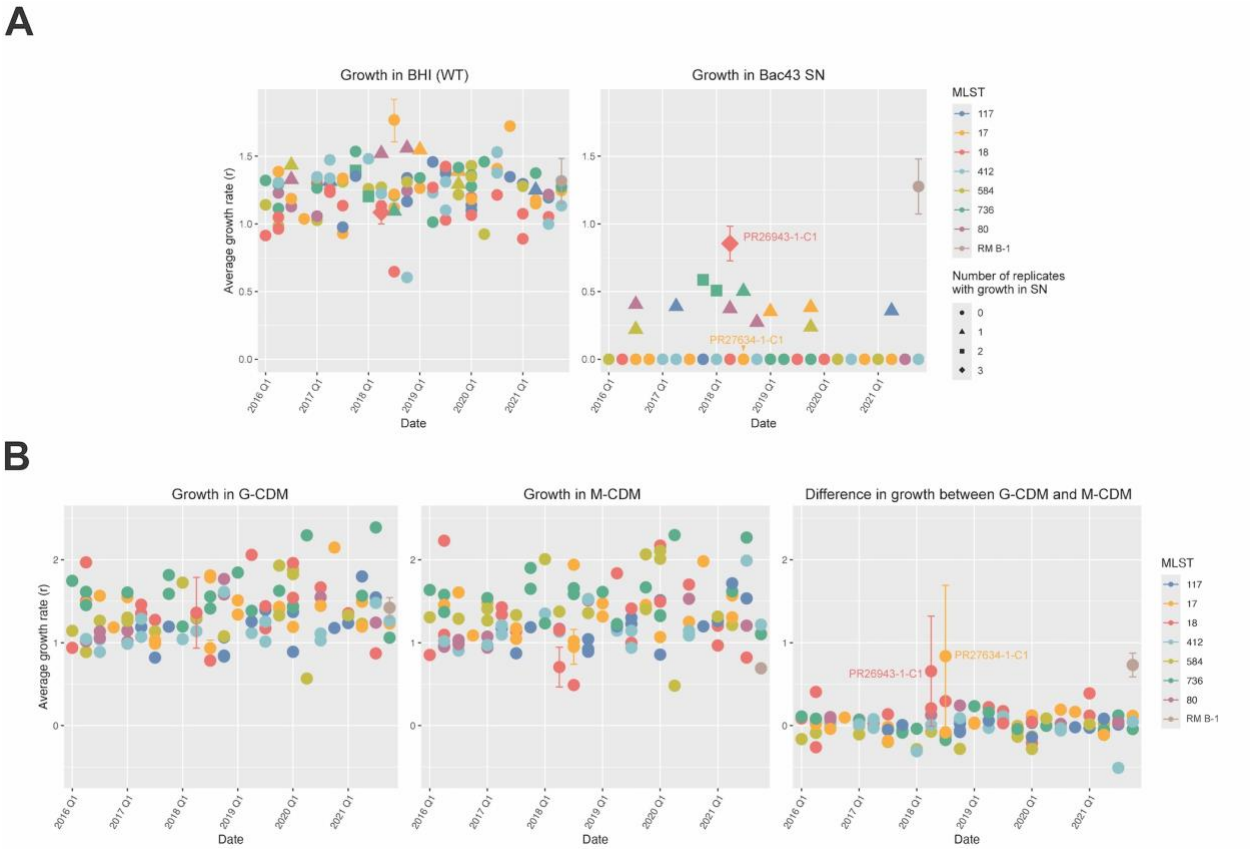


Figure 3.3: Representative screen for resistance and growth in mannose. Data points represent a growth rate average of three replicates and are colored by MLST. Resistant mutant (RM B-1) was used as a control and placed arbitrarily at time 2021 Q4. Error bars represent the standard deviation. (A) Isolate growth rates in nutrient rich media (BHI) and bac43 SN. Shape indicates the number of replicates (out of three) that presented growth in the SN. (B) Isolate growth rates in glucose (G-CDM), mannose (M-CDM), and the growth rate difference between glucose and mannose.

3.4.4 Clade 3 isolates present a 4-fold increase in resistance without growth defects in mannose.

To further explore resistance, we grew the more genetically divergent clade 2 and clade 3 isolates in two-fold dilutions of bac43 supernatant to calculate the half maximal effective concentration (EC50); an EC50 of 1 represents the highest concentration of bac43 SN used in these assays. Clade 2 isolates had slightly higher EC50 values compared to clade 1 isolates (t-test p-value 0.04148; 1.31x more resistant). Unlike the *in vitro* selected resistant strains, these isolates lacked any significant decrease in growth in mannose (Figure 3.4). Thirteen of the clade 3 isolates demonstrated consistently higher EC50 values compared to fifteen randomly selected clade 1 isolates (Welch t-test for unequal variance p-value 0.000345). Clade 3 isolates were 4.06-fold more resistant than clade 1 isolates and 3.11-fold more resistant than clade 2 isolates, but the difference in growth rate between glucose and mannose was again similar to the clade 1 isolates supporting no significant growth defects in mannose. One clade 3 isolate was found to have high level resistance along with a growth defect in mannose. However, this isolate produces another class IIa bacteriocin (enterocin P) and its cognate immunity protein. Cross-immunity has been documented between bacteriocins of the same subtype and we therefore cannot rule out cross-immunity to bac43 in this instance.[133]

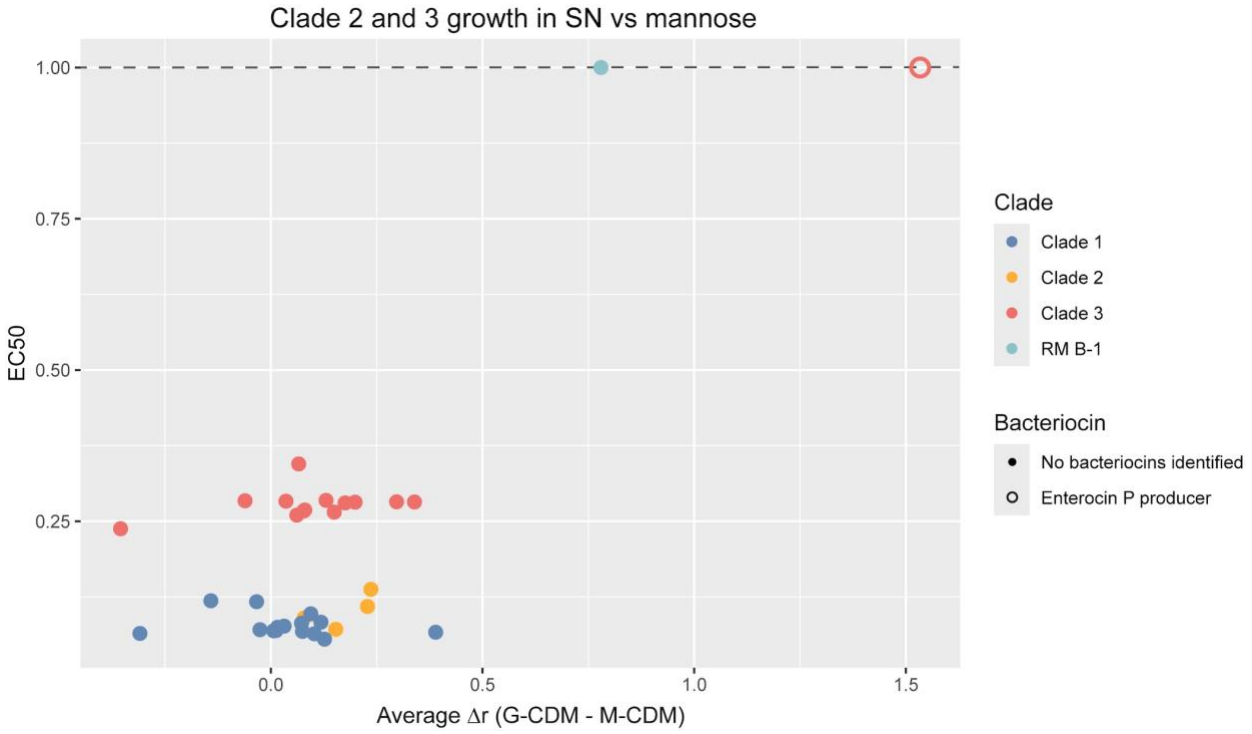


Figure 3.4: Clade 2 and clade 3 isolate growth in SN and in mannose. Isolates with unique sequences in clade 2 and clade 3 were grown in two-fold dilutions of bac43 SN to calculate EC50. EC50 is presented as the dilution of the bac43 SN; the limit of detection is 1 as the highest concentration of SN used in these assays. EC50 is plotted against the average growth rate difference between glucose and mannose. Points represent an average of three replicates and shape identifies a known enterocin P producer.

3.4.5 One clinical isolate with a stop gained mutation in IID demonstrated high level resistance with no growth defect in mannose.

Finally, we screened our collection of genomes for variants that could be highly impactful to the function of manPTS. This included frameshift, stop gained, and start lost mutations, and insertion elements or deletions in the manPTS genes. We identified fifteen isolates (with 12 distinct sequences) with these variants impacting *manR* in nine sequences, *manZ* in two, and *manX_2* in one. One isolate per sequence (12 total) was tested for bac43 resistance by measuring growth in two-fold SN dilutions and growth in mannose. Seven isolates demonstrated high level resistance to bac43 SN (Figure 3.5A). Six of these isolates, however, also encode bac43 and its cognate immunity protein. Only one isolate with a mutation resulting in a stop codon (nonsense mutation) in the gene encoding EIID (*manZ*) demonstrated high level

resistance without encoding any identified bacteriocin sequences (Figure 3.5B). Unexpectedly, this isolate did not show a decreased growth rate in mannose presenting a contrasting phenotype to our *in vitro* selected resistant mutants. Only three isolates (frameshift in *manR*, stop gained in *manR*, and frameshift in *manZ*) showed decreased growth rates in mannose and high-level resistance, but these isolates also encode bac43 so the contribution of mutations to bac43 resistance could not be independently assessed. In total, eight out of 12 isolates with high impact variants encode bac43. While bac43 production in the population as a whole is not significantly associated with these variations in manPTS (Fishers exact p-value 0.297), alteration of the manPTS may be a method employed by bac43 producers to gain resistance to other bacteriocins targeting the manPTS.

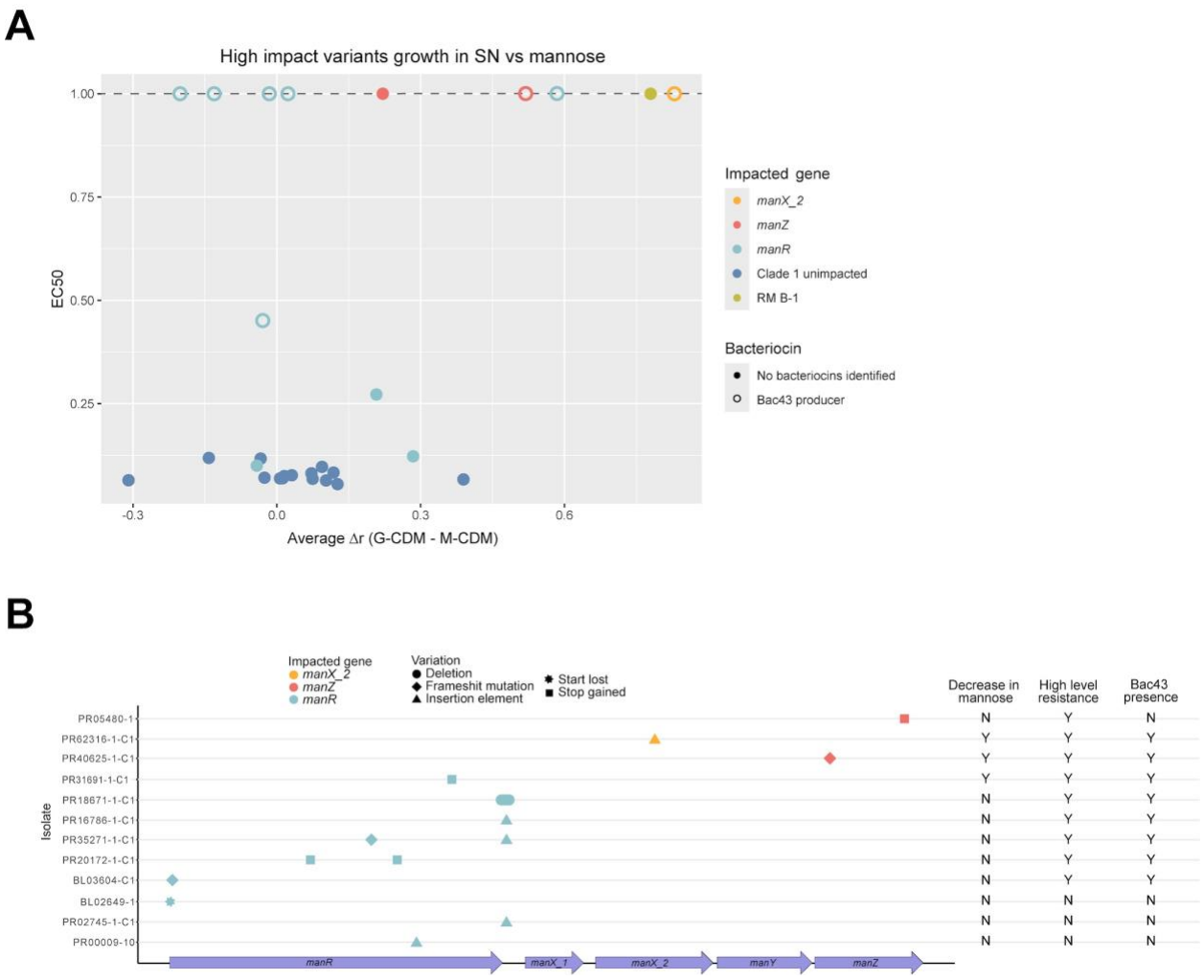


Figure 3.5: High impact variant influence on resistance and growth in mannose. (A) Isolates with high impact variants (frameshift, start lost, stop gained, insertion elements, or deletions) were grown in two-fold dilutions of bac43 SN to calculate EC50. EC50 is presented as the dilution of the bac43 SN; the limit of detection is 1 as the highest concentration of SN used in these assays. EC50 is plotted against the average growth rate difference between glucose and mannose. Points represent an average of three replicates and shape identifies known bac43 producers. (B) Map of the manPTS operon, the locations of high impact variants, and each isolates experimental results for resistance and growth in mannose.

3.5 Discussion

In bacteriocin-producing populations, resistant mutants can help in maintaining diversity of producer, resistant, and sensitive cells,[40,41] but knowledge is lacking on how this role unfolds in naturally occurring populations. The mannose phosphotransferase system (manPTS) impacts multiple functions in the cell including the uptake and phosphorylation of carbohydrates, carbon

catabolite repression, and conferring sensitivity to class IIa bacteriocins.[58] Resistance to these bacteriocins through downregulation of the manPTS operon has been observed arising readily *in vitro*,[54–57,63,64,120,121] but these mutants are most likely rare *in vivo* as manPTS has been implicated with providing critical contributions to colonization.[62,122–125] Previously, a six-year clinical cohort of *E. faecium* isolates from a single hospital system was found to contain a class IIa bacteriocin, bac43, with a rising prevalence over time.[119] Here we present an exploration of bac43 resistance within this cohort and investigate any associations between bac43 resistance and the rise in bacteriocin production.

A representative subsample of isolates stratified by time and MLST were tested for resistance to bac43 and for any potential growth defects in media in which mannose was the primary carbon source. Of 112 isolates tested from seven different MLSTs and from different time points over six years, we identified no isolates with high-level resistance, and thus no trend in resistance. A single isolate with a unique missense mutation in *manR* was identified to consistently grow in the presence of bac43, but with a level of growth much lower than seen in out *in vitro* selected mutants. This isolate did however demonstrate a growth defect in mannose similar to the *in vitro* selected mutants.

In examining the genetic sequence of the manPTS coding region, we identified three separate clades. Clade 3 isolates were the most divergent in the genes encoding subunits EIID, EIIC, and EIIAB, possibly as a result of a horizontal gene transfer event. Clade 3 isolates demonstrated 4-fold higher resistance to bac43 supernatant than clade 1 isolates with no impact on mannose nutrient utilization. Furthermore, of the 14 isolates in this clade, 13 were found to be more genomically related to *E. lactis*. One clade 3 sequence, however, was found in a *E. faecium*, supporting horizontal gene transfer between *E. faecium* and *E. lactis*. Our results are

consistent with these divergent subunits having functional mannose transportation but decreased bac43 binding presumably due to the altered protein sequence. While these sequences of manPTS provide a degree of bac43 resistance, we did not identify an increase in their prevalence during the collection period of our cohort suggesting this phenotype was not under positive selection during the study period.

Consistent with a prior study of isolates resistant to a cocktail of class IIa bacteriocins,[63] our *in vitro* selected bac43 resistant mutants consistently demonstrated high-level resistance and a defect in mannose utilization. These isolates differed from their parental sensitive strain only in the manPTS operon or regulatory region supporting the role of manPTS in resistance. However, this mannose growth defect was not consistently seen in the few cases of high-level or slight resistance we identified *in vivo*. All the high-level resistant isolates identified that demonstrated this growth defect also encoded another class IIa bacteriocin (either bac43 or enterocin P) muddling the source of resistance and phenotype/genotype assessment of changes in the manPTS. The one high-level resistant isolate without any identified bacteriocins still had no growth defect in mannose, even with a stop gained mutation in the gene encoding EIID that would be predicted to disrupt function. As bacteria can encode redundancies in manPTS,[134,135] we speculate additional encoded subunits or full operons of manPTS could be used for compensation.

While bac43 resistance arises readily *in vitro*, this investigation reveals that the phenotypic pattern seen in those mutants is not reflected in the *in vivo* isolates suggesting the associated cost is unsustainable *in vivo*. It is more likely cells have evolved on a longer time scale to have a slight level of resistance without incurring the same impacts to nutrient utilization. While resistance to class IIa bacteriocins have been explored *in vitro*, we still have

much to learn about resistance in *in vivo* populations. This study presents one of the first explorations of resistance *in vivo* by utilizing a six-year cohort of clinical *E. faecium* isolates collected from a single hospital system. Our *in vivo* findings support the selection against isolates with bac43 resistance associated with impaired mannose uptake and this study takes the first step in unraveling the ecological dynamics of bacteriocin-producing populations *in vivo*.

Chapter 4 Generation of Fluorescent Isogenic *E. faecium* Bac43 Producers and Competition in *C. elegans*

4.1 Preface

We present this chapter primarily as a methods paper. We provide an in-depth protocol for the generation of isogenic *E. faecium* strains resulting from the modification of fluorescently labeled plasmids with genes for bacteriocin production and immunity. We validate the expected bacteriocin activity of these strains and begin experimentation with a potential model to study bacteriocin competition.

4.2 Introduction

Vancomycin resistant enterococci (VRE, which refers primarily to *E. faecalis* and *E. faecium*) is a serious public health risk.[6,7] Gut colonization by VRE tends to proceed infection[14–17] and understanding the mechanisms of VRE gut colonization is an important step in ultimately slowing transmission. In a mouse model of enterococcal colonization, a bacteriocin producing *E. faecalis* strain was able to outcompete non-bacteriocin producing *E. faecalis* strains and establish long-term gut colonization demonstrating the competitive advantage that can be conferred by bacteriocins.[42] Previously, we identified the bacteriocin bac43 to be highly prevalent and phenotypically functional within a clinical *E. faecium* cohort collected from a single hospital system.[119]

In this protocol, we created and utilized fluorescently labeled *E. faecium* to study competition between bacteriocin and non-bacteriocin producing strains. We aimed to better

understand how bacteriocins influence population structures by visualizing the geography of competition and tracking strain changes over time. By generating isogenic strains that differ only in their bacteriocin genes and fluorescent markers, we could observe competition between different bacteriocin phenotypes. To this end, we utilized Gibson Assembly[136] to modify fluorescently labeled plasmids[137] to carry the genes for bac43 production and immunity (*bacAbacB*) and the immunity gene alone (*bacB*). Enterococcus species present many barriers to transformation with foreign DNA including a thick cell wall, presence of restriction-modification systems, and lack of natural cell competence.[138] However, using electroporation[139] we were successfully able to generate *E. faecium* and *E. faecalis* strains expressing our plasmids of interest.

Using these strains, we next attempted to devise an experimental model that would allow us to visualize the influence of bacteriocins in competition. The nematode model system *Caenorhabditis elegans* has previously been used to study bacterial communities[140,141] as well as virulence in *E. faecium* and *E. faecalis.*[142,143] Theoretically, *C. elegans* could provide and a spatially structured environment, like the human GI tract, where bacteria would be in close proximity for interaction. While we were able to successfully visualize our strains in the *C. elegans* gut, our experimental design left us unable to visually identify any bacteriocin influence on the population. Further work would be required to make *C. elegans* a successful model for studying bacteriocin competition. However, the strains generated here may prove useful for other investigations.

4.3 Methods

4.3.1 Key resources:

Reagent or Resource	Source	Identifier
Reagents		
BHI Gibco™ BBL™ Dehydrated Culture Media: Brain Heart Infusion Agar	Fisher	Cat #: 211065
BHI Broth- Gibco™ Bacto™ Brain Heart Infusion	Fisher	Cat #: 237500
Spectinomycin (100mg/mL)	Millipore-Sigma	Cat #: S0692-1ML
QIAprep Spin Miniprep Kit	Qiagen	Cat #: 27104
DreamTaq Green PCR master mix	ThermoFisher	Cat #: K1081
Q5 high-fidelity DNA polymerase	NEB	Cat #: M0491S
Gel Loading Dye Purple (6x), no SDS	NEB	Cat #: B7025S
Invitrogen SYBR Safe DNA Gel Stain	Fisher	Cat #: S33102
NEBuilder HiFi DNA assembly cloning kit	NEB	Cat #: E5520S
Agarose (Broad Separation Range for DNA/RNA/Genetic Analysis Grade), Fisher BioReagents™	Fisher	Cat #: BP1356-100
Bacteriological Agar, Ultrapure, Thermo Scientific Chemicals	Fisher	Cat #: AAJ10906P2
Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water	Fisher	Cat #: 10977015
PBS, Phosphate Buffered Saline, 1X Solution, pH 7.4, Fisher BioReagents	Fisher	Cat #: BP243820
Triton X-100	Fisher	Cat #: HFH10
Thermo Scientific Chemicals Paraformaldehyde, 16% w/v (PFA)	Fisher	Cat #: AA433689M
Vector Laboratories VECTASHIELD Antifade Mounting Medium	Fisher	Cat #: NC9265087
Bacterial Strains		
Enterococcus faecalis strain OG1RF	(Hallinen et al. 2020)[137]	NA
Enterococcus faecalis strain OG1RF + pBSU101-PP-GFP2 (*E. faecalis*+pBSU101-GFP)	(Hallinen et al. 2020)[137]	NA

Enterococcus faecalis strain OG1RF + pBSU101-PP-RFP2 (*E. faecalis*+pBSU101-RFP)	(Hallinen et al. 2020)[137]	NA
Enterococcus faecium BL02235-1 (Human blood isolate; no bacteriocins identified; MLST 80)	This study	NA
Enterococcus faecium PR46485-1-C1 (Human perirectal isolate; bac43 producer; MLST 117; GenBank accession number: JBEFKL000000000.1)	(Garretto et al. 2024)[119]	NA
Oligonucleotides		
pBSU101_OptimizedRudolph_fwd taaaattataGTCGCTCTTCTTGATGGAGC	IDT	NA
pBSU101_OptimizedRudolph_rev ttggagttaaAGGCTGCGCAACTGTTGG	IDT	NA
*bacA_bacB*_fwd tgcgcagcctTTAACTCCAAATACCAATAGAAG	IDT	NA
*bacA_bacB*_rev gaagagcgacTATAATTTTATTTAAACGATTATGAATTCAAAAC	IDT	NA
pBSU101_*bacB*only_fwd cctaccactaTTTCATGATTGTTTTTCCTTTTC	IDT	NA
pBSU101_*bacB*only_rev aatcatgaaaTAGTGGTAGGGAGGTTATTATAATG	IDT	NA
*bacAbacB*_insert_id_fwd cctgtaccgatgccagctag	IDT	NA
*bacAbacB*_insert_id_rev gctggcgaagatcgaagaga	IDT	NA
*bacB*_insert_id_fwd ccctggcgttacccaactta	IDT	NA
*bacB*_insert_id_rev tcatgaaatagtggtagggaggt	IDT	NA

4.3.2 Materials and equipment:

- Spectinomycin working stocks (2000ug/ml): Mix 1ml of spectinomycin (100mg/mL) with 49mL ultrapure water, sterilize through a 0.2-um filter, and store 1.5mL aliquots at −20°C. Once thawed aliquot must be used that day or discarded.
- BHI/spec broth (120ug/ml): BHI liquid media was supplemented with 120ug/mL spectinomycin.
- BHI/spec agar (120ug/ml): BHI liquid agar was supplemented with 120ug/mL spectinomycin.

Note: Used for selection of samples containing plasmids.

- 10% glycerol: Mix 400mL glycerol with 1600mL water (Milli-Q® system, Millipore) and autoclave by sterilizing.

  Note: Used for electrocompetent cell generation.

- Freezer stock media: Aliquot 1.5mL of BHI broth supplemented with of 20% glycerol into 2-mL screw cap tubes and sterilize by autoclaving.

  Note: Used for long term storage of bacterial clones.

- BHI soft agar: Mix BHI broth with 0.75% agar and sterilize by autoclaving.

  Note: Used for confirmation of bacteriocin activity.

- 1x PBST: Mix 100ml PBS with 0.1ml Triton X-100.

  Note: Used for washing *C. elegans*.

- 4% PFA (paraformaldehyde) fixative: Mix 200ul 5x sperm salts (50 mM Pipes pH 7, 25 mM KCl, 1 mM $MgSO_4$, 45 mM NaCl and 2 mM $CaCl_2$), 250ul PFA 16%, and 550ul Milli-Q water. Store at 4^{o}C and protect from light.

  Note: Used for fixing *C. elegans*.

- Dilution of oligonucleotides: Resuspend dry oligonucleotides in ultrapure water for 100uM concentration. Make 10uM working stocks with 50uL 100uM oligonucleotide and 450uL ultrapure water. Store at -20^{o}C.

4.3.3 Electrocompetent cell generation:

Note: Cells were generated for *E. faecium* BL02235-1 and *E. faecalis* OG1RF.

1. Grow 1L BHI broth of sample. Incubate overnight at 35^{o}C, 130rpm.
2. Aliquot samples into 50mL falcon tubes and centrifuge 15min at 3220rcf.
3. Remove supernatant and wash cells with 1L 10% glycerol (50ml per tube)

4. Repeat centrifugation.
5. Repeat steps 3 and 4 using 500mL 10% glycerol (25mL per tube).
6. Repeat steps 3 and 4 using 200mL 10% glycerol (10mL per tube).
7. Repeat steps 3 and 4 using 20mL 10% glycerol (1mL per tube).
8. Combine cell pellets and resuspend cells in 5mL 10% glycerol.
9. Store cells in 500ul aliquots at -80C (do not refreeze aliquots)

4.3.4 Transform pBSU101 plasmids into E. faecium *BL02235-1:*

Note: pBSU101-GFP and pBSU101-RFP were transformed into BL02235-1. Protocol is written for pBSU101-GFP

Note: Samples generated:

E. faecium BL02235-1 + pBSU101-GFP

E. faecium BL02235-1 + pBSU101-RFP

1. Extraction of plasmid
    a. Grow 10mL BHI/spec broth *E. faecalis* + pBSU101-GFP.
    b. Incubate overnight at 35ºC, 130rpm.
    c. Use QIAprep Spin Miniprep Kit following manufacturer's instructions to extract plasmid pBSU101-GFP from *E. faecalis* + pBSU101-GFP.
    d. Store plasmid DNA at -20ºC.
2. Electroporation
    a. Thaw electrocompetent cells on ice
    b. Mix 50ul cells with 1ul miniprep plasmid DNA
    c. Vortex briefly and add to ice chilled 0.2cm cuvette
    d. Give one pulse at 3.0kV (E=15kV/cm)

e. In cuvette mix 1mL BHI with cells

f. Transfer mixture to Eppendorf tube

g. Incubate shaking for 1hr at 37°C, 300rpm.

3. Extraction of plasmid and storage

a. Re-streak colonies from electroporation onto BHI/spec agar plates.

b. Use an isolated colony to grow 10mL BHI/spec broth. Incubate overnight at 35°C, 130rpm.

c. Store an isolated colony in tube with freezer stock media at -80°C.

d. Use QIAprep Spin Miniprep Kit following manufacturer's instructions to extract plasmid pBSU101-GFP from *E. faecium* + pBSU101-GFP.

e. Store plasmid DNA at -20°C.

f. Send 20ul for sequencing by Eurofins Genomics, whole plasmid sequencing.

4.3.5 Gibson assembly of plasmid with bac43 genes (pAMG100 and pAMG200):

Note: pBSU101-GFP was used as the starting plasmid to generate pAMG100 and pBSU101-RFP was used as the starting plasmid to generate pAMG200. Protocol is written for pAMG100.

1. Extraction of starting plasmid

a. Grow 10mL BHI/spec broth *E. faecalis* + pBSU101-GFP, and 10mL BHI broth of PR46485-1-C1

b. Incubate overnight at 35°C, 130rpm.

c. Use QIAprep Spin Miniprep Kit following manufacturer's instructions to extract plasmid pBSU101-GFP from *E. faecalis* + pBSU101-GFP, and pDT1 from PR46485-1-C1.

d. Store plasmid DNA at -20°C.

2. Amplify fragments

    a. Prepare PCR mix to linearize pBSU101-GFP:

DNA fragment generation		
Reagent	Final concentration	Amount
5X Q5 reaction buffer	1X	5ul
10mM dNTPs	200uM	0.5ul
10uM pBSU101_OptimizedRudolph_fwd	0.5uM	1.25ul
10uM pBSU101_OptimizedRudolph_rev	0.5uM	1.25ul
Q5 High-Fidelity DNA Polymerase	0.02U/uL	0.25ul
pBSU101-GFP DNA	-	1ul
Ultrapure water	-	15.75
Total		25ul

    b. Prepare PCR mix to isolate *bacAbacB* fragment from pDT1 using 'DNA fragment generation' mix with pDT1 DNA, and primers '*bacA_bacB*_fwd' and '*bacA_bacB*_rev'

    c. Incubate mixes using thermocycler conditions:

Fragment amplification conditions			
Step	Temperature	Time	Cycles
1. Initial denaturation	98°C	30s	1
2. Denaturation	98°C	10s	30 (Steps 2-4)
3. Annealing	66°C	20s	
4. Extension	72°C	3min	
5. Final extension	72°C	2min	1
6. Final incubation	4°C	hold	

3. Confirm band size by gel electrophoresis

    a. Make 2% agarose gel with 2g agarose, 100mL 1x TAE, and 5ul SYBRSafe

    b. Load wells with 5ul PCR product mixed with 1.5ul loading dye

    c. Run gel at 80V for 40min.

    d. pBSU101-GFP DNA fragment expected length = 6668bp

    e. pDT1 DNA fragment expected length = 696bp

4. HiFi DNA assembly

    a. Using NEBuilder HiFi DNA assembly cloning kit, mix components on ice:

b. Incubate sample in thermocycler at 50ºC for 15min

Reagent	Amount
pSBU101_GFP PCR product	1uL
pDT1 PCR product	2uL
HiFi master mix	10uL
Ultrapure water	7ul
Total	20ul

c. Store on ice (or -20ºC) for transformation

5. Transformation into *E. coli*

    a. On ice, thaw chemically competent *E. coli* (C2987) provided by NEBuilder HiFi DNA assembly cloning kit

    b. Mix chilled assembled product by pipetting and add 2ul to *E. coli* cells – do not mix.

    c. Place mixture on ice for 30min.

    d. Heat shock at 43ºC for 30min.

    e. Transfer tubes to ice for 2min.

    f. Add 950ul room temperature SOC media (NEBuilder HiFi DNA assembly cloning kit)

    g. Spread 200ul cells onto BHI/spec agar places

    h. Incubate overnight at 35ºC

6. Colony PCR

    a. Screen colonies by touching each colony into 50ul ultrapure water and onto BHI/spec agar plate.

    b. Incubate BHI/spec plate overnight at 35ºC.

    c. Incubate colony resuspensions for 10min at 98ºC

    d. Prepare PCR mix:

Colony PCR reaction		
Reagent	Final concentration	Amount
DreamTaq Green Master Mix	-	12.5ul
10uM *bacAbacB*_insert_id_fwd	0.5uM	1ul
10uM *bacAbacB*_insert_id_rev	0.5uM	1ul
Colony resuspension	-	1ul
Ultrapure water	-	9.5ul
Total		25ul

e. Incubate mixes using thermocycler conditions:

Colony PCR thermocycler conditions			
Step	Temperature	Time	Cycles
1. Initial denaturation	95ºC	2min	1
2. Denaturation	95ºC	30s	30 (Steps 2-4)
3. Annealing	66ºC	30s	
4. Extension	72ºC	1min	
5. Final extension	72ºC	5min	1
6. Final incubation	4ºC	hold	

7. Confirm band size by gel electrophoresis. Loading dye is not needed for PCR samples run with DreamTaq Green Master Mix

    a. Expected length = 540bp

8. Extract assembled product:

    a. From BHI/spec agar touch plate, identify colonies with confirmed assembled product.

    b. Re-streak confirmed colonies onto BHI/spec agar and incubate overnight at 35ºC.

    c. Use a single colony to grow 10ml BHI/spec broth, incubate overnight at 35ºC, 130rpm.

    d. Use QIAprep Spin Miniprep Kit to extract pAMG100.

    e. Store plasmid DNA at -20ºC.

    f. Use 20ul of DNA to send for sequencing.

4.3.6 Transform plasmid with bac43 genes (pAMG100 and pAMG200) into **E. faecium** *and* **E. faecalis***:*

Note: Both pAMG100 and pAMG200 were transformed into *E. faecium* BL02235-1 and *E. faecalis* OG1RF.

Note: Samples generated:

E. faecium BL02235-1 + pAMG100

E. faecium BL02235-1 + pAMG200

E. faecalis OG1RF + pAMG100

E. faecalis OG1RF + pAMG200

1. Electroporation
    a. Use electroporation to insert plasmid into strain of interest (see "Transform pBSU101 plasmids into *E. faecium* BL02235-1" step 2 "Electroporation")
2. Colony PCR
    a. Confirm insertion of plasmid using 'Colony PCR reaction' with primers '*bacAbacB*_insert_id_fwd' and '*bacAbacB*_insert_id_rev'. Incubate mixes using 'Colony PCR thermocycler conditions'
    b. Use gel electrophoresis to confirm band size.
    c. Expected band length = 540bp
3. Storage
    a. From BHI/spec agar touch plate, identify colonies with confirmed assembled product.
    b. Re-streak confirmed colonies onto BHI/spec agar and incubate overnight at 35ºC. Store colony off re-streak in tube with freezer storing media at -80ºC.

4.3.7 Gibson assembly of plasmid with bac43 immunity gene (pAMG101 and pAMG201):

Note: pAMG100 was used as the starting plasmid to generate pAMG101 and pAMG200 was used as the starting plasmid to generate pAMG201. Protocol is written for pAMG101.

1. Amplify fragment
    a. Prepare PCR mix linearize pAMG100 using 'DNA fragment generation' mix with pAMG100 DNA, and primers 'pBSU101_*bacB*only_fwd' and 'pBSU101_*bacB*only_rev.'
    b. Amplify fragment using 'Fragment amplification conditions' with an extension time of 3.5min.
2. Confirm band size by gel electrophoresis. Use 1.5ul loading dye mixed with PCR product.
3. HiFi DNA assembly
    a. Using NEBuilder HiFi DNA assembly cloning kit, mix components on ice:
    b. Incubate sample in thermocycler at 50^{o}C for 15min

Reagent	Amount
pAMG100 PCR product	1uL
HiFi master mix	10uL
Ultrapure water	9ul
Total	20ul

    c. Store on ice (or -20^{o}C) for transformation
4. Transform assembled product into *E. coli* (see "Gibson assembly of plasmid with bac43 genes (pAMG100 and pAMG200)" step 5 "Transformation into E. coli")
5. Colony PCR
    a. Confirm insertion of plasmid using 'Colony PCR reaction' with primers '*bacB*_insert_id_fwd 'and '*bacB*_insert_id_rev'. Incubate mixes using 'Colony PCR thermocycler conditions'

b. Use gel electrophoresis to confirm band size.

c. Expected length ~ 8100bp

6. Extract assembled product pAMG101 using QIAprep Spin Miniprep Kit.

    a. Use 20ul of DNA to send for sequencing.

4.3.8 Transform plasmid with bac43 immunity gene (pAMG101 and pAMG201) into E. faecium:

Note: Both pAMG100 and pAMG200 were transformed into *E. faecium* BL02235-1.

Note: Samples generated:

E. faecium BL02235-1 + pAMG101

E. faecium BL02235-1 + pAMG201

1. Electroporation

    a. Use electroporation to insert plasmid into strain of interest. (see "Transform pBSU101 plasmids into *E. faecium* BL02235-1" step 2 "Electroporation")

2. Colony PCR

    a. Confirm insertion of plasmid using 'Colony PCR reaction' with primers '*bacB*_insert_id_fwd' and '*bacB*_insert_id_rev'. Incubate mixes using 'Colony PCR thermocycler conditions'

    b. Use gel electrophoresis to confirm band size.

    c. Expected length = 426bp

3. Storage

    a. From BHI/spec agar touch plate, identify colonies with confirmed assembled product.

    b. Re-streak confirmed colonies onto BHI/spec agar and incubate overnight at

c. Store colony off re-streak in tube with freezer storing media at -80ºC.

4.3.9 Confirmation of expected activity:

Note: Strains used a bacteriocin sensitive:

E. faecium BL02235-1 + pBSU101-GFP

E. faecium BL02235-1 + pBSU101-RFP

E. faecalis OG1RF + pBSU101-GFP

E. faecalis OG1RF + pBSU101-RFP

Note: Strains tested for bacteriocin production:

E. faecium BL02235-1 + pAMG100

E. faecium BL02235-1 + pAMG200

E. faecalis OG1RF + pAMG100

E. faecalis OG1RF + pAMG200

Note: Strains tested for bacteriocin immunity:

E. faecium BL02235-1 + pAMG101

E. faecium BL02235-1 + pAMG201

1. Bacteriocin production
    a. Streak bacteriocin producer on BHI/spec agar, incubate overnight at 35ºC.
    b. Grow 3mL BHI/spec broth of bacteriocin sensitive strain, incubate overnight at 35ºC, 130rpm.
    c. Make sensitive lawn with 1mL overnight culture bacteriocin sensitive strain mixed with 3mL molten BHI soft agar spread on a BHI agar plate. Allow to dry (~1min).
    d. Using a toothpick poke a colony of bacteriocin producer into lawn.

e. Incubate overnight at 35ºC.

f. Confirm bacteriocin activity by the presence of a halo of inhibition surrounding the poke.

2. Bacteriocin immunity

a. Streak bacteriocin producer on BHI/spec agar, incubate overnight at 35ºC.

b. Grow 3mL BHI/spec broth of bacteriocin immune strain, incubate overnight at 35ºC, 130rpm.

c. Make immune lawn with 1mL overnight culture bacteriocin immune strain mixed with 3mL molten BHI soft agar spread on a BHI agar plate. Allow to dry (~1min).

d. Using a toothpick poke a colony of bacteriocin producer into lawn.

e. Incubate overnight at 35ºC.

f. Confirm immunity of lawn strain by the absence of a halo of inhibition surrounding the poke.

4.3.10 C. elegans *model of* E. faecium *competition:*

Note: Strains used:

E. faecium BL02235-1 + pBSU101-GFP (bac43 -)

E. faecium BL02235-1 + pBSU101-RFP (bac43 -)

E. faecium BL02235-1 + pAMG100 (bac43 +)

Note: Transfer sets (initial plate listed first, transfer plate listed second):

A: *E. faecium* BL02235-1 + pBSU101-RFP (bac43 -) → *E. faecium* BL02235-1 + pBSU101-GFP (bac43 -)

B: *E. faecium* BL02235-1 + pBSU101-GFP (bac43 -) → *E. faecium* BL02235-1 + pBSU101-RFP (bac43 -)

C: *E. faecium* BL02235-1 + pAMG100 (bac43 +) → *E. faecium* BL02235-1 + pBSU101-RFP (bac43 -)

D: *E. faecium* BL02235-1 + pBSU101-RFP (bac43 -) → *E. faecium* BL02235-1 + pAMG100 (bac43 +)

1. Initial plates
    a. Day 1: Grow 2ml BHI/spec broth of each of the four strains, incubate overnight at 35ºC,130rpm.
    b. Day 2: Spread 10ul overnight culture in the center of a BHI/spec agar plate. Incubate overnight at 35ºC
    c. Day 2: Grow 2ml BHI/spec broth of each of the four strains, incubate overnight at 35ºC,130rpm.
2. Set up *C. elegans*
    a. *C. elegans* strain N2 were grown and maintained on *E. coli* OP50 using standard techniques.[144]
    b. Day 3: Use a dissecting scope to pick and add 40 L4 *C. elegans* to each initial plate. Parafilm plates closed and incubate overnight at 20ºC.
    c. Day 3: Set up transfer plates. Spread 10ul overnight culture in the center of a BHI/spec agar plate. Incubate overnight at 35ºC
3. Transfer *C. elegans*
    a. Day 4: Transfer 20 worms from each initial plate to indicated transfer plate with alternative fluorophore.
    b. Day 4: After 10min sacrifice and fix five worms
        i. Use dissecting scope to pick five worms

ii. Add worms to a well with 100ul 4% PFA fixative

iii. Allow to sit for 10min

iv. Wash worms by transferring to a well with 100ul 1x PBST

v. Add a 5ul 1x PBST to slide

vi. Transfer worms to slide

vii. Add a drop of mounting media (~20ul) and add cover slip

c. Day 4: Repeat step b twice more (total time - 30min)

d. Day 5: Sacrifice and fix remaining five worms.

4.4 Results

4.4.1 Strains generated.

Here we have successfully transformed *E. faecium* with fluorescent plasmids from Hallinen et al.[145] (Figure 4.1A) as well as genetically modified versions of these plasmids containing the genes for bac43 production (*bacAbacB*)(Figure 4.1B-C) and for bac43 immunity (*bacB*)(Figure 4.1D). We also transformed *E. faecalis* with a plasmid containing the genes for bac43 production (*bacAbacB*)(Figure 4.1B-C). These plasmids were all sequenced to confirm maintenance of the plasmid backbone and proper insertion of expected genes (Figure 4.2A-F).

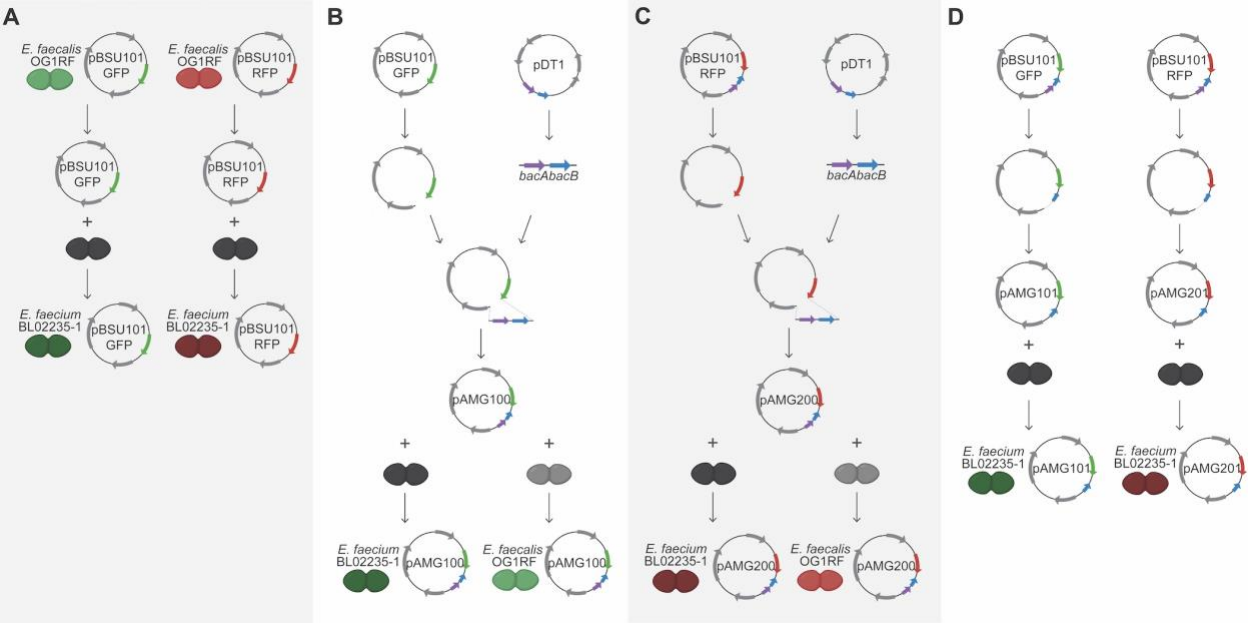


Figure 4.1: Strain generation overview. (A) Generation of *E. faecium* BL02235-1+pBSU101-GFP and *E. faecium* BL02235-1+pBSU101-RFP. The pBSU101-GFP and pBSU101-RFP plasmids were extracted from *E. faecalis* OG1RF and inserted into *E. faecium* BL02235-1 (B) Generation of *E. faecium* BL02235-1 (right) +pAMG100 and *E. faecalis* OGRF1+pAMG100 (left). The *bacAbacB* genes were cut from the pDT1 plasmid and inserted into the linearized pBSU101-GFP plasmid to create pAMG100. This plasmid was then inserted into *E. faecium* BL02235-1 and *E. faecalis* OG1RF. (C) Generation of *E. faecium* BL02235-1 (right) +pAMG200 and *E. faecalis* OGRF1+pAMG200 (left). The *bacAbacB* genes were cut from the pDT1 plasmid and inserted into the linearized pBSU101-RFP plasmid to create pAMG200. This plasmid was then inserted into *E. faecium* BL02235-1 and *E. faecalis* OG1RF. (D) Generation of *E. faecium* BL02235-1+pAMG101 and *E. faecium* BL02235-1+pAMG201. The *bacA* genes were removed from the pAMG100 and pAMG200 plasmids to create pAMG101 and pAMG201, respectively. These plasmids were then inserted into *E. faecium* BL02235-1. Figure created with BioRender.

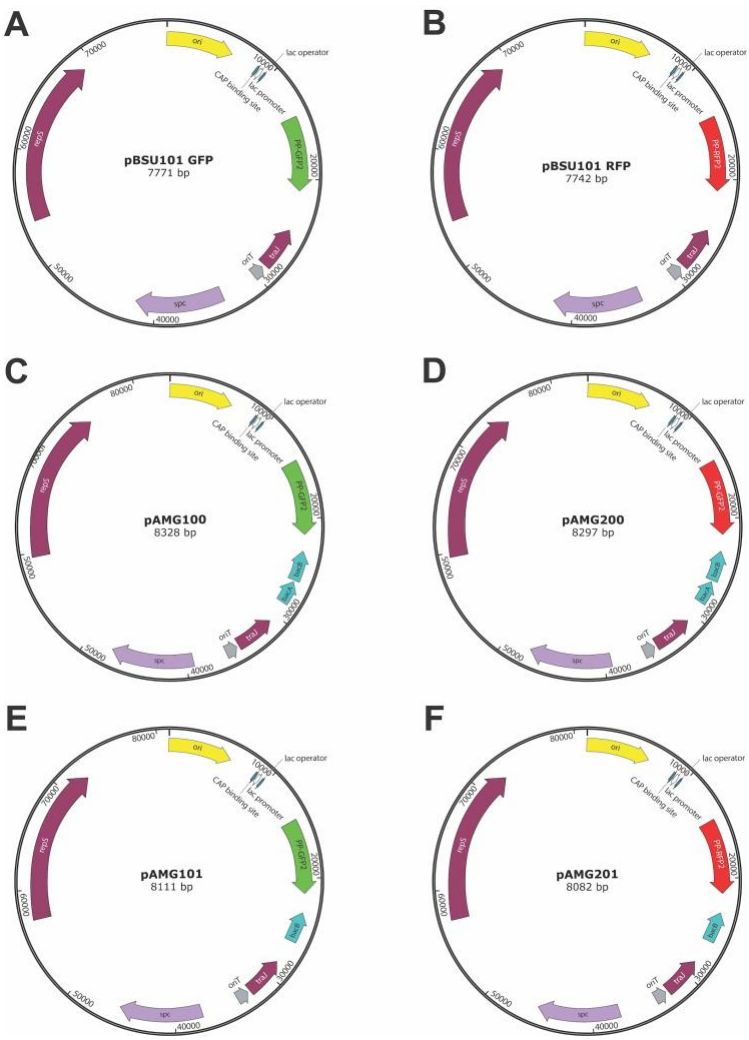


Figure 4.2: Plasmid maps. (A) pBSU101-GFP (B) pBSU101-RFP (C) pAMG100 (D) pAMG200 (E) pAMG101 (F) pAMG201. The fluorescence genes are colored in green or red respectively, the bac43 genes are in blue, and the gene for spectinomycin resistance is lavender. All other genes are for plasmid maintenance. Maps created with SnapGene.

4.4.2 Confirmation of expected activity.

Fluorescence for each of the generated strains could be visualized under a microscope. All strains demonstrated the expected fluorescent signal, however *E. faecium* and *E. faecalis* carrying the bac43 plasmids (pAMG100 and pAMG200) appeared to have a weaker fluorescent signal compared to strains carrying the non-bacteriocin plasmids (pBSU101-GFP and pBSU101-RFP; data not shown).

To assess bacteriocin activity, we used a poke assay where a bacterial colony is poked into a soft agar bacterial lawn. As expected, neither *E. faecalis* nor *E. faecium* carrying plasmids pBSU101-GFP and pBSU101-RFP demonstrated inhibitory activity (data not shown). We find our *E. faecium* strains producing bac43 (carrying plasmids pAMG100 and pAMG200) can kill both *E. faecium* and *E. faecalis* sensitive lawns (Figure 4.3A-B), though the halo of inhibition against *E. faecalis* lawn was smaller compared to the *E. faecium* lawn. The *E. faecalis* strains producing bac43 produced a small halo of inhibition against *E. faecium* sensitive lawns and have no activity against *E. faecalis* sensitive lawns (Figure 4.3C-D). Finally, our *E. faecium* strains that carry the bac43 immunity protein (carrying plasmids pAMG101 and pAMG201) are resistant to killing by *E. faecium* bac43 producing strains (Figure 4.3E-F). These results are summarized in Table 4.1.

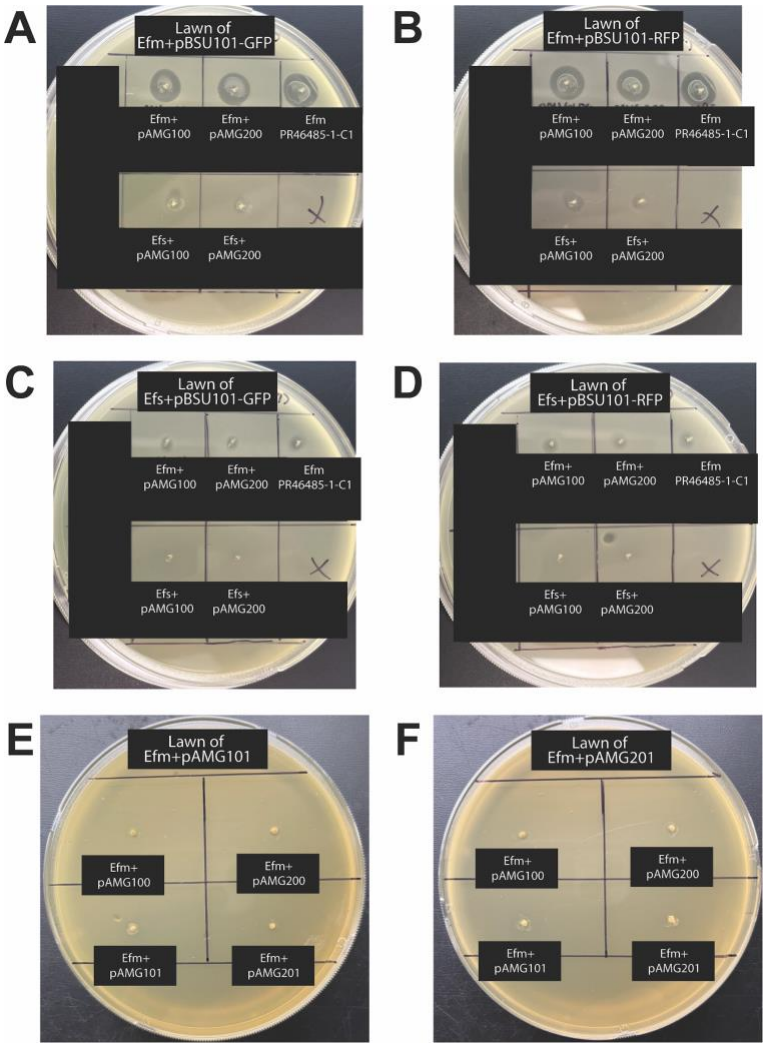


Figure 4.3: Spot assay confirming expected bacteriocin activity of generated strains. Lawns are as follows: (A) *E. faecium* (Efm)+pBSU101-GFP (B) *E. faecium* (Efm)+pBSU101-RFP (C) *E. faecalis* (Efs)+pBSU101-GFP (D) *E. faecalis* (Efs)+pBSU101-RFP (E) *E. faecium* (Efm)+pAMG101 (F) *E. faecium* (Efm)+pAMG201. In A-D, the top row represents pokes from *E. faecium* (Efm) +pAMG100, +pAMG200, and the original *E. faecium* bac43 producer PR46485-1-C1. The bottom row shows pokes from *E. faecalis* (Efs) +pAMG100 and +pAMG200. In E and F, the top row shows pokes from *E. faecium* (Efm) +pAMG100 and +pAMG200. The bottom row shows pokes from *E. faecium* (Efm) +pAMG101 and +pAMG201.

Table 4.1: Summary of strains created. Summary table of the eight strains generated, their fluorophore, bac43 genes, and activity in our confirmation assay.

Strains created	Fluorophore	Bac43 genes	Activity
E. faecium BL02235-1 +pSBU101-GFP	GFP	-	-
E. faecium BL02235-1 +pSBU101-RFP	RFP	-	-
E. faecium BL02235-1 +pAMG100	GFP	*bacAbacB*	Kills sensitive *E. faecium* Kills sensitive *E. faecalis*
E. faecium BL02235-1 +pAMG200	RFP	*bacAbacB*	Kills sensitive *E. faecium* Kills sensitive *E. faecalis*
E. faecalis OG1RF +pAMG100	GFP	*bacAbacB*	Kills sensitive *E. faecium* Does not kill sensitive *E. faecalis*
E. faecalis OG1RF +pAMG200	RFP	*bacAbacB*	Kills sensitive *E. faecium* Does not kill sensitive *E. faecalis*
E. faecium BL02235-1 +pAMG101	GFP	*bacB*	Immune to *E. faecium* producer
E. faecium BL02235-1 +pAMG201	RFP	*bacB*	Immune to *E. faecium* producer

4.4.3 C. elegans *transfer between lawns of* E. faecium.

We next attempted to use *C. elegans* as a study system for competition between bacteriocin-producing and non-bacteriocin producing strains. We aimed to track any interactions using RFP and GFP labeled *E. faecium*, with and without an encoded bacteriocin. In this preliminary study we allowed the worms to feed on a bacterial lawn with a single fluorophore for 24hrs before transferring to a bacterial lawn with the alternate fluorophore (Figure 4.4A-D). We sacrificed and imaged worms at three 10min time points after transfer as we previously noticed a quick turnover of fluorophore signal within the *C. elegans* gut (data not shown).

Transfer sets A and B included no bacteriocins from *E. faecium*+pBSU101-RFP to *E. faecium*+pBSU101-GFP (A, Figure 4.4A, Figure 4.5A) and vice versa (B, Figure 4.4B, Figure 4.5B). In the RFP to GFP worms, (Figure 4.4A, Figure 4.5A) we see by 30m some worms were almost entirely GFP and some had a mix of RFP and GFP. After 24hr on the GFP lawn, we noticed the worms were sluggish and barely moving. When fixed and imaged, the worms appeared to have ruptured, and the eggs can be seen leaking out of the body. This curious result was only found after feeding on the *E. faecium*+pBSU101-GFP lawn. After the initial feeding on *E. faecium*+pBSU101-GFP lawns, approximately 20% of the worms were found dead. For our

second transfer from GFP to RFP (Figure 4.4B, Figure 4.5B) we transferred any worms that still moved when poked. Once on the RFP lawn, the worms thrived and were all alive after 24hr. By 30m and after 24hr, RFP was the dominant signal in the *C. elegans* guts but puncta of GFP cells could still be identified. This transfer set shows us we see a majority of turnover from the initial fluorophore within the first 30m but a small number of cells with the initial fluorophore can still be found after 24hr.

In our second set of transfers (C and D), we looked for the influence of bac43 expression by transferring *E. faecium*+pAMG100 to *E. faecium*+pBSU101-RFP (C, Figure 4.4C, Figure 4.5C) and vice versa (D, Figure 4.4D, Figure 4.5D). With *C. elegans* starting on a bacteriocin producing lawn (Figure 4.4C, Figure 4.5C), we anticipated a slower replacement by the new bacteria when encountering a gut filled with a bacteriocin producer. When *C. elegans* first feeds on a non-bacteriocin lawn (Figure 4.4D, Figure 4.5D), we expected a faster turnover of the non-bacteriocin producer when transferred to a bacteriocin-producing lawn. In both these transfers, however, visually the turnover between fluorophores appeared no different when compared with two non-bacteriocin producing lawns. We suspect with this turnover occurring on the scale of minutes, there is most likely not enough time for the bacteriocin to be factor. Interestingly, however, feeding on the GFP lawn with the bacteriocin (*E. faecium*+pAMG100) did not result in *C. elegans* death like the non-bacteriocin GFP lawn (*E. faecium*+pBSU101-GFP). Further experimentation would be required for *C. elegans* to be an effective model to study bacteriocin competition.

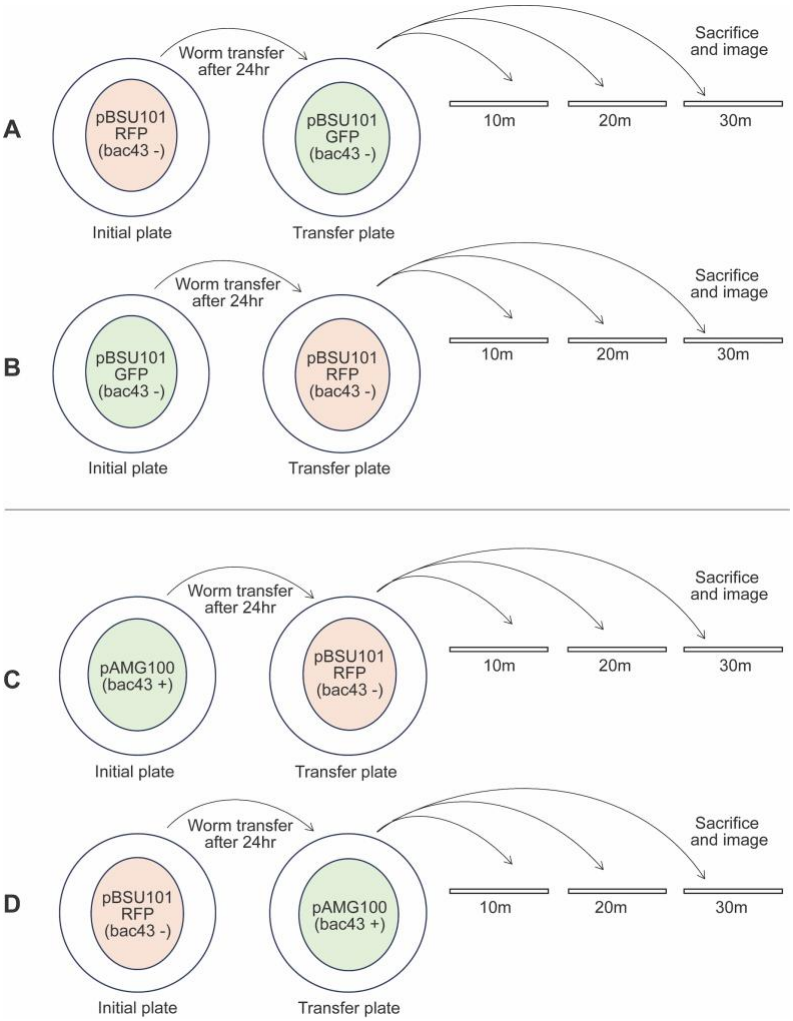


Figure 4.4: *C. elegans* transfer design between *E. faecium* lawns. *C. elegans* was transferred from the first lawn (initial plate) to the second (transfer plate) after 24hr. Transfers are as follows: (A) pBSU101-RFP to pBSU101-GFP (B) pBSU101-GFP to pBSU101-RFP (C) pAMG100 to pBSU101-RFP (D) pBSU101-RFP to pAMG100.

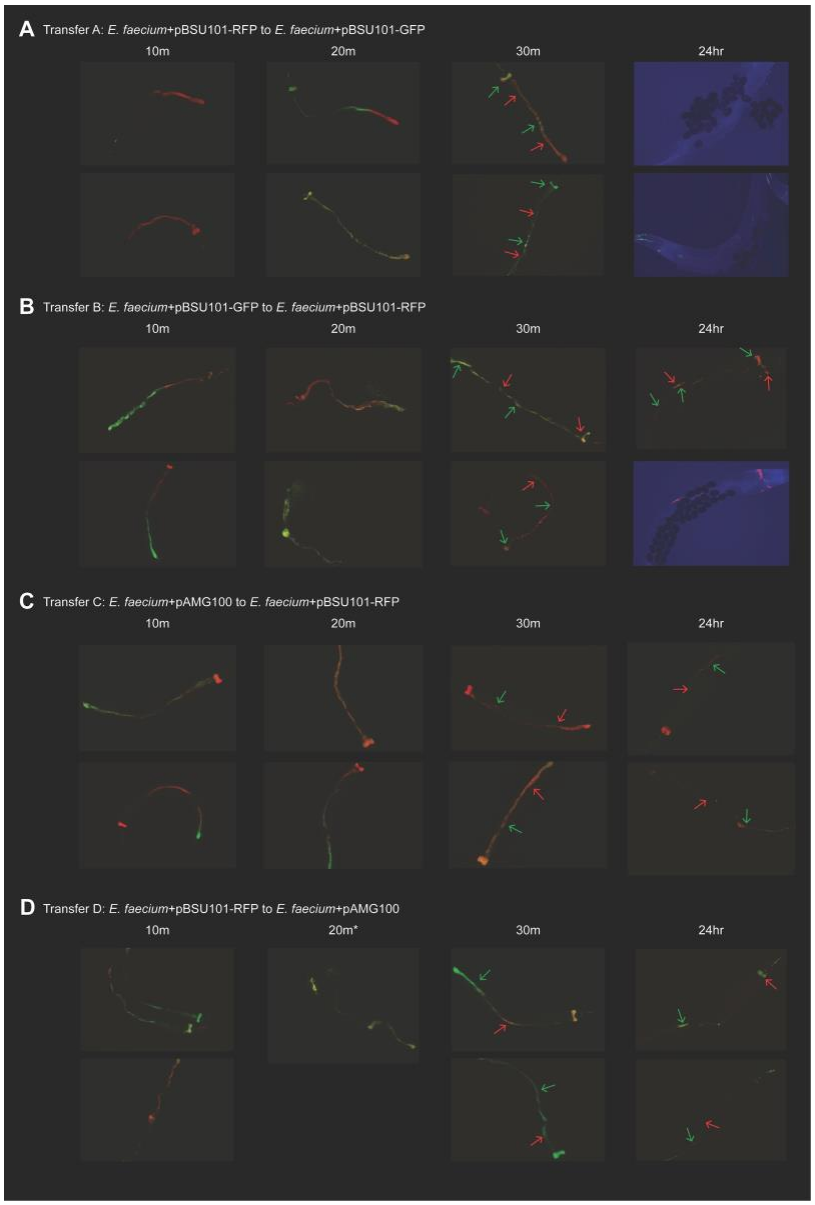


Figure 4.5: Fluorescence microscopy of *C. elegans* on *E. faecium* transfer experiment. Microscopy images of *C. elegans* at time points 10m, 20m, 30m, and 24hr. Transfers are as follows: (A) From pBSU101-RFP lawn to pBSU101-GFP lawn (B) From pBSU101-GFP lawn to pBSU101-RFP lawn (C) From pAMG100 lawn to pBSU101-RFP lawn (D) From pBSU101-RFP lawn to pAMG100 lawn. * indicates slide was dropped, and worms were damaged.

4.5 Conclusions

In this study we modified an existing shuttle vector that contained fluorescent markers[137] to encode genes for bac43 production and immunity (*bacA* and *bacB*, respectively). Using these isogenic strains, we were able to directly implicate bacA as the cause of the phenotypic halo of inhibition observed, and *bacB* also was able to confer immunity to *bacA*. Additionally, we demonstrated only the *bacAbacB* and the promoter region upstream *bacA* are sufficient for bac43 expression.

We successfully transformed the plasmids for bac43 production into *E. faecium* BL02235-1 and *E. faecalis* OG1RF and the plasmids for immunity into *E. faecium* BL02235-1. Our poke assays confirmed the expected activity for all the generated strains in *E. faecium*. While our *E. faecalis* bac43 producer was able to kill *E. faecium*, albeit to a lesser extent than our *E. faecium* producer, this strain had no activity against *E. faecalis*. These results suggest that the *E. faecalis* OG1RF producers are making less bacteriocin, or the bacteriocin is not being secreted as efficiently as in *E. faecium* BL02235-1. Furthermore, our *E. faecium* producers had a lesser killing effect against both *E. faecalis* than *E. faecium* strains we studied. These data prompt the idea that *E. faecalis* OG1RF could be less sensitive to bac43 than *E. faecium* BL02235-1. This could be due to possible differences in the cell wall[146] or in the bac43 target, the mannose phosphotransferase system.[54]

These fluorescently labeled strains were used in the model organism *C. elegans* as a way to study competition between bacteriocin and non-bacteriocin producing strains. We were able to successfully introduce *E. faecium* to *C. elegans* and monitor fluorescence. We did note, however, that *E. faecium*+pBSU101-GFP (non-bacteriocin producer) appeared to be mildly toxic to *C. elegans* as feeding on this lawn for 24hr resulted in the death 15% of the initial population. This was not the case after feeding on the RFP counterpart lawn. This prompts the question of the

influence of the GFP fluorophore itself, but this death result was also not replicated after feeding on the bacteriocin GFP counterpart lawn (*E. faecium*+pAMG100). While this phenotype was only observed in a single experiment that would need to be repeated for validation, one potential explanation is that the pBSU101-GFP plasmid could be present at a higher copy number compared to the pAMG100 plasmid as carrying pAMG100 would have a greater cost due to the bacteriocin. It is possible the GFP fluorophore is toxic to *C. elegans* at high densities but further work in this area is required.

While we were able to visualize the replacement of one fluorophore with another in the *C. elegans* gut, this turnover occurs within minutes and was highly variable from worm to worm. This left us unable visually identify any influence of bac43 production on turnover. Further experimentation would be required for *C. elegans* to be a reliable model for studying competition in this manner. Nevertheless, this work still provides a model that could prove useful in the study of other, more rapid interactions, and the fluorescent reporter strains generated hold value for future endeavors in visualizing competition.

Chapter 5 Discussion

This dissertation explores bacteriocin production and resistance in a clinical cohort of *E. faecium* isolates collected over a six-year time period from a single hospital system. We highlight the rising prevalence of bacteriocin bac43 and a rarity of resistant mutants identified *in vivo*.

In Chapter 2, we identified the bacteriocin bac43 as phenotypically functional and rising in prevalence in clinical *E. faecium* isolates. Notably, the rise of bac43 producers does not appear to be limited to our specific clinical system. Between 2016 and 2021 we identified bac43 to be highly associated with two Clade 1A *E. faecium* STs, 117 and 80, which came to dominate our sampled STs. Commensurate with our publication, Mills et al. demonstrated VRE faecium lineage replacement by STs 117 and 80 in a single hospital system between 2017 and 2022, and globally among VRE faecium isolates collected from human sources between 2002 and 2022.[117] Subsequently, bac43 presence was also found to rise over these study periods as bac43 was identified in the majority of these ST 117 and 80 isolates. Mills et al. also demonstrated an *E. faecium* clinical isolate carrying bac43 had a competitive advantage compared to a non-bacteriocin producing *E. faecium in vitro* in liquid culture as well as *in vivo* in a mouse model of the mammalian gut.[117] These findings demonstrate the importance of the emergence of STs 117 and 80 on a larger scale and confirm the association with bac43, suggesting bac43 production could be an influencing factor. Future work addressing this influence includes testing the inhibitory activity of bac43 against common gastrointestinal microbiota, comparing the gut

microbiota composition between patients with *E. faecium* positive and negative for bac43, and an analysis of a new set of *E. faecium* isolates collected after the end of the study period.

In Chapter 2, we also note the conflicting evolutionary story between enterocin A and bac43. While portions of the enterocin A gene cluster were found in 98% of our isolates, only six of the enterocin A sequences resulted in a phenotypically functional enterocin A. The bac43 genes however were highly conserved and almost always conferred phenotypic functionality. This contrast could be a result of bac43 persistence by being encoded on a conjugative plasmid, or a difference in regulation resulting in preservation of the bac43 locus. While enterocin A is encoded on the chromosome, bac43 is on a mobilizable plasmid. Bac43 production could therefore be increased through plasmid copy number, and the bac43 genes can be transferred to other cells through conjugation promoting gene persistence. In terms of regulation, most class IIa bacteriocins, like enterocin A, are regulated through quorum sensing involving a histidine protein kinase, an inducer peptide, and a response regulator.[68,147] In this system, the inducer peptide must be present in at sufficient concentration to trigger activation of the bacteriocin genes. The structural gene of bac43, however, contains a leader sequence directing export through the *sec*-pathway.[95] While regulation of *sec*-dependent bacteriocins still requires further investigation, no regulation genes have been associated with bac43 and nothing in our experiments indicate a mechanism of regulation. If bac43 is constitutively expressed, this could be a mechanism for self-preservation as loss of the locus would then result in cell death. The production of enterocin A is off before it gets triggered which could allow the locus to be lost without the cell dying. Further work would be required to determine if bac43 is transcribed and exported at a higher rate than enterocin A, and the prevalence of conjugation *in vivo*.

In Chapter 3, we explored resistance to bac43 through its targeting of manPTS. While resistance emerges readily *in vitro*, resistant mutants were rarely identified in our representative screen of *in vivo* isolates. This finding is consistent with the hypothesis that manPTS is an important factor in *E. faecium* colonization.[23,124,125] While bacteriocin population dynamics can be modeled as a rock-paper-scissors dynamic between bacteriocin producer-resistant-sensitive cells,[38,41] the rarity of resistant mutants suggests population dynamics of bac43 production *in vivo* may be better represented primarily as a two-player system of producers and sensitives with resistant mutants present only transiently. This would support the noted surge in presence of bac43 as there are rarely resistant mutants helping maintain balance among the players. Possible future work to investigate this could include introducing *in vitro* generated resistant mutants to a gut mouse model to assess survival.

Additionally, in Chapter 3, we assessed growth with mannose as the primary carbon source. *In vitro* generated bac43 resistant mutants consistently demonstrated growth defects in mannose supporting alteration of the bacteriocin target, manPTS, as the source of resistance. In class IIa bacteriocin producers, the immunity protein is proposed to protect the producer by blocking the pore created in the manPTS by the bacteriocin.[49–53] This would also, theoretically, impair the receptor for mannose uptake. However, while eight bac43 producers had significant mutations in their manPTS operons (e.g. frameshift or stop gained mutations, deletions, or insertion elements), only three demonstrated a growth defect in mannose while being immune to bac43. This suggests either immunity does not interfere with manPTS function, or many bac43 expressing strains have additional systems for mannose uptake that are not targeted by the bac43 immunity protein. Further work will be needed to gain a greater understanding of the specific interactions and implications involved in bacteriocin immunity.

In Chapter 4, we generated fluorescently labeled *E. faecium* strains carrying the genes for bac43 production and attempted to visually track geographic changes during competition between bacteriocin-producing and non-bacteriocin producing strains in the model organism *C. elegans*. The *C. elegans* gut proved to have a turnover rate too rapid for our purposes, but we did identify a curious phenomenon where *C. elegans* feeding on *E. faecium* with a GFP fluorescent plasmid tended to die while those feeding on *E. faecium* with the bacteriocin GFP plasmid survived. Special attention will have to be paid to *C. elegans* in regard to enterococci and GFP in future studies. While this model would require significant additional work to be useful for these purposes, the isogenic fluorescent strains generated still hold potential for competition studies. For example, population changes could be tracked through fluorescence by competing the producer, immune, and sensitive strains *in vitro* on agar plates using various initial population sizes and arrangements of each strain. *In vivo* these strains could be used to compare differences between two-player and three-player interactions if the model system presented a slower gut transit time or had a stable gut microbiome.

By understanding the factors influencing *E. faecium* gastrointestinal colonization, we can begin to mitigate the serious health threat posed by vancomycin resistant enterococcus. In this dissertation, we advance our knowledge of bacteriocin prevalence and resistance. Bac43 production is highly associated with emergent *E. faecium* STs and could be a potential factor increasing *E. faecium* colonization. We have also begun to improve our understanding of bacteriocin resistance *in vivo*. In the current era of rampant antibiotic resistance, bacteriocins are being investigated as alternatives against multi-drug resistant organisms. It is therefore imperative to understand existing bacteriocin presence in target organisms and their mechanisms

of bacteriocin resistance. This work lays a foundation of knowledge about *E. faecium* bacteriocin dynamics in healthcare settings and prompts future studies to complete this picture.

Appendices

Appendix A: Supporting Information for Chapter 2

Appendix Table A.1: Genes for enterocin A, bac43, and enterocin NKR-5-3B.

Bacteriocin.	Gene	Length (bp)
Enterocin A (Genbank accession number AF099088.1)	*entA*	198
	entI	312
	entF	147
	entK	1284
	entR	753
	entT	2154
	entD	1368
Bac43 (Genbank accession number AB178871.1)	*bacA*	225
	bacB	288
	mobC	381
	mobA	915
	repA	945
	repB	534
Enterocin NKR-5-3B (Genbank accession number LC068607.1)	*enkB*	264
	enkB1	1140
	enkB2	519
	enkB3	591
	enkB4	498

Appendix Table A.2: PCR primers used for confirming bacteriocin gene presence.

ID	Sequence	Description
bacA_fwd	TTGTCTAGCTGGCATCGGTA	Forward PCR primer targeting *bacA*
bacA_rev	GGACCATGATTAACCCAACC	Reverse PCR primer targeting *bacA*

Appendix Table A.3: All bacteriocin cluster hits and the number of isolates in which they are found.

Bacteriocin cluster	Isolate presence
Enterocin A cluster	2395
Bac43 full cluster	1402
Bac43 partial cluster	988
Enterocin NKR-5-3B cluster	466

Enterocin SE-K4 cluster	69
Enterocin P cluster	14
Enterocin B cluster	13
Bac32 cluster	12
Enterocin Xα cluster	10
Bac32 truncated cluster	5
Enterocin Xβ cluster	4
Enterocin L50b cluster	3
Enterocin L50a cluster	3
Enterocin Q cluster	2
Pneumolancidin cluster	1
Bac31 cluster	1
Enterocin P-like cluster	1

Appendix Table A.4: Bacteriocin presence between perirectal (PR) and blood (BL) isolates. Significance values: p-value < 0.05 *, p-value < 0.001 **.

Bacteriocin	Percentage of PR isolates (n=2150)	Percentage of BL isolates (n=278)	p-value	Odds ratio
Enterocin A	0.09%	1.9%	0.00028 **	0.049
Bac43	59.1%	50.4%	0.0086 *	1.42

Appendix B: Supporting Information for Chapter 3

Appendix Table B.1: Genes used as query sequences for blast analysis

Query	Locus tag (genome accession NZ_CP013994.1)	Location in NZ_CP013994.1 genome
manR (sigma 54-interacting transcriptional regulator)	AWJ25_RS09865	Complement (1957024..1959831)
manX_1 (subunit EIIB)	AWJ25_RS09860	Complement (1956342..1956833)
manX_2 (subunit EIIAB)	AWJ25_RS09855	Complement (1955250..1956239)
manY (subunit EIIC)	AWJ25_RS09850	Complement (1954414..1955217)
manZ (subunit EIID)	AWJ25_RS09845	Complement (1953479..1954393)
Region of manR to manZ		Complement (1953479…1959831)

Appendix Table B.2: Chemically Defined Media (CDM) composition:

The following formulation was ordered from Gibco through Thermo-Fisher Scientific using their custom media service:

Name	Concentration (g/L)
Adenine	0.02
L-alanine	0.1
L-arginine	0.1
L-aspartic acid	0.1
D-biotin	0.0002
Calcium chloride, anhydrous	0.0051
Calcium D-pantothenate	0.002
Cyanocobalamin	0.0001
L-cystine disodium salt	0.0629
Ferric nitrate, nonahydrate	0.001
Ferrous sulfate, heptahydrate	0.005
Folic acid	0.0008
L-glutamic acid	0.1
L-glutamine	0.2
Glycine	0.1
Guanine hydrochloride, anhydrous	0.0182
L-histidine	0.1
trans-4-hydroxy-L-proline	0.1
L-isoleucine	0.1
L-leucine	0.1

L-lysine	0.1
Magnesium sulfate, anhydrous	0.3419
Manganese(II) sulfate, monohydrate	0.005596
L-methionine	0.1
Niacinamide	0.001
Beta-NAD hydrate	0.0025
4-aminobenzoic acid	0.0002
L-phenylalanine	0.1
Potassium phosphate, dibasic, anhydrous	0.2
Potassium phosphate, monobasic, anhydrous	1
L-proline	0.1
Pyridoxal hydrochloride	0.001
Pyridoxine hydrochloride	0.000853
Riboflavin	0.002
L-serine	0.1
Sodium acetate, anhydrous	2.7126
Sodium phosphate, dibasic, anhydrous	7.35
Sodium phosphate, monobasic, monohydrate	3.195
Thiamine hydrochloride	0.001
L-threonine	0.2
L-tryptophan	0.1
L-tyrosine disodium salt	0.144
Uracil	0.02
L-valine	0.1
Total	17.092949
1.709 g CDM powder, 0.25 g sodium bicarbonate, 0.075 g L-cysteine hydrochloride, and 1 g glucose or mannose, 1mL of 10% choline chloride were added to a final volume of 100mL ddH2O. Media was filter sterilized, stored at 4ºC.	

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