IDENTIFICATION OF NOVEL SELF-LETHALITY PHENOTYPE OF CLASS IIA BACTERIOCIN-PRODUCER *ENTEROCOCCUS MUNDTII* CUGF08 AND GENOMIC COMPARISONS OF CLOSELY RELATED STRAINS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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January 2015

IDENTIFICATION OF NOVEL SELF-LETHALITY PHENOTYPE OF CLASS IIA BACTERIOCIN-PRODUCER *ENTEROCOCCUS MUNDTII* CUGF08 AND GENOMIC COMPARISONS OF CLOSELY RELATED STRAINS

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Cornell University 2015

There are several strains of *Enterococcus mundtii* that produce a class IIa bacteriocin, mundticin, along with a cognitive immunity protein and ABC transporter. However, *E. mundtii* CUGF08 and ATO6 are not immune to their own bacteriocin production, like the other bacteriocinogenic strains. Since it was found that the presence of proteinase K inactivated the self-lethality phenotype, peptides produced by *E. mundtii* CUGF08 were isolated from its supernatant though chromatography (solid-phase extraction, cation-exchange, and RP-HPLC). The intact mass of one causative agent was 6 kDa and its trypsin-digested fragment sequence was determined to be AIGIIGNNSAANLATGGAAGWK. The fragment is 100% identical to the C-terminal sequence of mundticin L, but the intact mass corresponds more closely to the precursor peptide. Conversely, SDS-PAGE analysis displayed only a 4-kDa peptide from the supernatant that showed activity to *E. mundtii* CUGF08. After Edman degradation was performed on the band, the N-terminal sequence was found to be KYYGNGLSXNKKGXSVDX(G)(K)A(I)(G)(I), which matches with mundticin L. Since there are several strains that react differently to mundticin homologs, the genomes of four mundticin producers and one non-mundticin producer were compared in order to identify potential genes involved with mundticin immunity or target specificity. The two aforementioned strains, along with non-producer strain *E. mundtii* ATCC 882, are sensitive to mundticin,. Two producers in literature, *E. mundtii* QU 25 and CRL35, were reported to be immune to their purified mundticin,

There are 28 genes unique to the immune strains and 3 genes unique to the sensitive strains. Many of those found in the immune strains are phage proteins. One unique gene from the immune strains may be a secreted protein or a lipoprotein, which could be involved with the additional immunity. One unique gene from both sensitive strains shows homology to a transmembrane protein, which may be an additional cell membrane receptor for mundticin. Further work with complementation studies with those genes may determine additional, uncharacterized mechanisms of immunity and action, especially since many of those mechanisms are poorly understood for class IIa bacteriocins.

BIOGRAPHICAL SKETCH

Giselle Kristi P. Guron was born to Merlyn Guron and Revelino Guron in Phoenix, Arizona. As an undergraduate at Cornell University, she was selected for the Food Science Summer Scholars Research Program in 2008. She started her work under Dr. Randy Worobo learning about antimicrobial peptide research, and she continued the work her last year of undergraduate studies for her honors thesis. She received her B. S. in Food Science in 2009 in Ithaca, New York, and continued antimicrobial peptide research as a graduate student in Geneva, New York, pursuing a Ph. D. After completing her graduate program, she plans to pursue her interests in microbial ecology as a post-doctorate fellow.

This dissertation is dedicated to my mother Merlyn Guron

and father Revelino Guron

for always pushing me to do my best

ACKNOWLEDGEMENTS

I would like to thank my adviser, Dr. Randy W. Worobo, for his patience and guidance. He has helped grow to be a more independent researcher that I strove to become. I would like to thank Dr. Anthony Hay for introducing new approaches to incorporate into my research. Moreover, I appreciate the additional technical support from members of his lab group. I also want to thank Dr. David Wilson for his ideas and insight. Lastly, I want to thank Dr. Ray Glahn for being my field appointment member.

I want to acknowledge our lab technician, John Churey, who has always served as a great example of a hard-working and generous scientist. Additionally, I would like to thank all my lab mates for support, especially Dr. David Manns and Dr. Guoping Feng who were additional mentors for me throughout my research. There were numerous other students, post-doctorate fellows, technicians, and professors from the New York State Agricultural Experiment Station, and specifically the Student Association of the Geneva Experiment Station, who have contributed to the quality of my graduate research program in all ways. In addition, I would like to acknowledge the Cornell Food Science Department in Ithaca. I especially appreciate the help of Janette Robbins, as well as Dr. Alícia Orta-Ramírez, my undergraduate adviser. Moreover, I would like to thank Dr. Martin Wiedmann and the several members of his lab for additional technical support.

Lastly, I want to thank the extension educators from whom I gained valuable experience and knowledge. I learned immensely about public speaking from Dr. Betsy Bihn and the rest of the team in the Good Agricultural Practices program. I also want to acknowledge the New York State Food Venture Center for allowing me to learn about how they help food entrepreneurs.

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CHAPTER 1

LITERATURE REVIEW: THE FUNCTIONS AND MECHANISMS OF CLASS IIA BACTERIOCINS AND THEIR ASSOCIATED PROTEINS

Consumers demand for wholesome, healthful, and safe products containing only "natural" ingredients has been a driving force behind research and development in the food industry. Bacteriocins are antibacterial peptides that are naturally produced by bacteria, including generally recognized as safe (GRAS) status lactic acid bacteria (LAB) (Bourdichon et al. 2012, FDA 2014), and can potentially be used to enhance the safety of foods (Balciunas et al. 2013). There are several classes and subclasses of bacteriocins consisting of lantibiotics and non-lantibiotics. Class IIa bacteriocins are unmodified, non-lantibiotics that are similar to pediocin (Klaenhammer 1993). These bacteriocins are stable under extreme temperatures and pHs, and target a wide variety of gram-positive bacteria, including the foodborne pathogen *Listeria monocytogenes*, so they can be well suited as "natural" additives for foods (Belguesmia et al. 2011).

Unfortunately, how class IIa bacteriocins specifically interact with their targets is still poorly understood (Belguesmia et al. 2011). In order to assure that the usage of class IIa bacteriocins in foods is only beneficial for consumers, more information is needed with regards to their mechanism of action, the maturation process, and the mechanism of the immunity protein.

Class IIa bacteriocin structure and mode of action. Class IIa bacteriocins share common characteristics to pediocin PA-1, the first characterized class IIa bacteriocin and the first commercially available bacteriocin for use in the food industry (Chen et al. 2004). They are generally small (< 10 kDa), unmodified, positively-charged, heat-resistant peptides. They also

contain a YGNG[V/L] motif "pediocin box" or "*Listeria* box" near the N-terminus, and contain at least one disulfide bridge. They are synthesized as pre-bacteriocins, often containing a leader sequence separated by a double glycine motif, which is the cleavage site to form the mature peptide that is secreted (Drider et al. 2006).

The secondary structure of the hydrophilic N-terminal region consists of antiparallel β-sheets with a conserved disulfide bridge. The less conserved, hydrophobic C-terminal region contains one or two α-helices and a tail to form a hairpin structure, which may provide specificity for the bacteriocin's target. Connecting the N- and C-termini is a flexible hinge (Belguesmia et al. 2011). Their targets are mostly *Listeria* spp. and other species closely related to the producer strain (Belguesmia et al. 2011). Though these bacteriocins generally target Gram positive species, some combinations of technologies can be used synergistically with a bacteriocin to combat Gram negative species (Deegan et al. 2006).

Bacteriocins cause leakage of small molecules in sensitive bacteria, suggesting that the bacteriocin targets the cell membrane and forms pores to induce leakage. One proposed mechanism is that the positively-charged N-terminal region can electrostatically interact with the negatively-charged membrane, which can then allow the C-terminal region to penetrate into the lipid bilayer of the cell membrane. However, it is unclear as to how many molecules of the peptide are required for pore formation, or which receptor or docking molecule that the N-terminal region recognizes. The overall cell surface composition seems to be involved in bacteriocin attachment (Belguesmia et al. 2011). Many have proposed that the receptor of interest is the mannose phosphotransferase system (MptABCD) (Belguesmia et al. 2011). Moreover, expression of *mpt*ABCD in insensitive strains leads to sensitivity to bacteriocins (Arous et al. 2004, Kjos et al. 2009). The subunits IIC and IID may be most directly involved

(Kjos et al. 2009). Nonetheless, the interaction of mptABCD may not be the only mechanism of action for bacteriocins in certain target species.

Genetic organization. The genes associated with bacteriocin production may be located in one or more operons, and encode for the structural peptide, immunity protein, ABC transporter, and the three components for regulation (Belguesmia et al. 2011). The immunity gene is generally co-transcribed and downstream the structural gene. The ABC transporter is necessary for all class IIa bacteriocins, but some also produce accessory transport proteins. The optional regulatory proteins include an inducer, histidine kinase, and response regulator. However, not all class IIa bacteriocins require regulatory proteins, such as mundticin KS & L produced by *Enterococcus mundtii* strains.

This diversity in essential genes among class IIa bacteriocins is also exemplified by the different loci of the operons. Most bacteriocin-related genes are plasmid-encoded, but a few are encoded on the chromosome. Some species encode for the bacteriocin on a plasmid, but also transcribe genes on the chromosome for production, like carnobacteriocin BM1 & B2 produced by *Carnobacterium piscicola* LV17A (Quadri et al. 1997) Interestingly, *Lactobacillus sakei* 5 produces sakacin P, T, & X, but it may also encode some of the genes for an additional bacteriocin, brochocin-C (Vaughan et al. 2003).

Maturation and secretion. Most bacteriocin producers produce an ABC transporter for maturation and secretion, but some bacteriocins are *sec*-dependent (Drider et al. 2006). The ABC transporter consists of a hydrophobic transmembrane domain and a hydrophilic nucleotide-binding domain in the cytoplasm. For class IIa bacteriocins, there are generally 5 to 6 transmembrane α-helices and an N-terminal peptidase, grouped as SunT-type ABC transporters (Gebhard 2012). There are two N-terminal consensus sequences that may be involved with the

peptidase activity, $QX_4[D/E]CX_2AX_3MX_4[Y/F]GX_4[I/L]$ and $H[Y/F][Y/V]VX_{10}[I/L]XDP$ (Figure 1.1) (Håvarstein et al. 1995). It was found that the cysteine in the first consensus sequence and the histidine and aspartic acid in the second consensus sequence are directly involved in the cleavage activity (Ishii et al. 2010). The papain-like cysteine peptidase recognizes the double-glycine motif in the N-terminal leader sequence of the bacteriocin and cleaves it (Barrett and Rawlings 2001) to yield the mature peptide, which is then secreted (Håvarstein et al. 1995). Additionally, some bacteriocin producers utilize an accessory protein for transport (Drider et al. 2006).

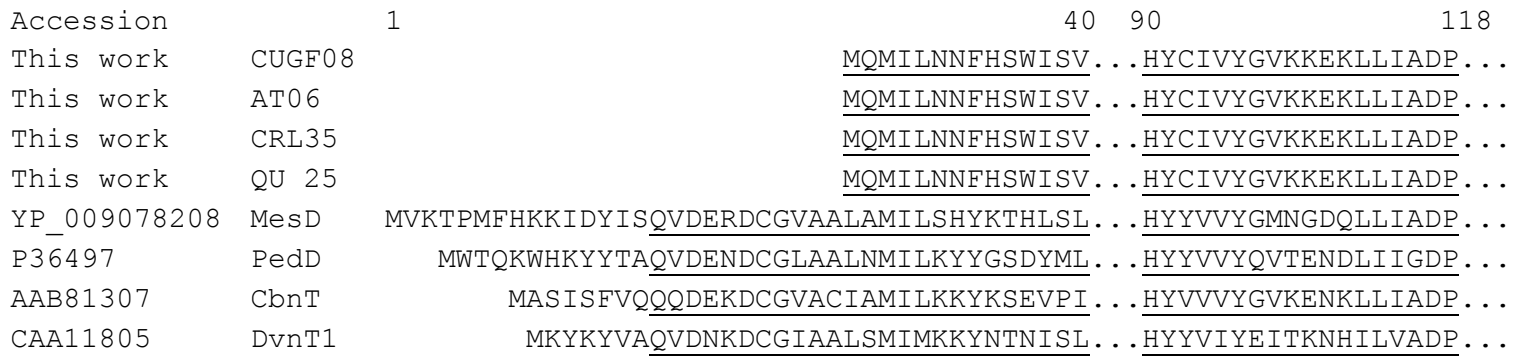

Figure 1.1. The N-termini of putative cognitive ABC transporters for mundticin (munT), produced by the four producer strains (CUGF08, ATO6, CRL35, and QU 25), and other class IIa bacteriocin ABC transporters: MesD from *Leuconostoc mesenteroides*, PedD from *Pediococcus acidilactici*, CbnT from *Carnobacterium maltaromaticum*, and DvnT1 from *Carnobacterium divergens*. The NCBI accession numbers are indicated for the latter four transporters. Underlined are the motifs necessary for peptidase activity (QX_4[D/E]$CX_2AX_3MX_4$[Y/F]GX_4[I/L] and H[Y/F][Y/V]VX_{10}[I/L]XDP) (Håvarstein et al. 1995).

Accession		* * * **	
P29430	pediocin PA-1 precursor	MKKNEKLTEKEMANIIGG	KYYGNGVTCGKHSCSVDWGK...
ACQ77507	mundticin L precursor	MKKLTSKEMAQVVGG	KYYGNGLSCNKKGCSVDWGK...
AAQ95741	enterocin CRL35 precursor	MKKLTSKEMAQVVGG	KYYGNGVSCNKKGCSVDWGK...
AAK69419	piscicolin 126 precursor	MKTVKELSVKEMQLTTGG	KYYGNGVSCNKNGCTVDWSK...
EHE85053	sakacin X precursor	MEAIKKLDLQAMKGIVGG	KYYGNGLSCNKSGCSVDWSK...
CAA88428	sakacin P precursor	MEKFIELSLKEVTAITGG	KYYGNGVHCGKHSCTVDWGT...
CAA11804	divercin V41 precursor	MKNLKEGSYTAVNTDELKSINGG	TKYYGNGVYCNSKKCWVDWG...
1RY3_A	carnobacteriocin B2 precursor	MNSVKELNVKEMKQLHGG	VNYGNGVSCSKTKCSVNWGQ...
YP_009074374	curvacin A precursor	MNNVKELSMTELQTITGG	ARSYGNGVYCNNKKCWVNRG...
AAA68003	leucocin A precursor	MMNMKPTESYEQLDNSALEQVVGG	KYYGNGVHCTKSGCSVNWGE...
P38577	mesentericin Y105 precursor	MTNMKSVEAYQQLDNQNLKKVVGG	KYYGNGVHCTKSGCSVNWGE...

Figure 1.2. The precursor peptides of various class IIa bacteriocins. The important hydrophobic residues for sectretion (positions -1, -2, -4, -7, -12) are indicated by an asterisk (*). The cleavage site is indicated by the vertical line after the conserved GG-motif

N-terminal sequences vary in length, but most of them contain a consensus sequence of the double-glycine since it is the cleavage site for the ABC transporter (Figure 1.2). One hypothesis is that this leader sequence is to prevent leakage activity within the cell prior to secretion. The precursor peptides have varying activities, but it is generally less potent than the mature counterparts for differing indicator species (Drider et al. 2006). In addition to the importance of the double-glycine motif, the hydrophobic residues at positions -4, -7, and -12 may be vital for secretion (Aucher et al. 2005).

Immunity. Immunity proteins are synthesized by bacteriocin-producing strains in order to protect the producer cells from the action of the bacteriocin it produces. They are generally specific towards the corresponding bacteriocin, though the determined 3D structures display a conserved bundle of four α-helices consisting of positively charged residues. The specificities of the immunity proteins seem to be located in the loops connecting the α-helices. Even though the 3D structures look similar, the sequence homology varies widely from 5% to 85% (Drider et al. 2006, Jeon et al. 2009).

There are three distinct groups of immunity proteins based on sequence alignments. The crystalized structure of one mundticin immunity protein (Mun-im), which is a subgroup B immunity protein, shows that it is comprised of a four-helix bundle with flexible N- and C-terminal ends, and includes the conserved IRYGY motif. Within the helices is a hydrophobic pocket, which is surrounded by positively charged residues on the surface. One side of the surface of the four-helix bundle is negatively charged, while the opposing side is positively charged. When the IRYGY motif is eliminated, the target producing the mutated protein is no longer immune to the corresponding bacteriocin (Jeon et al. 2009).

It is not fully clear whether the immunity gene generally downstream the structural gene is the only factor preventing activity against the producer strain. For example, mundticin L-producer *Enterococcus mundtii* CUGF08 is not fully immune to mundticin L or mundticin KS, despite producing an immunity protein (Feng et al. 2009). Even after homologous expression in sensitive strains *Listeria monocytogenes* 10403S and *E. faecalis* FSL23-140, the strains were only partially immune to mundticin L (unpublished work). *E. mundtii* QU 25 also produces mundticin KS and an immunity protein, but in contrast, is fully immune to mundticin KS (Shiwa et al. 2014). The mundticin immunity proteins are 100% identical, so there must be an additional factor involved in the immunity mechanism.

Regulation. For many producers, there are three components associated with inducing bacteriocin production when the bacterial density threshold is achieved (Drider et al. 2006). The inducer peptide is secreted at a constant level by all producers. The concentration of the peptide indicates the cell density. At the cell density threshold, the histidine kinase is activated and phosphorylates a response regulator. The activated response regulator then induces the transcription of the bacteriocin gene, as well as the regulation genes (Drider et al. 2006). While most producers encode genes for regulation of bacteriocin production, there are some that lack any of the genes, like mundticin KS (Shiwa et al. 2014), mundticin L (Feng et al. 2009), and mesentericin Y105 (Aucher et al. 2004).

Because of their potency and tolerance to a myriad of environments, there is potential in the use of class IIa bacteriocins in foods to increase safety and quality. There are numerous studies elucidating their mechanisms of biosynthesis, action, and immunity, but more work needs to be performed. It is especially important to determine how bacteriocins specifically interact with their target species since it may provide clues to how resistance occurs. There are several

examples where genes involved in bacteriocin production are in multiple operons (Ennahar et al. 2000, Drider et al. 2006).

Perhaps there are additional, uncharacterized genes that have not been discovered among all of the bacteriocins known. Many studies have determined the flanking open reading frames of the structural gene (Ennahar et al. 2000), in an attempt to elucidate additional genes involved in bacteriocin production, immunity or regulation. Whole genome sequencing and genomic comparisons of bacteriocin producers can potentially supplement the known information of the genetic organization of bacteriocins.

There are numerous advantages to studying novel bacteriocin-producers, such as the partially self-lethal mundticin L-producer *E. mundtii* CUGF08. This phenotype has not been observed with other bacteriocin producers reported to date. Similar to other producers, this strain produces an immunity protein without atypical sequences or structures (Feng et al. 2009, Jeon et al. 2009). The gene cluster is also unique in that the structural gene and the immunity gene are downstream different promoters, but both, as well as the ABC transporter, are plasmid encoded (Figure 1.3).

Figure 1.3. The genetic organization of mundticin L (*munL*), the ABC transporter (*munT*), and the immunity protein (*munI*) of *E. mundtii* CUGF08. Putative promoter regions are indicated by the thin, bent arrows and the putative *rho*-independent terminators are indicated by the octagons. This genetic organization is the same for all mundticin producer strains.

The goal of this study was to identify the additional, uncharacterized gene(s) involved with the mechanism of action of MunL. Initially, it was determined that *E. mundtii* CUGF08 may be affected by its own bacteriocin production via overlay (Figure 1.4). Since the activity is lost with the application of proteinase K, peptide column purification methods were performed in order to partially isolate the causative agent. Additionally, to determine uncharacterized genes involved with the self-lethality and immunity, first random insertion mutagenesis utilizing the pLTV3-*Tn*917 was performed as a preliminary study. Subsequently, the whole genome of two mundticin KS-producers, *E. mundtii* QU 25 (Shiwa et al. 2014) and *E. mundtii* CRL35 (Bonacina et al. 2014), were sequenced. Using the Illumina high-throughput sequencing (HTS) MiSeq system, the genomes of *E. mundtii* CUGF08 and mundticin KS-producer *E. mundtii* ATO6 were sequenced and assembled. While all four mundticin-producers share nearly identical genes for mundticin, immunity protein, and ABC transporter, two strains are partially self-lethal while the other two are fully immune to mundticin (Shiwa et al. 2014, Acuña et al. 2012). Together with non-mundticin-producer *E. mundtii* ATCC 882, the genomes of all five strains were compared. Overall, these studies will help further understand how class IIa bacteriocins specifically target closely-related strains and identify other potential immunity genes.

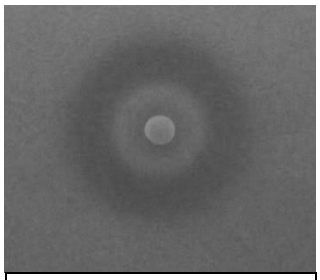

Figure 1.4. *E. mundtii* CUGF08 as the producer strain overlaid with the same strain.

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CHAPTER 2

IDENTIFICATION OF THE CAUSATIVE AGENT FOR SELF-LETHAL PHENOTYPE OF BACTERIOCIN-PRODUCER *ENTEROCOCCUS MUNDTII* CUGF08

ABSTRACT

Enterococcus mundtii CUGF08 is a lactic acid bacterium (LAB) isolated from alfalfa sprouts that produces mundticin L (munL), a class IIa bacteriocin. Similar to other class IIa bacteriocins, it is active against closely related bacteria and *Listeria* spp., including *L. monocytogenes*. However, unlike other bacteriocin-producing strains, this bacterium exhibits self-lethality, resulting in a halo-shaped inhibition zone surrounding the producer strain when using the deferred inhibition assay. This self-lethality phenotype is rarely observed in bacteriocinogenic bacteria, because immunity proteins are generally co-transcribed with the bacteriocin structural gene. No mutations or atypical gene arrangements were observed within the gene cluster for munL. Therefore, the objective of this study was to identify the component(s) causing the self-lethality phenotype. Mass spectroscopy data suggests that a 6-kDa, precursor mundticin L was the causative agent for self-lethality, while SDS-PAGE images illustrate that a 4-kDa mature mundticin L also targets its producer. This data may be used to elucidate more information on the mechanism of action, and specificity of class IIa bacteriocins, the maturation process, and the mechanism of the immunity protein.

INTRODUCTION

Enterococcus mundtii CUGF08 is a lactic acid bacterium (LAB) isolated from alfalfa sprouts and produces mundticin L (MunL), a pediocin-like, or class IIa, bacteriocin (Feng et al. 2009). Mundticin-producing *E. mundtii* strains can also be found in other food sources, such as soy beans and other vegetables (Bennik et al. 1998, Kawamoto et al. 2002, Zendo et al. 2005).

Similar to other class IIa bacteriocins, MunL is highly active against *Listeria* spp. Co-synthesized with munL, is an immunity protein (MunL-im), which prevents the bacteriocin from exerting its lethal effects against the producer, as well as an ABC transporter, which cleaves a N-terminal leader sequence at a double-glycine motif and secretes the mature peptide (Feng et al. 2009). However, unlike any reported bacteriocin-producing species, *E. mundtii* exhibits partial self-lethal activity (Figure 1.4). This phenotype has not been observed with bacteriocin producers because immunity proteins are usually synthesized to protect against the lethal effects of its own bacteriocin. Since MunL-im contains no atypical sequences (Feng et al. 2009, Jeon et al. 2009), it is likely that additional uncharacterized mechanisms exist with class IIa bacteriocin sensitivity or resistance. The elucidation of these mechanisms may provide more insight for class IIa bacteriocin target specificity, mechanism of action, immunity, and how target species confer resistance.

METHODS

Cultures and media. *Enterococcus mundtii* CUGF08 was grown in de Man Rogosa Sharpe (MRS) broth (Difco, Becton Dickinson and Company) at 30 °C at 200 rpm except after transformation. For protein purification, *E. mundtii* was incubated for 18 hours, which is the maximum production of mundticin L (Feng et al. 2009). *E. coli* HB101 containing pLTV3 (Fig. 5) was grown in Luria broth (Fisher Scientific) containing 20 μg/mL kanamycin at 37°C at 300 rpm. *Listeria monocytogenes* 10403S was grown on tryptic soy broth (Difco, Becton Dickinson and Company) at 37°C at 200 rpm. Indicator strains were inoculated into soft agar (0.75%) with compatible broth.

Electrocompetent *E. mundtii* cells. The protocol used was modified from that described by Shepard and Gilmore (1995) with *E. faecalis*. A single colony of *E. mundtii* was inoculated in 5

mL M17 (Difco, Becton Dickinson and Company) broth overnight (12 – 15 hours) without aeration at 37°C. One milliliter of that overnight inoculum was added to 100 mL M17 broth supplemented with 0.5 M sucrose and 3% glycine and incubated for 18-24 hours without aeration at 37°C. Cells were collected by centrifugation (10000 x *g* for 10 min) at 4°C and washed with 1 volume of ice cold electroporation buffer (0.5 M sucrose and 10% glycerol) twice. The cells were resuspended in minimum electroporation buffer and divided into 40-µL aliquots to be stored at -80°C.

Transformation of pLTV3 and transposon insertion into chromosome. The extraction of pLTV3 was performed using the Qiagen midi-kit (Valencia, CA). The plasmid solution was stored in 30 µL aliquots at -20°C or thawed overnight at 4°C before use.

Then pLTV3 was electroporated into wild-type *E. mundtii*. The cells were thawed on ice while the 0.2 mm cuvettes and pLTV3 chilled at 4°C. After thawing, 1 µL of pLTV3 solution was added to the cells and mixed by gently stirring with a sterile pipette tip. The cell mixture was added to the bottom of the cuvette and pulsed at 12.5 kV/cm, 25-µF capacitance, and 200-Ω resistance. Afterwards, 1 mL of ice-cold M17 broth supplemented with 0.5 M sucrose, 3% glycine, 10 mM $MgCl_2$, and 10 mM $CaCl_2$ was added to the cuvette and chilled on ice for at least 5 min. The broth and cells were transferred to sterile test tubes and incubated for 2 hours at 37°C without agitation. The cells were spread plated (100 µL each) on streptococcal regeneration (SR) plates. One liter of SR plate agar contained 10 g tryptone, 200 g sucrose, 5 g yeast extract, 25 g gelatin, 10 g glucose, 15 g agar, 0.25 g MgCl2, and 0.14 g CaCl2. The plates were also supplemented with 5 mg tetracycline (Tet) and incubated at 37°C for 48 hours (Holo and Nes 1989, Shepard and Gilmore 1995).

Second, the transposon was integrated into the host genome by temperature shift. The transformants generated were inoculated into 5 mL tubes of MRS broth containing 5 μg/mL Tet and incubated overnight at 30°C with agitation. Then, 6.25 μL (~1/800) of the overnight culture was added to fresh 5 mL MRS broth with 0.04 μg/mL erythromycin (Em) followed by incubation at 41°C, a nonpermissive temperature, for 48 hours. Each transformant was then spread plated on MRS plates containing 1 μg/mL Em at 41°C until colonies appeared.

Digestion and ligation of mutant genomes. Each mutant cell of interest was lysed. After the protein was degraded by proteinase K, the cell lysate was subjected to phenol/chloroform extraction, followed by precipitation with isopropanol, and then the DNA was dissolved in 10 mM Tris, 1 mM EDTA (TE) buffer (pH = 8.0) (Sambrook and Russell 2001). The DNA was digested with *Xba*I to cleave off part of Tn917 and erythromycin resistance, leaving left flanking DNA, lacZ, chloramphenicol resistance, neomycin phosphotransferase II, and bleomycin resistance. After another DNA extraction to eliminate the enzyme, the DNA is then ligated with ligase (Camilli et al. 1990). The interrupted gene was amplified by inverse PCR and sequenced using primers derived from pLTV3, one from the monoclonal site (5'-CCG GGG ATC CTC TAGA-3') and the other upstream *lacZ* (5'-GTT AAA TGT ACA AAA TAA CAG CGA-3') (Martin et al. 2003). The PCR product was then sent to the Cornell Biotechnology Resource Center for Sanger sequencing.

Spot diffusion assay. Producer strains *E. mundtii* CUGF08 and ATO6 was grown for 18 hours, and then centrifuged. The supernatant was filter-sterilized using 0.22-μL PES. Cell-free supernatant samples were serial diluted 2-fold with sterile Milli-Q water, and 100 μL of each dilution was spotted onto the agar plate, 20 μL at a time. Eight milliliters of soft agar (0.75%)

inoculated with 100 µL of overnight culture of indicator species were then poured carefully onto the spotted plate.

Peptide purification and sequencing. The 18-hour, 200-mL *E. mundtii* culture in MRS was centrifuged and the supernatant was applied to a C18 SepPark cartridge that was equilibrated with 100% methanol. Milli-Q water, 100% methanol, 20% acetonitrile, and 20% isopropanol were then applied to elute salts and fractions without bacteriocin activity. The active fraction was eluted with 50% isopropanol and applied to a cation exchange column of Amberlite IR120 Na+ (Sigma-Aldrich) that was equilibrated with 5 mM sodium phosphate (pH =7). The eluent was injected into the HPLC (Agilent) equipped with a Jupiter C5 200Å column (Phenomenex). The active eluent collected from the HPLC at 30% acetonitrile in 0.1% trifluoroacetic acid and was sent to the Proteomics & Mass Spectrometry Facility at the Donald Danforth Plant Science Center (St. Louis, MO) for amino acid sequencing and intact mass determination. For sequencing, the sample was first digested with trypsin, and then it was run through LC-MS/MS with an 1-hour gradient. Each purification step underwent spot diffusion assays in order to quantify self-lethal activity.

Tricine SDS-PAGE. A tricine-polyacrylamide gel, consisting of a loading layer (4% acrylamide, 30% solution, 29:1 Bio-Rad), a stacking layer (10% acrylamide), and a resolving layer (15% acrylamide, 9% glycerol), was hand casted using the mini Protean III gel platform (Bio-Rad). The gels were loaded and ran at 120 mV for about 2 hours (Manns et al. 2012). Gels for staining were then rinsed briefly (5-10 s) in distilled water and then fixed in 5% glyceraldehyde for 1 hour. The glyceraldehyde solution was then rinsed from the gel three times for 5 minutes in distilled water followed by staining with 0.025% Coomassie blue G-250 in 10%

acetic acid. Lastly, the gel was destained three times with 10% acetic acid overnight (Schägger 2006).

The gels for overlaying were fixed in a 10% acetic acid and 50% methanol solution for 30 minutes, and rinsed three times in sterile Milli-Q water for 15 minutes each time. The gel was placed onto an MRS agar plate and overlaid with 8 mL soft agar inoculated with 100 μL of overnight indicator species.

Edman degradation. Gels were subjected to SDS-PAGE as described, then electroblotted onto a polyvinylidene difluoride membrane (PVDF) for 1 hour at 100 V, 400 mA, and 4 °C. The blot was then stained with 0.1% Coomassie blue R-250 for 15 minutes and destained with 10% methanol (Burnette 1981, Towbin et al. 1979). Bands to be sequenced were excised and stored at -20 °C. The sample was sent to the Synthesis and Sequencing Facility at Johns Hopkins University (Baltimore, MD) for Edman degradation to determine the N-terminal sequence of the peptide responsible for activity.

RESULTS AND DISCUSSION

Identification and amplification of Tn*917* interrupted genes by inverse PCR.

Approximately 3000 mutants were screened. Five were found to be fully sensitive to bacteriocin production (Figure 2.1), so it is likely a gene that contributes to immunity was interrupted. *Xba*I was used to cleave the chromosomal DNA, leaving the sequences flanking the Tn*917* insertion site. Fragments were then circularized by a ligation reaction. The interrupted gene was amplified by inverse PCR and sequenced using primers derived from pLTV3. One of the PCR fragments was successfully sequenced using a Sanger method, and the best tBLASTx match (E-value = 1.4) was for a synthetic AraC construct from *E. coli*, which is a repressor protein of the arabinose operon (Figure 2.2) (Schleif and Favreau 1982). It might be possible that the disrupted

gene is a repressor protein for another that acts as a target for mundticin L. A high-fidelity *Taq* polymerase would have improved the quality of the PCR product for sequencing.

Although there were multiple mutants that displayed the same phenotype, it is possible that they were all daughter cells of the same mutant. Additionally, this approach may not have been viable since the disruption of one or more immunity genes for a self-lethal strain may have exasperated the phenotype, rendering it challenging to culture and select for such a mutant. Moreover, if the causative agent were essential for survival, the interruption of the gene may have prevented culturing of the mutant. To more effectively determine the causative agent, a peptide isolation approach was used since it is known that protease K can inactivate the causative agent of self-lethality (results not shown).

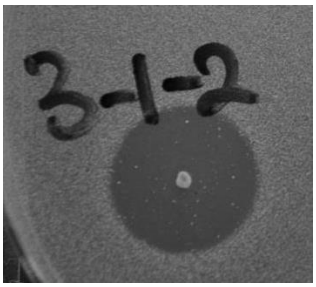

Figure 2.1. Mutants that were found to be fully self-lethal, as opposed to the wild-type, which still reproduces near the producer colony (Figure 1.4).

```
Search Query   CERITISHRKQ--P-PGI-RGS-S-R
AraC           CERITISHRKQPDPAPALLQNAFNKR
```

Figure2.2. Alignment of the translated PCR product to its best tBLASTx hit (E-value = 1.4), AraC.

Mundticin L and precursor mundticin L both cause self-lethality in *E. mundtii* CUGF08. The peptide responsible for the self-lethality was partially purified using the steps mentioned in Table 2.1, and the final eluent was sequenced using mass spectroscopy. Results from the LC-MS/MS indicated a peptide of intact mass of Mr = 6028.3 Da (Figure 2.4). The sequence of the trypsin-digested fragment is AIGIIGNNSAANLATGGAAGWK (Figure 2.5), which is 100% identical to munL and munKS (Figure 2.6).

The intact mass conflicted with the calculated mass of MunL that Feng et al. (2009) determined (Mr = 4.3 kDa), but closely resembled the precursor mass of MunL. The precursor form of the MunL may be partially responsible for causing lethality to its producer, *E. mundtii* CUGF08. This precursor form of MunL was also found to be active against *L. monocytogenes* 10403S.

Generally, the ABC transporter cleaves class IIa bacteriocins after the GG motif (Figure 2.6) (Håvarstein et al. 1995). However, there may be another factor necessary in order for cleavage to occur or proteolytic activity is low. Alternatively, because of the self-lethality, there may be detectable presence of precursor peptide that was not cleaved by the ABC transporter. If the precursor peptide is responsible for self-lethality, one explanation is that the additional N-terminal residues may prevent the action of immunity protein cognitive to MunL.

Table 2.1. Antimicrobial activity of fractions against *L. monocytogenes* (*Lm*) and *E. mundtii* (*Em*). The volume normalized activity unit (AU/mL) was calculated as the reciprocal of the highest dilution displaying an inhibition zone divided by the volume utilized.

	Volume (mL)	***Lm* Activity (AU/mL)**	***Lm* Total Activity (AU)**	***Lm* Activity Recovery**	***Em* Activity (AU/mL)**	***Em* Total Activity (AU)**	***Em* Activity Recovery**
***E. mundtii* Supernatant**	7.00×10^{2}	6.40×10^{3}	4.48×10^{6}	1.00	4.00×10^{1}	2.80×10^{4}	1.00
C18 SepPak Eluent	6.00	2.56×10^{4}	1.54×10^{5}	3.43×10^{-2}	1.60×10^{2}	9.60×10^{2}	3.43×10^{-2}
Ion Exchange Eluent	3.00×10^{1}	4.00×10^{2}	1.20×10^{4}	2.68×10^{-3}	2.78×10^{-1}	8.34	2.98×10^{-4}
HPLC Eluent	2.00×10^{-2}	5.00×10^{1}	1.00	2.23×10^{-7}	3.48×10^{-2}	6.95×10^{-4}	2.48×10^{-8}

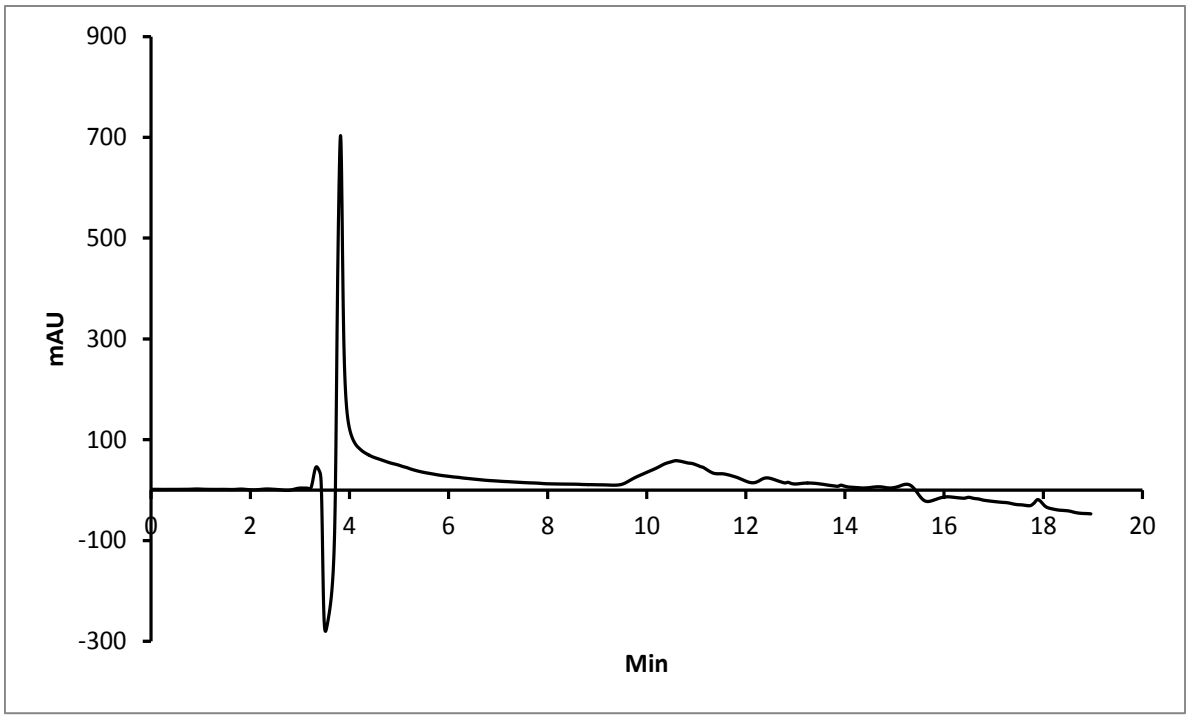

Figure 2.3. HPLC chromatograph for mundticin L after it was eluted from the cation exchange column. The steps are also highlighted in Table 1. The active fraction was collected after 3.4 min (30% acetonitrile and 0.1% trifluoroacetic acid), and then sequenced.

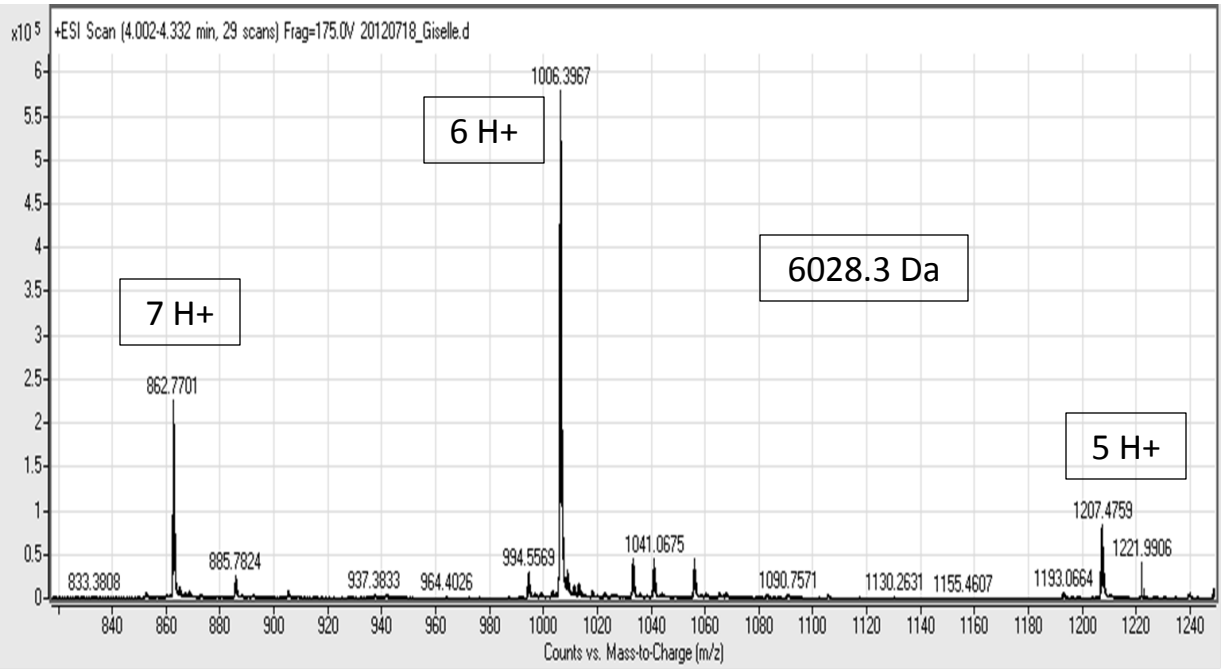

Figure 2.4. The intact mass of the peptide as determined by LC-MS prior to trypsin digestion.

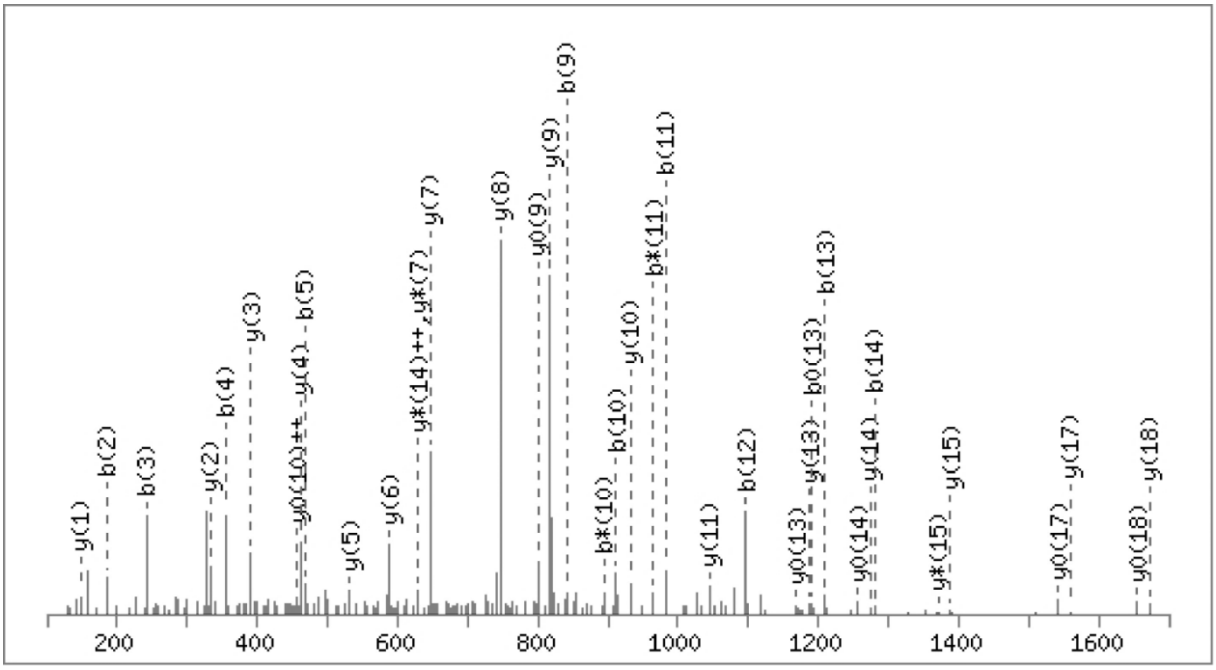

Figure 2.5. MS2 spectrum determining sequence of trypsin-digested munL. See bolded sequences in Figure 2.6.

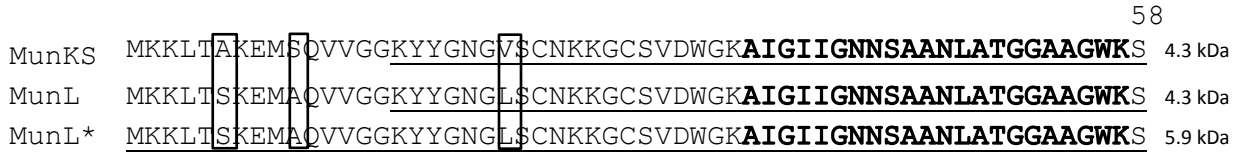

Figure 2.6. Sequence alignment of MunKS (Kawamoto et al. 2002), MunL (Feng et al. 2009), and MunL*, which is the projected primary sequence based on the flanking DNA sequence of munL The amino acid substitutions are boxed, the peptide sequenced by the MS2 is in bold, the secreted peptides are underlined, and the calculated masses of the secreted peptides are indicated.

SDS-PAGE and N-terminal sequencing. The supernatant from the 18-hour, 200-mL culture was applied to a C18 SepPak Cartridge, and the final, 50% isopropanol eluent was used for SDS-PAGE. Despite finding that a 6 kDa peptide is a causative agent for the self-lethality of *E. mundtii* CUGF08, the SDS-PAGE overlay only had a zone of inhibition around 4 kDa. After electroblotting and Edman degradation, the N-terminal sequence of this peptide was KYYGNGLSXNKKGXSVDX(G)(K)A(I)(G)(I). This discrepancy between the mass spectroscopy result and the SDS-PAGE result may be due to a higher concentration of the smaller peptide and that the concentration found for the 6 kDa peptide is not sufficient through this preparation to display its activity. Additionally, the 6 kDa peptide may have had a greater affinity for the material of the Eppendorf tubes in which the samples were stored, which may have decreased the concentration during the purification process and assay procedures. Regardless, the mature peptide was also a causative agent for self-lethality for this strain (Figure 2.7).

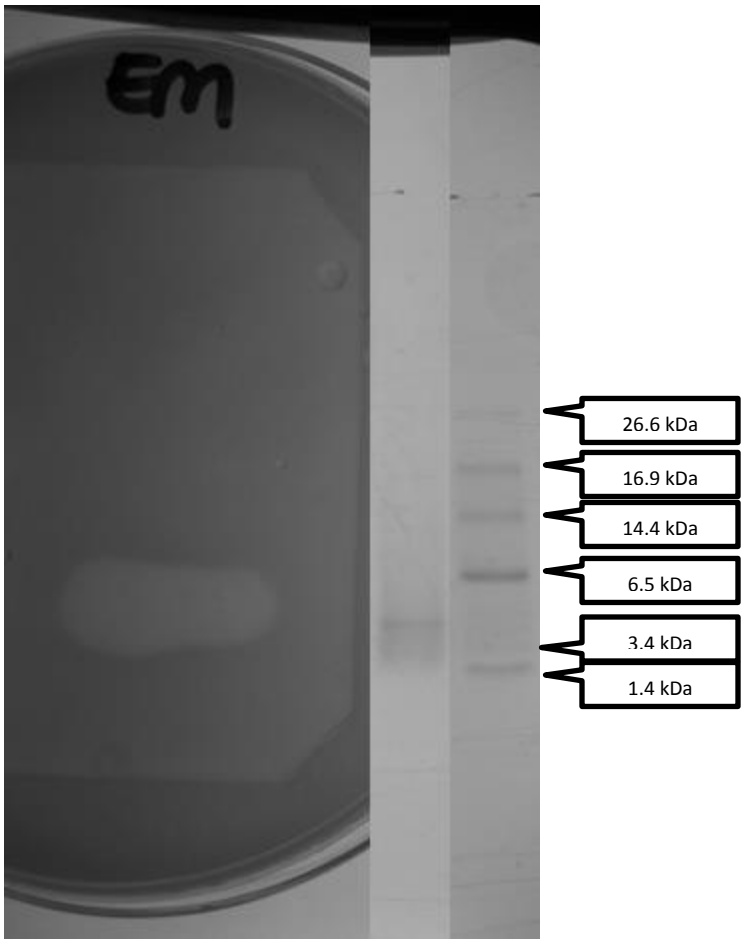

Figure 2.7. SDS-PAGE stained and overlay to scale. The gel was placed onto an agar plate and overlaid with soft agar inoculated with overnight *E. mundtii* CUGF08. The marker lane (right stained) from the bottom are 1.4, 3.4, 6.5, 14.4, 16.9, 26.6 kDa (Bio-Rad). The C18 SepPak eluent was applied the left lane.

CONCLUSIONS

Both the precursor and mature mundticin L may be responsible for causing lethality to the producer, *E. mundtii* CUGF08. The sequence of the purified self-active fraction was found to be nearly identical to the C-terminal sequences of the other known mundticins. The intact mass conflicts with the calculated mass of MunL that Feng et al. (2009) determined (Mr = 4.3 kDa), but closely resembles the pre-protein mass of MunL. Generally, the ABC transporter cleaves class IIa bacteriocins after the GG motif (Håvarstein et al. 1995). However, there may be another factor necessary in order for cleavage to occur. Conversely, the self-lethality also causes cell lysis, which would also release precursor munL that could not be processed by the ABC transporter.

The purification process of both the precursor and mature MunL was challenging, most likely due to the MRS medium in which the producer was cultured. If the self-lethality phenotype could be exhibited using a different medium containing fewer ingredients, such as tryptic soy, then the purification process may not require as many steps. Fewer elution steps would increase the final yield.

While there are some N-terminal substitutions between mundticin L and mundticin KS, both appear to be active against both *E. mundtii* CUGF08 and ATO6 (results not shown). This phenotype has not been observed in any other bacteriocin-producer, so the elucidation of this mechanism may be used to discover more information on the mechanism of action of class IIa bacteriocins, the maturation process, and the mechanism of the immunity protein.

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CHAPTER 3

IN SILICO GENOMIC COMPARISONS OF CLASS IIA BACTERIOCIN-PRODUCING *ENTEROCOCCUS MUNDTII* STRAINS TO IDENTIFY ADDITIONAL GENES INVOLVED WITH MECHANISMS OF ACTION AND IMMUNITY

ABSTRACT

Several *Enterococcus mundtii* strains produce a class IIa bacteriocin, mundticin KS or other homologs. However, two strains, *E. mundtii* CUGF08 and ATO6, are sensitive to their own bacteriocins, which is a phenotype not yet been observed in any other class IIa bacteriocin-producer, despite encoding a downstream immunity gene. The genomes of four mundticin-producers, the two sensitive strains and two fully immune strains *E. mundtii* QU 25 and CRL35, and one non-mundticin-producer, *E. mundtii* ATCC 882, were compared to identify additional genes potentially involved with the immunity and target specificity of mundticin mechanisms of action. There were no correlations among the translated sequences of the bacteriocin-associated genes, between the immune and sensitive strains. There are 28 genes unique to the fully immune strains, and 3 genes unique to the sensitive strains. Many of the unique genes of the immune strains encode for phage-related proteins, while the unique genes shared by the sensitive strains may be involved with the mechanism of action. Elucidation of the roles of these genes may further the understanding of how class IIa bacteriocins interact with the producer strain, specifically target closely-related strains, identify other potential immunity genes or accessory immunity genes.

INTRODUCTION

Class IIa bacteriocins are widely produced by various lactic acid bacteria isolated from all commodities of foods (Belguesmia et al. 2011, Klaenhammer 1993), making them potential

agents to control the growth of pathogenic and spoilage bacteria. However, the mechanisms of action, specificity, and immunity are not fully characterized. This may be because there may be genes encoded outside of the known bacteriocin gene cluster that have not been identified as vital genes involved in bacteriocin production or susceptibility.

There are several strains of *Enterococcus mundtii* that produce mundticin and encode for transport and immunity genes, but some are partially sensitive to mundticin (Feng et al. 2009) (Figure 1.4) while other producers are immune as predicted (Shiwa et al. 2014, Acuña et al. 2012). This is unusual since all strains encode for a cognitive immunity protein.

In order to identify additional genes involved with immunity or mechanism or action, the whole genomes of five strains were compared. Two strains, *E. mundtii* CUGF08 (Feng et al. 2009) and ATO6 (Bennik et al. 1998) were found to be partially self-lethal while *E. mundtii* QU 25 and CRL35 are reported to be completely immune to their respective mundticin bacteriocins (Shiwa et al. 2014, Acuña et al. 2012). Another strain, *E. mundtii* ATCC 882, does not produce mundticin, but was also compared to the other four strains. By comparing closely related strains, it allows for simpler elimination of core genes that are likely not involved with bacteriocin production or action.

METHODS

Cultures and media. Strains used in this study are indicated in Table 3.1. *Enterococcus mundtii* was grown in de Man Rogosa Sharpe (MRS) broth (Difco, Becton Dickinson and Company) at 30 °C at 200 RPM. For MRS agar plates, 1.5% agar (Difco, Becton Dickinson and Company) was added to broth. For MRS soft agar plates, 0.75% agar was added to broth. All MRS media was autoclaved for 7 minutes at 121 °C and 15 psi.

Deferred inhibition zones. A colony of a producer strain was spotted onto an MRS agar plate from an isolated colony and incubated overnight 30 °C. Then 8 mL of soft agar was inoculated with 100 µL overnight culture of the indicator strain and poured slowly onto the plate containing the bacteriocin producing colony.

Spot diffusion assay. Producer strains *E. mundtii* CUGF08 and ATO6 was grown for 18 hours, and then centrifuged. The supernatant was filter-sterilized using 0.22-µL PES. Cell-free supernatant samples were serial diluted 2-fold with sterile Milli-Q water, and 100 µL of each dilution was spotted onto the agar plate, 20 µL at a time. Eight milliliters of soft agar (0.75%) inoculated with 100 µL of overnight culture of indicator species were then poured carefully onto the spotted plate.

Table 3.1. Strains used for comparison studies. Strains indicated with an asterisk (*) were sequenced for this work. The genomes of the other strains were downloaded from the genome database from NCBI.

E. mundtii strains	Size (Mb)	Coding Sequences	Scaffolds	Origin	Reference
QU 25	3.35106	3221	6	Ovine faeces	Shiwa et al. 2014
CRL35	2.86632	2757	69	Argentinian cheese	Bonacina et al. 2014
CUGF08*	3.472719	3221	30	Alfalfa sprouts	Feng et al. 2009
ATO6*	3.209928	2891	46	Chicory endive	Bennik et al. 1998
ATCC 882	3.06559	2913	2	Dairy products	Broad Institute

Whole genome sequencing and assembly. Library preparation was performed using the Nextera® XT DNA Sample Preparation Kit (Illumina, Inc.) for *E. mundtii* CUGF08 and ATO6, and sequencing was performed at the Cornell University Biotechnology Research Center Genomics Facility using the Illumina Miseq with four other strains using 2 x 250 bp paired-end reads. Reads were trimmed using Trimmomatic (Bolger et al. 2014) and assembled using SPAdes (Bankevich et al. 2012).

Genomic comparisons, alignments, and data mining. Annotation was performed using RAST (Aziz et al. 2008, Overbeek et al. 2014), and comparisons were visualized using the SEED Viewer and BRIG (Alikhan et al. 2011). Genes of interest were selected based on an identity percent cutoff of 90% between those present in the strains fully immune to mundticin (*E. mundtii* QU 25 and CRL35) and susceptible to mundticin (*E. mundtii* CUGF08, ATO6, and ATCC 882). Other putative bacteriocin genes were identified using BAGEL3 (van Heel et al. 2013). Each gene of interest was searched using the default settings for Position-Specific Iterative BLAST (PSI-BLAST), using one or more iterative searches until no new hits were returned. Genes of interest were also searched and aligned with Pfam, the Protein Families database, in order to identify conserved domains (Finn et al. 2014). Local alignments were performed using ClustalX2 (Larkin et al. 2007).

RESULTS AND DISCUSSION

***Enterococcus mundtii* strains and genomes.** Three genomes of the selected *E. mundtii* strains (QU 25, CRL35, ATCC 882) were found in the NCBI database. The chromosome and plasmids of *E. mundtii* QU 25 have been completely mapped (Shiwa et al. 2014), while all the other genomes are draft genomes. *E. mundtii* CRL35 was selected because it produces enterocin CRL35, which is nearly identical to mundticin KS and mundticin L (Farías et al. 1996, Feng et

al. 2009) (Figure 3.2). *E. mundtii* ATCC 882 is a non-mundticin producer that was obtained from the ATCC: The Global Bioresource Center, to verify sensitivity to mundticin L. *E. mundtii* CUGF08 and ATO6 were sequenced using the Illumina MiSeq. This yielded 2,403,107 reads for *E. mundtii* CUGF08 and 2,640,849 reads for *E. mundtii* ATO6, for a coverage of 345x and 411x, respectively.

Among the five strains, the pan-genome consists of 3992 genes and the core-genome consists of 1662 genes (Figure 3.1). While the pan-genome increases with each subsequent strain, the core-genome steadily drops. However, the slope of the core-genome graph never seemed to approach zero between each additional strain, implying that additional strains may help in determining a more complete core-genome. These five strains were selected because their sensitivities to a mundticin KS homologue are known.

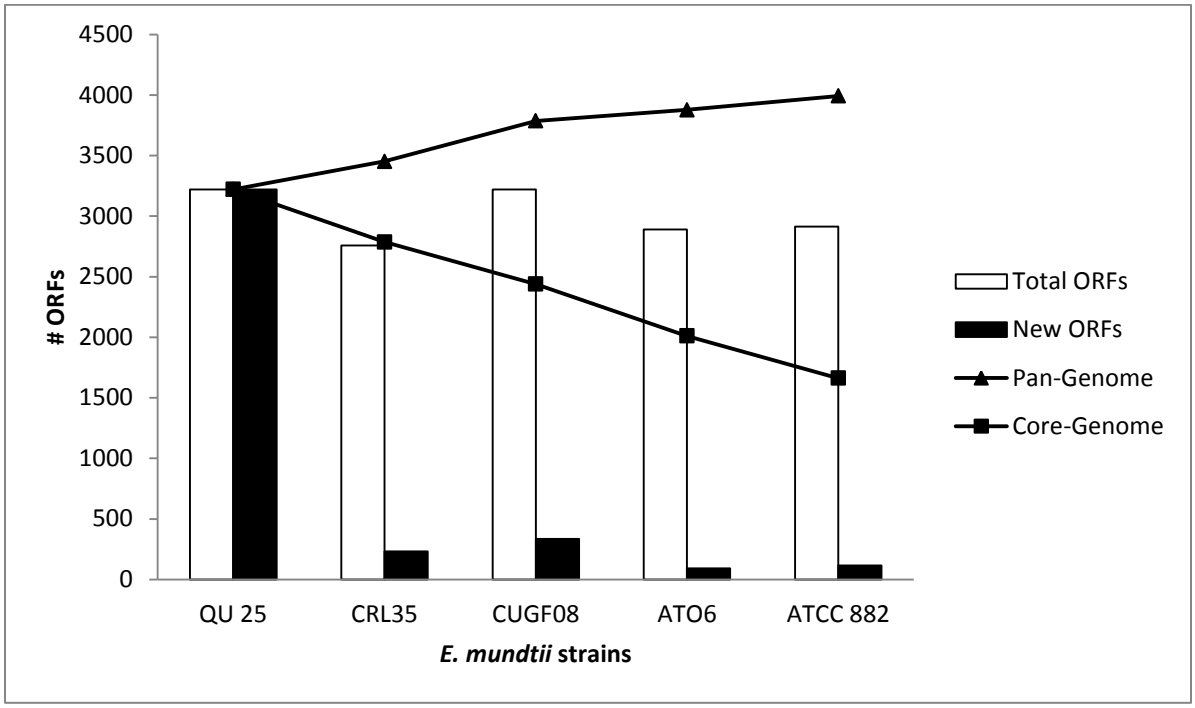

Figure 3.1. Genome analysis of the five *Enterococcus mundtii* strains.

Mundticin production and alignment of characterized mundticin genes. When the purified mundticin was applied to QU 25 and CRL35, no indication of self-lethality was reported (Shiwa et al. 2014, Bonacina et al. 2014). However, when deferred inhibition zones were performed for *E. mundtii* CUGF08 and ATO6 against themselves, a partial zone of inhibition was formed (Figure 1.4). In addition, the supernatants from both producers are active against each other (Table 3.2), implying that both strains are sensitive to mundticin L and mundticin KS. *E. mundtii* ATCC 882 was confirmed not to produce antimicrobial agents active against *E. mundtii* strains using the deferred inhibition zone method. As predicted, the strain is sensitive to bacteriocin production of *E. mundtii* CUGF08, as it exhibited a complete zone of inhibition.

Table 3.2. The susceptibility of the two self-lethal strains against sterilized supernatant.

Supernatant	*E. mundtii* Indicator Strain Activity (AU/mL)	
	CUGF08	ATO6
CUGF08	160	160
ATO6	20	20

E. mundtii strains QU 25, CRL35, CUGF08, and ATO6 all produce class IIa bacteriocin mundticin (Shiwa et al. 2014, Farías et al. 1996, Feng et al. 2009, Bennik et al. 1998), but there are some N-terminal substitutions in the leader sequence (Figure 3.2). The substitutions are not conserved mutations (A→S or S→A), but each mundticin contains both an alanine and serine in positions -6 and -10. Those residues may serve similar, polar to non-polar interaction, regardless of their order, as long as they occupy those positions and there is always one of each residue. There is also one V→L mutation, but it was shown that it does not significantly affect the level of susceptibility in indicator species, most likely because it is a conserved mutation (Feng et al. 2009).

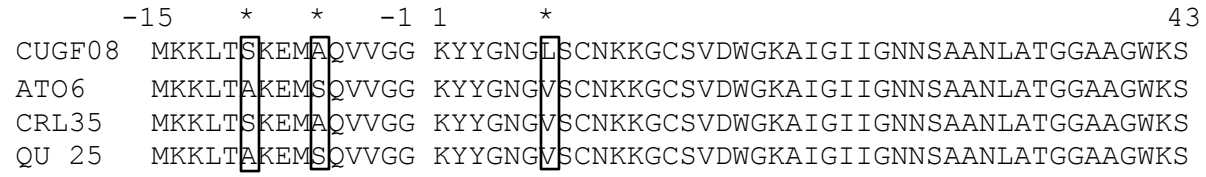

Figure 3.2. Precursor mundticin sequences from the four producer strains. The space between signifies where the ABC transporter cleaves, after the conserved GG-motif. Boxed are the 3 mutations.

There are two additional genes downstream the plasmid-encoded structural gene: the cognitive ABC transporter (*munT*) and immunity (*munI*) genes. The genetic organization of the three components is the same for all four strains (Figure 3.3). With the exception of one G→T mutation in *E. mundtii* CRL35, the promoters upstream both the structural gene and *munTI* are identical among the four producer strains (results not shown). Most other class IIa bacteriocin transporters contain the N-terminal motifs QX_4[D/E]$CX_2AX_3MX_4$[Y/F]GX_4[I/L] and H[Y/F][Y/V]VX_{10}[I/L]XDP, which are important for peptidase activity at the GG motif of precursor class IIa bacteriocins (Håvarstein et al. 1995). However, the ABC transporters for mundticin do not fully contain the first motif, and therefore, may contain only an incomplete active site (Figure 3.4). Since mature mundticin can be isolated from the cell-free supernatant, it is possible that the ABC transporter still functions as a peptidase, or there is an additional factor involved in the cleavage. The immunity proteins conform to α-helices that form a four-bundle shape, with a hydrophobic inner portion and a hydrophilic outside, and the conserved IRYGY motif is necessary for immunity function (Jeon et al. 2009). With the exception of one D → E conserved mutation in the cognitive immunity protein for mundticin L produced by *E. mundtii* CUGF08, the immunity proteins are identical (Figure 3.5).

Figure 3.3. The genetic organization of mundticin L (*munL*), the ABC transporter (*munT*), and the immunity protein (*munI*) of *E. mundtii* CUGF08. Putative promoter regions are indicated by the thin, bent arrows and the putative *rho*-independent terminators are indicated by the octagons. This genetic organization is the same for all four producer strains.

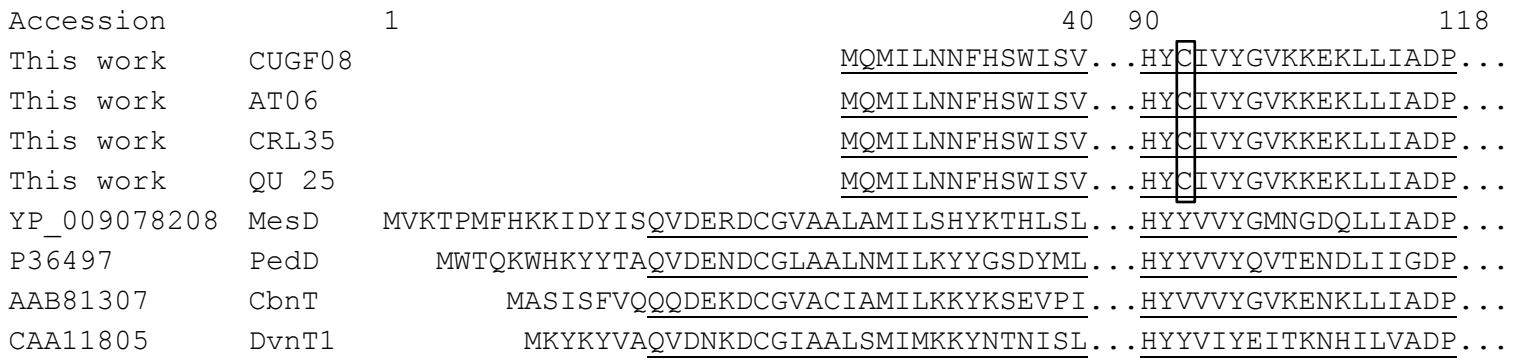

Figure 3.4. The N-termini of putative cognitive ABC transporters for mundticin (munT), produced by the four producer strains (CUGF08, ATO6, CRL35, and QU 25), and other class IIa bacteriocin ABC transporters: MesD from *Leuconostoc mesenteroides*, PedD from *Pediococcus acidilactici*, CbnT from *Carnobacterium maltaromaticum*, and DvnT1 from *Carnobacterium divergens*. The NCBI accession numbers are indicated for the latter four transporters. Underlined are the motifs necessary for peptidase activity (QX_4[D/E]$CX_2AX_3MX_4$[Y/F]GX_4[I/L] and H[Y/F][Y/V]VX_{10}[I/L]XDP) (Håvarstein et al. 1995). Boxed is a single base substitution found within the conserved motif in the mundticin transporters.

```
        1                              *                   50
CUGF08  MSNLKWFSGGDDRRKKAEVIITELLDDLEIELGNESLRKVLGSYLKKLKN
ATO6    MSNLKWFSGGDDRRKKAEVIITELLDDLEIDLGNESLRKVLGSYLEKLKN
CRL35   MSNLKWFSGGDDRRKKAEVIITELLDDLEIDLGNESLRKVLGSYLEKLKN
QU 25   MSNLKWFSGGDDRRKKAEVIITELLDDLEIDLGNESLRKVLGSYLKKLKN

       51                                                98
CUGF08  EGTSVPLVLSRMNIEISNAIKKDGVSLNENQSKKLKELISISNIRYGY
ATO6    EGTSVPLVLSRMNIEISNAIKKDGVSLNENQSKKLKELISISNIRYGY
CRL35   EGTSVPLVLSRMNIEISNAIKKDGVSLNENQSKKLKELISISNIRYGY
QU 25   EGTSVPLVLSRMNIEISNAIKKDGVSLNENQSKKLKELISISNIRYGY
```

Figure 3.5. Plasmid-encoded immunity proteins from the four producer strains. Underlined is the conserved C-terminal motif IRYGY, found in subgroup B immunity proteins (Jeon et al. 2009, Drider et al. 2006).

Identification of additional antimicrobial compounds. Additional bacteriocin genes were searched using BAGEL3, which uses hidden Markov models to identify sequences that may be related to bacteriocins in the BAGEL3 database, to determine whether an uncharacterized bacteriocin may be responsible for self-lethality. Even though the genomes were annotated, this additional search may help identify other putative ribosomally synthesized antimicrobial peptide ORFs that may be too small for many automatic annotation programs (van Heel et al. 2013).

Among the five strains, there were 17 ORFs identified as putative antimicrobial peptides (Table 3.3). It correctly identified the four mundticin-producers. The four mundticin homologs are the only peptides among the 17 total that share homology. The two self-lethal strains encode for two different sactipeptides, which is a new class of ribosomally-synthesized, posttranslationally modified antimicrobial peptides that contain thioester bonds (Fluhe and Marahiel 2013). However, *E. mundtii* QU 25 and ATCC 882 also produce different sactipeptides, and neither strain is self-lethal. Most of the putative identified bacteriocins also matched for an ORF previously identified, but most of the annotations were different than the antimicrobial peptide proposed by BAGEL3. There were some putative bacteriocins that were not identified as an ORF at all. Even though the two self-lethal strains do not share an additional antimicrobial peptide, it is possible their phenotype is caused by independent antimicrobial peptides.

Table 3.3. The list of total putative antimicrobial peptides and proteins encoded within the five genomes as determined by BAGEL3, which searches potential peptides through whole genomes (van Heel et al. 2013).

Strain	Antimicrobial peptide/protein	Type	Corresponding ORF
QU 25	Sactipeptide 15	Sactipeptide	28 Decarboxylase
	Enterocin SE-K4	Class II bacteriocin	n/a
	Mundticin KS	Class II bacteriocin	3208 Hypothetical protein
	Enterolysin A	Class III bacteriocin	3131 Cell wall surface anchor family protein
CRL35	Enterocin CRL35	Class II bacteriocin	2527 Hypothetical protein
ATCC 882	UviB	Class II bacteriocin	2057 Hypothetical protein
	Zoocin A	Class III bacteriocin	2061 Membrane proteins related to metalloendopeptidases
	Sactipeptide 22	Sactipeptide	2687 lysine decarboxylase family
CUGF08	Enterolysin A	Class III bacteriocin	2035 Phage lysin
	Enterolysin A	Class III bacteriocin	3058 Enterolysin A
	Mundticin L	Class II bacteriocin	1405 Hypothetical protein
	Sactipeptide	Sactipeptide	n/a
	Enterolysin A	Class III bacteriocin	1167 Phage lysin
	Lactococcin 972	Class II bacteriocin	3122 Hypothetical protein
	Lanthipeptide 28	Lanthipeptide	1262 Hypothetical protein
ATO6	Mundticin KS	Class II bacteriocin	2604 Hypothetical protein
	Sactipeptide 25	Sactipeptide	n/a

Additional chromosome-encoded immunity genes. All five strains encode for an additional, putative mundticin immunity protein. In *E. mundtii* QU 25, this protein is chromosomal-encoded. With the exception of the *E. mundtii* ATO6 immunity protein, all five contain the C-terminal conserved IRYGY motif (Figure 3.6).

The chromosomal immunity proteins for sensitive strain *E. mundtii* CUGF08 and immune strain *E. mundtii* QU25 are 99% identical. There are no differences between the immunity proteins of the mundticin-sensitive and -immune strains. Even though *E. mundtii* ATCC 882 is sensitive to mundticin L-production by *E. mundtii* CUGF08, their chromosomal immunity proteins are 100% identical. It is possible that this immunity protein is not involved with mundticin immunity. The C-terminal IRTGY motif is present, but since there are several mutations (Figure 3.7), the residues involved in specific immunity to mundticin may not be present in the chromosomal immunity protein.

```
          1 *     * **   *  ** *   *  ** ** *   *  *** **   *    *** 60
ATO6      MSNLSWFSGGDDRRKKAEALITELIGDLEVDSENQSLRVVLKSNLRNLQNEGASVLLLLS
CUGF08    MSHLRWFSGGNDRRKTAETIINELIADLALDLGNESLREVLHAYLEKLQNDGASVPFILS
ATCC 882  MSHLRWFSGGNDRRKTAETIINELIADLALDLGNESLREVLHAYLEKLQNDGASVPFILS
QU 25     MSHLRWFSGGNERRKTAETIINELIADLALDLGNESLREVLHAYLEKLQNDGASVPFILS
CRL35     MSHLRWFSSGNDRRKRAETIINELIADLALDHGNESLREVLHAYLEKLQNDGASVPFILS

         61  ** *  *    *  * ** *  *  **  *    98 %Identity
ATO6      RMNVEIANAIKKDGVSLDEAYSKKLQELISISNIRYG-  74%
CUGF08    RMNLDISNALKKDGASLNEHQSEKLRELMAISSIRYGY  71%
ATCC 882  RMNLDISNALKKDGASLNEHQSKKLRELMAISSIRYGY  n/a
QU 25     RMNLDISNALKKDGASLNEHQSEKLRELMAISSIRYGY  72%
CRL35     RMNLDISNALKKDGASLNEHQSEKLHELMAISSIRYGY  69%
```

Figure 3.6. Putative additional mundticin immunity proteins that are encoded by all five strains in this study. Underlined is the IRYGY motif that is conserved in immunity proteins cognitive for mundticin (Jeon et al. 2009). Indicated are the percent identities to munI of the same strain, which is encoded downstream *munKS* or *munL*.

```
             1 * *     *    *  *  *  **  ***       *  **  *  * * *   ** 60
Chromosomal  MSHLRWFSGGNDRRKTAETIINELIADLALDLGNESLREVLHAYLEKLQNDGASVPFILS
Plasmid      MSNLKWFSGGDDRRKKAEVIITELLDDLEIELGNESLRKVLGSYLKKLKNEGTSVPLVLS

            61  **         *    *  *     **  *    98
Chromosomal  RMNLDISNALKKDGASLNEHQSEKLRELMAISSIRYGY
Plasmid      RMNIEISNAIKKDGVSLNENQSKKLKELISISNIRYGY
```

Figure 3.7. Comparison of the two mundticin immunity proteins produced by *E. mundtii* CUGF08. They are 71% identical to each other.

Mannose phosphotransferase systems encoded in all genomes. It has been reported that the transmembrane domains of the mannose phosphotransferase system (ManPTS) is a potential target for class IIa bacteriocins, especially since the gene is down regulated in resistant strains (Kjos et al. 2009, Arous et al. 2004). The ManPTS consists of a hydrophilic domain, IIAB, and two transmembrane domains, IIC and IID (Mao et al. 1995). By cloning hybrids of ManPTS from sensitive *L. monocytogenes*, Kjos et al. (2010) found that a 40-amino acid region containing the conserved GGQG motif of an extracellular loop of IIC is involved with class IIa bacteriocin activity.

The IIC domains from all five strains contain the GGQG motif and are 100% identical. Therefore, it is unlikely that this domain is involved with the self-lethality. It is still unclear how any class IIa bacteriocin specifically targets the cognitive sensitive strains. It is possible that among the mundtin-producing *E. mundtii* strains, an additional factor can be identified that allows mundticin to specifically target only select strains as opposed to all *E. mundtii* strains.

Identification of unique genes between sensitive and immune groups of strains. BRIG, which uses a BLASTn function to compare entire genomes against a reference (Alikhan et al. 2011), was first used to visualize the entire map of *E. mundtii* QU 25 against the other four strains (Figure 3.8). The sequence-based compare function in the SEED Viewer was then used to determine unique genes between the two different groups (Aziz et al. 2008, Overbeek et al. 2014). Identified genes must be 90% identical within the group and below 90% identical in the other group. There are 28 unique genes among the immune strains (Table 3.4) and 3 unique genes among the sensitive group (Table 3.5). Putative functions were inferred using PSI-BLAST, and databases CDD (Marchler-Bauer et al. 2011) and Pfam (Finn et al. 2014).

Hypothetical proteins contained no known conserved domains, but were encoded by a putative ORF.

E. mundtii QU 25 was used as the reference genome using the SEED compare genomes function since it is both completely immune to mundticin and completely mapped. Unique genes from the immune group are chromosomal-encoded for *E. mundtii* QU 25 (Table 3.4).

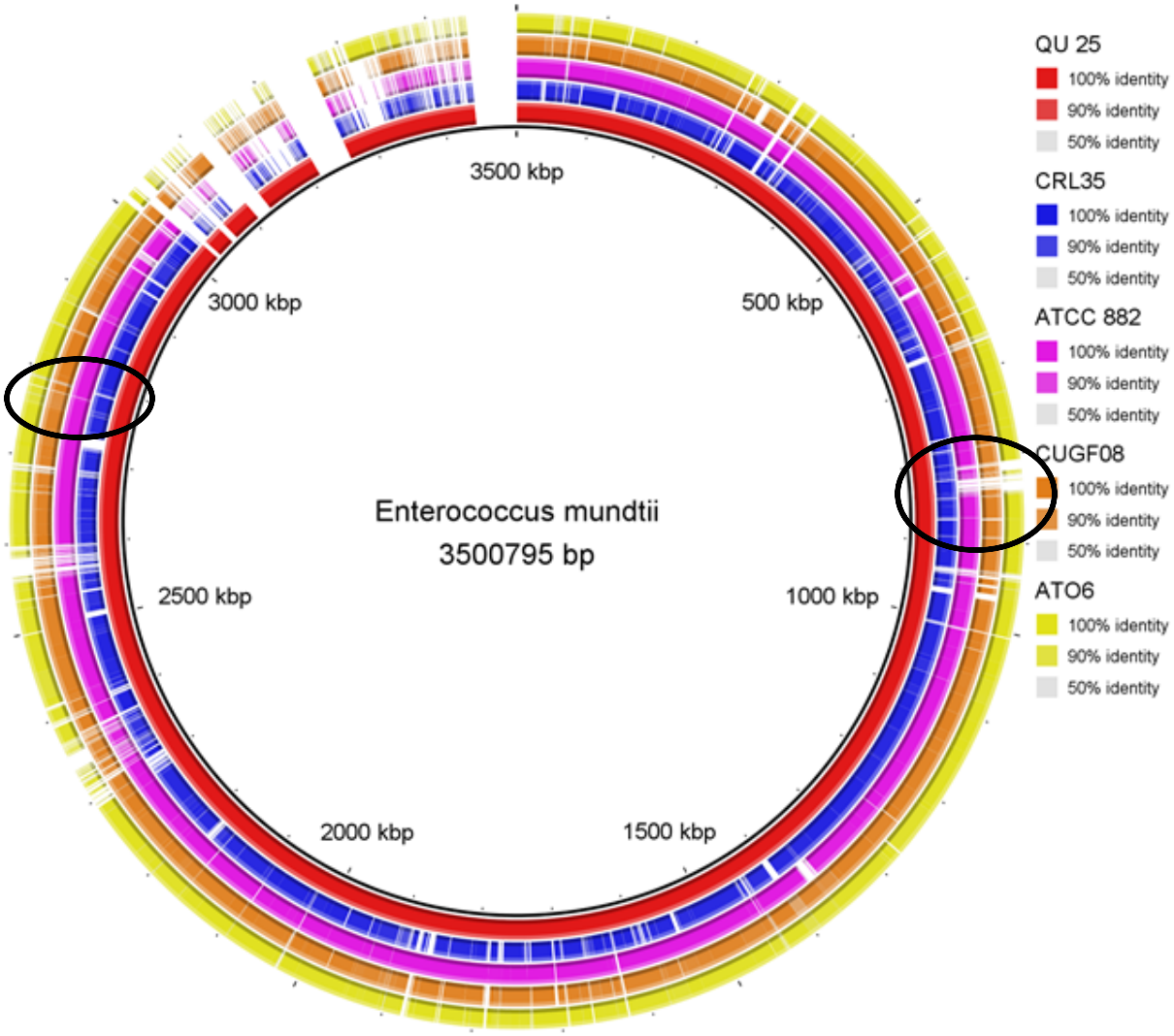

Figure 3.8. The mapped genome of *E. mundtii* QU 25 BLAST compared to the other four genomes, done with BRIG (Alikhan et al. 2011). Many of ORFs 754-805 and ORF 2688 are visibly unique to *E. mundtii* QU 25 and CRL35.

While several of the genes are of unknown function, most encode for phage-related proteins. The phage proteins unique to the immune group are homologous to phage proteins from a wide variety of *Enterococcus* and non-Enterococcal Gram positive Firmicutes. The presence and proximity of the tail proteins (ORFs 798, 801, 804, 805), as well as several structural and phage-related regulatory genes, implies that at least one prophage from the order Caudovirales is unique to the immune strains (Ackermann and Kropinski 2007). There are several phages that produce proteins that modify the cell envelope in order to prevent superinfection of other, similar phages. In particular, those infecting Gram positives can produce cell membrane-associated proteins to block the invasion of superinfecting DNA (Bondy-Denomy and Davidson 2014). Furthermore, it is possible for some bacteriocin genes to be associated with phage genes (Chavan et al. 2005). Perhaps one or more of the hypothetical proteins may be membrane-associated and can provide for the additional immunity necessary to provide full immunity against mundticin.

ORF 755 is a hypothetical protein that shares homology with a putative secreted protein (31% identity) or putative lipoprotein (27%). This may function as an accessory protein for the membrane-bound immunity protein (Jeon et al. 2009).

In addition, ORF 2688, a hypothetical protein, is unique to the immune strains. It is 20% identical to a twin-arginine protein translocation pathway signal sequence, which translocates proteins from the cytoplasm across the membrane with or without a co-factor. Proteins that use this system contain a specific N-terminal motif, S/T-RRXFLK (X is any polar amino acid). However, Gram positive species generally require at least two membrane proteins, TatA and TatC, in order to form a docking complex (Goosens et al. 2014). The immune strains do not encode for a second Tat protein, so it may be likely this hypothetical protein does not function as

a Tat pathway component. Regardless, the immunity proteins contain the RR signal sequence required for Tat-pathway translocation. It may be possible this single Tat protein could be a novel transport system that interacts with the immunity protein.

There are two genes potentially involved in transcription regulation. ORF 778 is DNA-cytosine methyltransferase, which forms 5-methylcytosine at CG dinucleotides outside of CG-rich regions. Especially when found in promoter regions, 5-methylcytosine prevents the transcription of certain genes (Attwood et al. 2002). ORF 756 contains an N-terminal helix-turn-helix motif, which functions as a xenobiotic response element (XRE)-family transcriptional regulator that binds to DNA, causing repression of a gene (Matsushita et al. 1993, Luscombe et al. 2000). In addition, it contains a C-terminal S24 or LexA-like peptidase motif. This family of proteins binds to DNA until it undergoes a conformational change and self-cleavage event from an SOS response, allowing the transcription of genes that were initially repressed (Luo et al. 2001). The SOS box (CGAACRNRYGTTYC) to which a LexA-homolog may bind in *Bacillus subtilis* was not found in either *E. mundtii* QU 25 or CRL35 (Winterling et al. 1998), but there *Enterococcal* SOS box most likely varies. It may be possible that repression is preventing the expression of genes that are targets of mundticin.

Lastly, there are seven hypothetical proteins of unknown function, most of which are homologous to other hypothetical proteins from *Enterococcus* spp. One ORF 773 is 26% identical to 2-methylcitrate dehydratase, which catalyzes the dehydration of 2-methylcitrate to 2-methyl-cis-aconitate during propionate metabolism (Tabuchi et al. 1981). It is unlikely that an enzyme involved in propionate metabolism also contributes to bacteriocin immunity of a producer cell.

Table 3.4. Genes unique only to immune strains. *E. mundtii* QU 25 was used as the reference genome. Genes found in *E. mundtii* CRL35 that were above 90% but were also below 90% in the other three genomes were identified as unique genes.

			Percent Identity					
ORF	RAST Annotation	Length	CRL35	CUGF08	ATO6	ATCC 882	PSI-BLAST Hit	E-Value
Putative membrane-associated proteins								
755	Hypothetical protein	215	98.13	n/a	n/a	n/a	*Lactobacillus salivarius* putative secreted protein; *Lactobacillus oris* putative lipoprotein	1e-09; 2e-08
2688	Hypothetical protein	182	97.81	n/a	n/a	n/a	*Faecalibacterium prausnitzii* Tat pathway signal sequence	5e-09
Phage proteins								
754	Phage integrase	376	99.47	28.34	28.53	32.26	*E. faecium* site-specific recombinase, phage integrase family	6e-144
757	Conserved hypothetical protein, phage associated	78	98.7	n/a	n/a	n/a	*E. faecium* XRE family transcriptional regulator	1e-24
759	Hypothetical protein	105	100	n/a	n/a	n/a	*Staphylococcus aureus* phage protein	6e-10
789	Phage protein	114	96.46	n/a	n/a	n/a	*Enterococcus faecalis* HNH endonuclease domain protein	3e-49
790	Phage terminase, small subunit	155	98.7	n/a	n/a	n/a	*Streptococcus parauberis* Phage Terminase Small Subunit	2e-59
791	Phage terminase, large subunit	581	100	18.72	n/a	18.97	*Enterococcus casseliflavus* putative phage terminase, large subunit	0
793	Phage portal	421	98.81	n/a	n/a	n/a	*E. casseliflavus* phage portal protein, HK97 family	0

Table 3.4 (Continued)

794	Phage head maturation protease	207	98.54	n/a	n/a	n/a	*E. casseliflavus* phage prohead protease, HK97 family	2e-114
795	Phage capsid protein	405	97.77	n/a	n/a	n/a	*E. casseliflavus* phage major capsid protein, HK97 family	0
797	DNA packing protein, phage associated	118	98.04	n/a	n/a	n/a	*E. casseliflavus* phage DNA packaging protein	3e-40
798	Hypothetical protein	112	96.4	n/a	n/a	n/a	*E. faecalis* putative phage head-tail adaptor	2e-42
799	Phage protein	122	100	n/a	n/a	n/a	*E. faecium* phage protein, HK97 gp10 family	2e-48
800	Phage protein	139	98.55	n/a	n/a	n/a	*Enterococcus* sp. C1 Phage protein	1e-53
801	Phage major tail protein	193	100	n/a	n/a	n/a	*E. faecium* phage major tail protein, phi13 family	1e-106
802	Hypothetical protein	109	99.07	n/a	n/a	n/a	*Streptococcus anginosus* phage protein	2e-05
804	Phage tail length tape measure protein	1532	98.63	47.8	n/a	46.55	*Enterococcus* sp. C1 phage-related minor tail protein	0
805	Putative tail or base plate protein gp17 [Bacteriophage A118]	274	96.34	61.76	n/a	49.6	*E. faecalis* phage tail component protein	3e-115
Regulatory proteins								
756	Transcriptional regulator, xre family	280	100	n/a	24.35	n/a	*E. faecium* peptidase S24	6e-153
778	DNA-cytosine methyltransferase	353	96.6	36	n/a	n/a	*E. faecalis* DNA (cytosine-5-)-methyltransferase	0

Table 3.4 (Continued)

Other								
760	Hypothetical protein	56	94.55	n/a	n/a	n/a	*E. faecium* hypothetical protein	7e-16
772	Hypothetical protein	145	95.18	n/a	n/a	n/a	*E. faecium* hypothetical protein	4e-50
773	Hypothetical protein	191	99.47	75.79	n/a	74.74	*Bacillus thuringiensis* 2-methylcitrate dehydratase	4e-12
788	Hypothetical protein	100	96.97	48.96	n/a	52.81	*Enterococcus gallinarum* hypothetical protein	8e-27
792	Hypothetical protein	57	96.43	n/a	n/a	n/a	*Enterococcus* spp. hypothetical protein	3e-16
796	Hypothetical protein	98	95.88	n/a	n/a	n/a	*E. faecalis* hypothetical protein	9e-14
803	Hypothetical protein	55	98.15	n/a	n/a	n/a	*E. casseliflavus* hypothetical protein	2e-10

E. mundtii CUGF08 was the sensitive strain used as the reference strain to compare against the other strains using RAST since this will determine genes that are not present in the other two strains (Table 3.5). There are 3 genes unique to the sensitive group.

Pfam was used to determine that ORF 1017 encodes for conserved C-terminal sequences for a TrbL/VirB6 plasmid conjugal transfer protein family, as determined by Pfam (Finn et al. 2014). VirB6 is a transmembrane protein involved with forming a channel for the Type IV secretion system (Liang et al. 1998, Zhang et al. 2012). All five strains encode for a homolog of ORF 1017. However, this protein is included since the two immune strains are less than 90% identical to the one encoded by *E. mundtii* CUGF08, while the ones encoded by *E. mundtii* ATO6 and ATCC 882 are over 98% identical, thus fitting the criteria to be a unique gene. There are several mutations that correlate between the two different groups, including non-conserved mutations (Figure 3.9). The protein from the sensitive group contains negatively charged residues while some of the residues mutated to uncharged residues. Mundticin is a positively charged peptide that may have affinity for these negatively charged residues of this protein. The different residues in the sensitive group may alter affinity for mundticin, especially since this protein is membrane-bound.

Table 3.5. Genes unique only to sensitive strains. *E. mundtii* CUGF08 was used as the reference genome. Genes found in *E. mundtii* ATO6 and ATCC 882 that were above 90% but were also below 90% in the other two genomes were identified as unique genes.

			Percent Identity					
ORF	RAST Annotation	Length	ATO6	ATCC 882	QU 25	CRL35	PSI-BLAST Hit	E-Value
1017	Hypothetical protein	275	99.64	98.54	89.42	89.05	*Streptococcus dysgalactiae* conjugal transfer protein TrbL	2e-16
1087	Hypothetical protein	206	95.12	91.49	81.82	n/a	*E. faecium* hypothetical protein	7e-61
2429	Hypothetical protein	41	97.5	100	n/a	n/a	*Clostridium botulinum* fosfomycin resistance protein	0.043

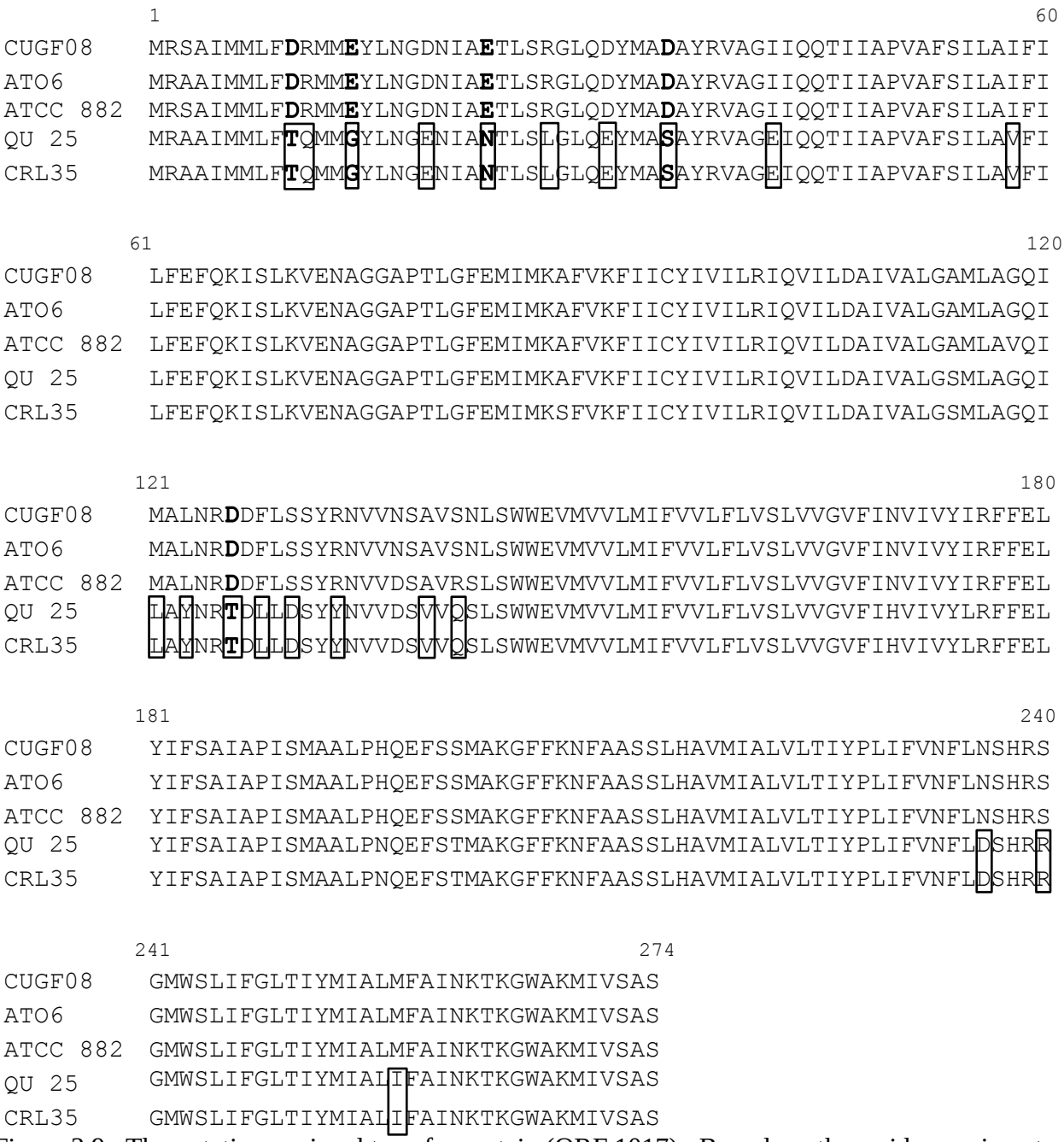

Figure 3.9. The putative conjugal transfer protein (ORF 1017). Boxed are the residues unique to the fully immune strains. In bold non-conserved mutations between negatively charged residues in the sensitive strains in comparison to the immune strains.

The functions of ORFs 1087 and 2429 are unknown. The product of ORF 2429 contains an N-terminal conserved fosfomycin resistance protein (FosX) domain (1.08e-03), as determined by the Conserved Domain Database (Marchler-Bauer et al. 2013). Fosfomycin interferes with the synthesis of the cell wall, but FosX modifies the antibiotic by hydration, inactivating the activity. However, the active site of FosX from *Listeria monocytogenes* requires a Mn(II) ion that interacts with two histidines and one glutamate residues (Fillgrove et al. 2007). ORF 2429 lacks a second histidine. Regardless of the predicted BLAST function, it may still be involved in mundticin target specificity or action.

Conversely, the absence of one of the 28 genes unique to the immune group may have the most effect on the sensitive group. For example, all five strains may encode for the same genes that affect bacteriocin specificity, but the sensitive group may not produce the proper repression factors that the immune group could produce. Moreover, the sensitive strains may lack all the immunity factors involved.

CONCLUSIONS AND FUTURE RESEARCH

The genomic comparison of five closely related strains of *E. mundtii* provided more insight as to the cause of self-lethality. Further experimentation is required to elucidate whether the differences observed in the sequences code for mechanisms of immunity or action, or if they are even involved with bacteriocin function at all.

E. mundtii CUGF08 and ATO6 are sensitive to each other's mundticin, implying that the amino acid substitutions in the structural and immunity proteins do not affect the extent of sensitivity. Additionally, ManPTS was found to be identical across all five genomes. This suggests that the cause of the self-lethality of *E. mundtii* CUGF08 and ATO6 must be from an uncharacterized gene. The addition of sensitive strain *E. mundtii* ATCC 882 allows for further

elimination of genes most likely not involved in bacteriocin activity. Genes found unique to these three strains may contribute to the specifity of a mundticin target, but it is also possible that the absence of genes unique to the immune group contributes fully to sensitivity.

Additionally, the mechanism of immunity of *E. mundtii* QU 25 and CRL35 requires further characterization since the immunity proteins are 100% identical to that produced by *E. mundtii* ATO6. All five strains encode for a putative mundticin immunity protein as determined by homology and the presence of the conserved C-terminal sequence IRYGY, but the levels of sensitivity differ among the strains and may be due to sequence variations in this immunity protein. The immunity proteins from *E. mundtii* QU 25 or CRL35 can be cloned into non-producer strain *E. mundtii* ATCC 882 to determine whether this protein alone functions to reduce the sensitivity to mundticin.

Transmembrane protein ORF 1017 may be a potential target for sensitive strains. ORF 1017 from an immune strain could be cloned into a sensitive strain in order to determine if it sensitivity. If it does, then point mutations could pinpoint which residues or regions in the protein are directly involved. Since class IIa bacteriocins cause leakage in target cells (Drider et al. 2006), it is likely that mundticin would dock itself on the cell envelope in order to form a pore, so it seems reasonable to consider this transmembrane protein as a potential target.

Regulatory genes found in the genomes of immune strains may also play a vital role in immunity. It may be beneficial to determine which genes are regulated by the two regulatory genes unique to the immune strains. Either of the two genes could be disrupted to first determine if the strains lose immunity. If so, then whole transcriptome analysis could be performed and compared between mutants and wild types to determine which genes are regulated. Moreover, a

transcription analysis of the mundticin operon could be compared among the four producer strains to assure the rates of transcription are equal.

The presence of at least one unique prophage within the genomes of the immune group may also contribute to the immunity, which would be a novel factor in bacteriocin mechanism research. The phage could be activated to the lytic cycle, and isolated to subsequently infect the sensitive cells, to determine whether sensitivity reduces as a result of phage infection.

The additional bacteriocins found among the sensitive strains may contribute to self-lethality. However, when incubated in MRS media for 18 hours, there was only one bacteriocin produced at sufficient concentrations that targets *E. mundtii* CUGF08 according to an overlay over a SDS-PAGE gel containing cell-free supernatant (unpublished work). Therefore, it may not be likely that an additional bacteriocin contributes to self-lethality.

This approach is especially insightful for a phenotype so distinct from all other characterized bacteriocin-associated mechanisms, so it expected that genes of unknown function or other unpredicted genes would be identified in this study. However, study of the novel genes presented can further the overall understanding of class IIa bacteriocins.

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CHAPTER 4

CONCLUSIONS AND PROSPECTUS

CONCLUSIONS

Class IIa bacteriocins may be used as a "natural" GRAS ingredient for the control of foodborne pathogens in foods (Balciunas et al. 2013). However, in order to assure that adding class IIa bacteriocins to foods is beneficial, additional information is needed with regards to their mechanism of action, the maturation process, the mechanism of the immunity protein, and how sensitive strains can develop resistance. It is especially concerning that target species develop resistance after exposure to class IIa bacteriocins (Kaur et al. 2011). Understanding how class IIa bacteriocins interact with target species is essential before using them against pathogens of interest.

Enterococcus mundtii CUGF08 is a unique class IIa bacteriocin producer since it is susceptible to its own mundticin L, as well as mundticin KS produced by *E. mundtii* ATO6. Since the immunity protein of CUGF08 is 99% identical to fully immune strain, *E. mundtii* QU 25, there may be an additional factor preventing the latter strain from cell leakage when exposed to mundticin L. Even though there are point mutations in the structural and immunity protein when compared to those produced by *E. mundtii* QU 25, these point mutations are not shared by *E. mundtii* ATO6, which is also sensitive to mundticin L and mundticin KS. By studying a novel class IIa bacteriocin-producer like *E. mundtii* CUGF08, additional, uncharacterized mechanisms may be identified.

Both the precursor and mature mundticin L are active against the producer strain, though to what extent is still unknown, since the products were not completely purified from one another or all of the supernatant components. The function of the N-terminal sequence is still poorly

understood, but it may be a signal sequence to allow the ABC transporter to cleave the conserved GG-motif. It may be possible that the ABC transporter for mundticin L does not contain one of the necessary motifs for the peptidase activity (Håvarstein et al. 1995, Ishii et al. 2010). This may explain why the precursor mundticin L was found in the supernatant of the producer in MRS broth. Conversely, the presence of precursor peptide in the cell-free supernatant may be a result of the lysis that is caused by self-lethality.

All five strains encode for an additional protein that is homologous to the mundticin immunity protein, regardless of their sensitivity to mundticin. This supplemental immunity gene is not in close proximity to the mundticin operon. More work needs to be performed to determine whether this putative immunity protein confers any immunity against mundticin, or if it has any role in mundticin phenotype. Even non-producer strain *E. mundtii* ATCC 882 encodes for this gene, so it may not be transcribed, or the essential residues may be absent, or the protein may only impart partial immunity if any.

There were multiple unique genes found among both immune and sensitive groups that may be additional factors involved with immunity and target receptors. While most of those unique genes are phage-associated or hypothetical, there are also several genes whose products are homologous to membrane-associated proteins. The immune strains share a unique gene encoding for a potential lipoprotein and another homologous to a transmembrane protein. Both or either of those may be accessory proteins to the membrane-bound immunity protein. The phage proteins shared by the immune strains may also affect the cell envelope composition (Bondy-Denomy and Davidson 2014), which may also affect the function of the immunity protein since it is membrane-bound. The two proteins involved in regulation, the LexA-like repressor and the cytosine-methyltransferase, may also be causing the repression of genes that

might be targeted by mundticin. The sensitive strains share a unique gene encoding for a transmembrane protein, which may be an additional receptor for mundticin to target. Not all the unique genes identified may be involved with mundticin, but they provide starting points for further experimentation.

Class IIa bacteriocins are thought to be an example of interference competition, providing the producer a competitive advantage over closely related species by causing cell leakage (Cornforth and Foster 2013, Drider et al. 2006). *Enterococcus mundtii* CUGF08 is an evolutionary exception in that it is decreasing its own fitness, yet it is still able to grow normally in culture broth. Nevertheless, despite self-lethality caused by its plasmid-encoded bacteriocin (Feng et al. 2009), the plasmid is not cured. Bacteriocin production and immunity may play a role in plasmid maintenance in a similar fashion as toxin-antitoxin systems (Inglis et al. 2013). There may be other factors involved with maintenance of this plasmid, which may provide additional benefits that outweigh the cost of producing a bacteriocin that targets the producer.

While there are viable mechanisms that have been proposed for the mechanism of action and immunity for class IIa bacteriocins, it is clear that there are other mechanisms yet to be determined. It is likely that there are multiple factors or genes involved in the mechanism of action, especially the specificity of the target of each individual class IIa bacteriocin. It is also likely the immunity protein does not act alone. Future experiments with *E. mundtii* strains should be aimed to determine these additional factors involved. The steps afterwards should also determine whether other class IIa bacteriocins utilize the same factors. Overall, the genes identified in this study uncover more clues concerning class IIa bacteriocin function.

PROSPECTUS

The characterization of a novel class IIa-bacteriocin producer may help determine additional mechanism of immunity and action that are poorly understood. Future experiments are aimed to characterize the genes identified through genomic comparisons.

Growth and production curve. To determine whether all four producer strains secrete mundticin at the same rate, a growth and production curve can be compared. Throughout a 24-hour incubation period, the optical density can be measured every hour and small aliquots of cell-free supernatant can be quantified for activity using the spot diffusion assay against indicator strains.

Transcriptional analysis of mundticin operon. The rate of transcription of the three mundticin genes of all four producers has yet to be determined. This can be performed by quantitative real-time PCR of the structural gene and the immunity gene.

Degradation of mundticin L. It is possible there is more proteolytic activity caused by the immune strains than that of the sensitive strains, thus degrading secreted mundticin. Purified mundticin can be applied to the cell-free supernatant from both groups. An aliquot of the solution every hour can be used for a spot diffusion assay to quantify activity of mundticin.

Characterization of truncated ABC transporter. The ABC transporter is essential for N-terminal cleavage of precursor class IIa bacteriocins. However, the one for mundticin may not contain the conserved motifs reported necessary for peptidase activity. Both precursor peptides and mature mundticin L were purified from the supernatant of *E. mundtii* CUGF08. Heterologous expression of the structural, immunity, and transporter genes may determine whether this ABC transporter is able to cleave mundticin L. N-terminal sequencing will also confirm whether the motifs reported to be involved in peptidase activity are completely present.

Additionally, the *Enterococcus mundtii* ABC transporter could be expressed in a different class IIa bacteriocin-producing strain. First, the wild-type ABC transporter should be knocked out, and then *Enterococcus* ABC transporter should be cloned into the mutant. The N-terminal sequences of the purified bacteriocin can then determine whether the peptides were cleaved or not.

Characterization of chromosomal immunity protein. All five *E. mundtii* strains in this study encode for a putative immunity protein homologous to the plasmid-encoded mundticin immunity gene, even if the strain was sensitive to mundticin. With the exception of the chromosomal immunity protein of *E. mundtii* ATO6, all the other proteins contain the conserved C-terminal motif IRYGY. In order to determine whether this protein is an immunity protein, the plasmid encoding for mundticin should first be cured from *E. mundtii* CUGF08 and ATO6. This plasmid-cured strain should be tested for change in sensitivity to mundticin L. Another vector containing the putative immunity gene should then be transformed into a sensitive *Enterococcus faecalis* strain. This strain can then be tested for any change in sensitivity against mundticin L. Alternatively, the putative chromosomal immunity protein from *E. mundtii* QU 25, CRL35 or ATO6 can be cloned into *E. mundtii* ATCC 882. This will help determine whether the putative immunity protein provides protection against mundticin L, and which residues could be involved with immunity function.

Characterization of transmembrane ORF 1017 as potential mundticin target. All five strains encode for a homolog of ORF 1017 from *E. mundtii* CUGF08, which may be the membrane channel component of the type IV secretion system. However, the ones for the other two sensitive strains are over 98% identical, whereas the ones for the immune strains are about 89% identical. There are 21 amino acid substitutions unique to the immune strains, so

complementing the homolog from either *E. mundtii* QU 25 or CRL35 into a sensitive strain may help determine if sensitivity decreases as a result. If it does, then point mutations of the 21 unique residues will determine which ones are directly involved with mundticin specificity.

Transcriptome analysis to determine genes regulated by ORF 756, LexA-like repressor, and ORF 778, DNA cytosine methyltransferase. The regulatory proteins found to be unique to the immune strains may be affecting the transcription of genes involved with bacteriocin immunity. To determine which genes are regulated by these proteins, they can first be disrupted. Afterwards, the transcriptome of the mutants can be compared to that of the wild type.

Effect of phage on sensitivity to mundticin. The phage genes that are unique to the immune strains indicate that they share at least one prophage. The phage should be isolated and infected into a sensitive strain in order to determine if the phage reduces the sensitivity to mundticin. First, the prophage needs to be induced in order to be lysed from the cell, which can be done by exposing the infected cells with mitomycin C. Phage can then be isolated from the cell-free supernatant (Stevens et al. 2009). The phage can then be used to infect the sensitive cells, and bacteriocin phenotypes can be determined for the phage infected cells, to determine the effect, if any on the newly phage infected strains.

Future studies on these novel class IIa bacteriocin producers are aimed to characterize unknown mechanisms of secretion, action, immunity, and specificity. They will provide insight for a class of bacteriocins that is poorly understood. This is significant since further understanding will allow the usage of class IIa bacteriocins in foods to be more effective against target foodborne pathogens or spoilage organisms.

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