ABSTRACT

DOBSON, ALLESON ELAINE. Identification of an operon involved in the production of Lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. (Under the direction of Professor T.R. Klaenhammer.)

Lactacin B is a class II bacteriocin produced by *Lactobacillus acidophilus* NCFM (Barefoot and Klaenhammer 1983). Mutational, nucleotide sequence, and transcriptional analyses revealed that the genetic determinants responsible for lactacin B regulation and production are located on a 9.5 kb polycistronic region (LBA1803-LBA1791) of the *L. acidophilus* NCFM chromosome. The *lab* operon comprised 12 putative open reading frames (ORFs) organized into three clusters: a production and regulation cluster encoding putative proteins that resemble two component signal transduction systems of the AgrC-AgrA type; an export cluster encoding putative proteins that resemble ATP-binding cassette (ABC) transporters and accessory proteins, and the final cluster composed of three putative proteins of unknown functionality. Each cluster was separated by an intrinsic terminator, the strongest terminators flanked the entire lactacin B region (ΔG = -13.4kcal/mol and ΔG = -17.0kcal/mol respectively). A total of 7 genes with unknown functionality were situated in this region, each containing a double-glycine leader motif characteristic of bacteriocin structural genes and their precursors. Insertional inactivation of the gene believed to encode an ABC transporter *(labT)* completely abolished bacteriocin activity, implicating this region in lactacin B production. Cloning of the first four genes within this region (LBA1803-LBA1800) onto a high copy number plasmid resulted in markedly higher levels of lactacin B activity compared to the control. These ORFs encoded proteins typical to bacteriocin peptides; small, cationic peptides, each with an N-terminal double glycine leader motif. Experiments with chemically synthesized peptides revealed that LBA1800 was not inhibitory, but induced lactacin B production in broth cultures. The genetic organization of the region indicates that lactacin B production is regulated through the three component regulatory system common to many class II bacteriocin systems.

IDENTIFICATION OF AN OPERON INVOLVED IN THE PRODUCTION OF LACTACIN B, A BACTERIOCIN PRODUCED BY LACTOBACILLUS ACIDOPHILUS

by

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

FOOD SCIENCE

Raleigh

2005

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ACKNOWLEDGEMENTS

I would first like to thank Professor Todd Klaenhammer for his support, guidance, encouragement, and humor throughout my graduate career. I feel quite honored to have been given the opportunity to be a member of his lab; it has been a dubious but truly wonderful and enlightening experience. I deeply appreciate his confidence and sincerity, which has enabled me to succeed in my research.

I would like to thank the following people in the lab for their helpful, entertaining, and insightful discussions and for their support: Rodolphe Barrangou, Tri Duong, Mike Miller, Eric Altermann, Evelyn Durmaz, B. Logan Buck, Andrea Azcarate-Peril, Rosemary Dawes, and Nicole Souther. I am particularly thankful for their guidance, for their confidence, and will greatly miss the laughter. A special thanks is extended to Evelyn Durmaz and Rosemary Dawes, they are truly the heart and soul of the lab. Both are invaluable and indispensable.

I would like to acknowledge the members of my advisory committee, Professors Robert Kelly and Fred Breidt for their guidance during my graduate studies. I would also like to acknowledge the Molecular Biotechnology Training Fellowship, Southeast Dairy Foods Research Center, and the North Carolina Dairy Foundation for their support of my research.

Finally I would like to thank my family for their neverending eccentricities and their comedy.

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LITERATURE REVIEW

MECHANISMS OF BACTERIOCIN REGULATION BY LACTIC ACID BACTERIA

Introduction

Lactic acid bacteria are a diverse group of microorganisms found in various ecological niches including foods, the oral cavity, the urogenital tract and the gastrointestinal tract of humans, animals, and insects (22, 202). Lactic acid bacteria (LAB) are members of the *Clostridium-Bacillus* sub-division of Gram-positive eubacteria whose main fermentation product is lactate. These microorganisms are typically catalase-negative, non-sporulating, and include *Lactobacillus* spp., *Carnobacterium* spp., *Enterococcus* spp., *Lactococcus* spp., *Streptococcus* spp., *Oenococcus* spp., *Leuconostoc* spp., *Pediococcus* spp., and *Vagococcus* spp. (44) LAB are commonly found in food and animal feed and, as such, are considered food grade or generally recognized as safe (GRAS) microorganisms. Due in part to their GRAS status, the use of LAB is widespread and can be seen in food, pharmaceutical, and manufacturing industries. The majority of LAB inhabit extremely population dense environments, for example the human gastrointestinal tract alone houses between 500-1000 different species of bacteria reaching populations as high as 10^{11} organisms per gram of contents (217). These populations include pathogenic and enteric microorganisms, as well as selected members of the LAB. In these environments where the competition for nutrients is fierce, LAB have evolved a cache of defense mechanisms that act against neighboring, competitive species. Bacteriocins are among the most powerful defense mechanisms that microbes can employ in the struggle for survival.

Bacteriocins are small, ribosomally synthesized antimicrobial peptides produced by both gram-positive and gram-negative bacteria (166). LAB in particular produce a wide array of these peptides active against gram-positive spoilage and pathogenic

microorganisms, as well as closely-related species that inhabit the same environmental niches (17, 56). In general, bacteriocins range in size from 20-60 amino acids, possess a net positive charge, and are typically inhibitory to closely-related species with notable exceptions including pediocin, nisin, and lacticin 3147 (107, 121, 133). Bacteriocins of LAB exhibit antagonistic activity by creating pores in the membrane of target cells. This effect results in a dissipation of proton motive force, ATP depletion, and leakage of nutrients and metabolites (1). The formation of pores in the cytoplasmic membrane is a universal feature among bacteriocins, yet their size and stability vary from bacteriocin to bacteriocin (137). Pore formation is partly governed by electrostatic interactions between the positively-charged bacteriocin molecule and the anionic lipids of the gram positive membrane. This interaction is usually affected by the membrane potential and pH. The higher the concentration of anionic phospholipids, the lower the threshold potential for dissipation of the proton motive force (27). Therefore, bacteriocin efficacy may be a function of the physiological state of target cells.

Evidence suggests that bacteriocin activity may also be the result of a specific interaction of a membrane receptor to that of the bacteriocin molecule. Nisin, a bacteriocin produced by *Lactococcus lactis*, was found to recognize a precursor lipid II molecule that facilitated pore formation (16). The role of specific receptors in other bacteriocins is still unresolved. A number of mode of action studies demonstrated that bacteriocins act toward whole cells or vesicles containing cellular proteins, whereas no activity was detected towards protein-free vesicles (30). Using a bacteriocin produced by *Leuconstoc mesenteroides*, Yan et al (220) found that interaction between the bacteriocin and the membrane was stereospecific suggesting a chiral target receptor molecule. Taken together,

bacteriocin action appears to be the result of, or enhanced by, interaction with a specific receptor on the cellular membrane of sensitive cells (137).

Genomic studies reveal that bacteriocins of the LAB compose a large, heterologous group of antimicrobial peptides (175). Based on biochemical and genetic analyses (105), these bacteriocins are organized into three distinct classes: class I, known as lantibiotics, are composed of elongated or globular molecules that require extensive post-translational modification for biological activity; class II are composed of small, heat stable peptides that undergo minimal modification resulting in biological activity; while class III bacteriocins are composed of larger bacteriocin proteins.

Although the bacteriocins of LAB are extremely diverse in terms of their size, structure, inhibitory spectrum, and genetic organization, a common feature throughout many of these systems lies within their mechanism of regulation. Most bacteriocins possess a two component regulatory system that serves as a basic sense-response mechanism allowing the organism to adapt to changes in their environment (212). First observed approximately 20 years ago, two component regulatory systems have been implicated in a number of adaptive responses including bacterial chemotaxis, sporulation in *Bacillus subtilis*, and aerobic/anaerobic regulation in *Escherichia coli*. The prototype for these systems is composed of a histidine protein kinase (HPK) and a response regulator (RR). HPKs are a diverse family of proteins that range in size from less than 40 kDa to more than 200 kDa. These proteins function as a receptor, identifying changes in the environment through a variety of input signals. They are typically composed of an N-terminal sensing domain, a transmembrane domain, and a C-terminal cytoplasmic kinase domain. Response regulators are the terminal component of the signal transduction

pathway, essentially functioning as a molecular switch effecting gene expression underlying adaptive responses. RRs compose a diverse superfamily with a highly conserved regulatory domain and a C-terminal DNA-binding effector domain. Stimulated by the sensing domain, HPKs attach a phosphate group from an ATP molecule to a conserved histidine residue in the kinase core (189). This phosphate group is then transferred to a conserved aspartate residue located in the regulator domain of the RR. This phosphorylation results in a conformational change that facilitates intermolecular and intramolecular interactions. It is these interactions that eventually yield a response, typically through the DNA binding domain that functions to activate or repress transcription of a set of genes (189). Recent evidence has elucidated the nature of these input signals that stimulate this signal transduction pathway, the nature of these input signals, and the molecular mechanisms used to regulate LAB bacteriocin production.

Genetics of bacteriocin production by Lactic acid bacteria (LAB)

Class I – The lantibiotics

Lantibiotic bacteriocins, a complex group of ribosomally synthesized antimicrobial peptides, typically have a broad spectrum of inhibition compared to other bacteriocin classes (98). Members of *Lactococcus*, *Enterococcus*, *Streptococcus*, and *Lactobacillus* of the lactic acid bacteria family (LAB) reportedly produce at least one lantibiotic. Examples of known lantibiotics produced by LAB are listed in Table 1. Lantibiotics display unusual amino acids, including lanthionines and β-methyllanthionines and the unsaturated residues dehydroalanine and dehydrobutyrine (107, 132). Lanthionines and β -methyllanthionines contribute to the unique polycyclic structure characteristic of class I bacteriocins. As a

whole, lantibiotics of LAB form a relatively small group of heterologous peptides. Based on marked variations in size, structure, biosynthetic machinery, and mode of action, lantibiotics are organized into two distinct subgroups, type A and B (98). Type A lantibiotics include elongated, cationic peptides that inhibit sensitive cells by disruption of membrane integrity. Nisin produced by *Lactococcus lactis* and mutacin II produced by *Streptococcus mutans* are classified within this group (28, 32). Type B lantibiotics are composed of globular peptides that act to impede essential enzymatic functionality i.e. cell wall biosynthesis. Mersacidin produced by *Bacillus subtilis* and cinnamycin produced by *Streptomyces cinnamoneus* are among group B lantibiotics (21, 127). To date, LAB bacteriocins of type B lantibiotics have not been identified and, therefore, will not be discussed in this review.

The genetic determinants for several LAB lantibiotics have been elucidated in detail and are summarized in Figure 1. Comparison of the genetic clusters reveals a number of conserved genes believed to encode proteins with similar functionality. These clusters may be chromosomally encoded, found on large plasmids, or contained within transposible elements (6, 133, 180). Using the generic nomenclature established by de Vos (41), these conserved genes include a precursor peptide (LanA), proteins involved in regulation and sensory (LanR, K), enzymes responsible for post-translational modifications (LanB,C/LanM), a protease responsible for cleavage of leader peptides (LanP), an ATP binding cassette (ABC) transporter protein responsible for cleavage of the leader peptide and/or translocation of the mature peptide from the cell, and finally a cognate immunity protein to protect the producer cell from the antagonistic effects of the mature bacteriocin molecule. In addition to these conserved genes, additional putative genes of unknown

function are usually present in lantibiotic gene clusters (177). The biosynthesis of lantibiotics is summarized in Figure 2. The production of a lantibiotic begins with the formation of the pre-lantibiotic, containing a leader sequence between 23 to 30 amino acids in length (REF). The pre-lantibiotic is post-translationally modified through dehydration and cross-linkage reactions of specific residues within the prepeptide, followed by cleavage of the leader sequence, and finally export from the cell.

Precursor peptides

Lantibiotic precursor peptides are biologically inactive and contain an N-terminal leader sequence attached to the C-terminal propeptide. Leader peptides do not possess a hydrophobic transmembrane core and processing site characteristic of *sec*-dependent signal sequences and are, therefore, exported from the cell via a different transport mechanism. Studies of nisin, lacticin 481 produced by *Lc. lactis*, and mutacin II produced by *S. mutans* found that these post-translationally modified peptides with the leader sequence attached showed little or no antagonistic activity. These data suggest that the presence of the leader sequence protects the producer cell from intracellular antagonistic activity.

Type A lantibiotics can be further subdivided based simply on their leader peptide characteristics including size, charge, and sequence (27). Additional functions of leader peptides remain to be elucidated but may depend on the type of leader sequence analyzed. Type AI leaders are hydrophilic peptides containing the conserved 'FLNDL' box. The amino acids serine and proline at positions -6 and -2 respectively, are also highly conserved. These sequences possess a large proportion of charged amino acids and have a net negative or slightly positive charge (132). Evidence suggests that the importance of specific leader peptide sequences is based upon protease action. This difference is

exemplified in the Pep5 bacteriocin system produced by *Staphylococcus epidermidis* and nisin produced by *Lc. lactis*. Neis et al (145) found that mutations in the type AI leader sequence led to a reduction in Pep5 biosynthesis, whereas mutations in the type AI leader sequence of nisin led to a complete abolition of nisin production ((145, 204). The authors speculated that this discrepancy was the result of the location of processing by the cognate protease. In the Pep5 system, the action of the protease occurs intracellularly, while in the nisin system, the protease is an extracellular membrane protein (145).

Leader sequences of type AII lantibiotics are negatively charged peptides containing the so-called "double glycine" GG/GA/GS leader motif. This same motif is also present in class II bacteriocins and is typically positioned proximal to the cleavage site (148). A peptidase similar to those present in class II bacteriocins is thought to cleave the leader sequence upon export (82). Group AII leader sequences are proposed to protect the producer against intracellular bacteriocin activity in a manner similar to type AI, as well as to direct protein translocation across the cellular membrane, or interact with the propeptide domain to facilitate enzyme substrate interaction (146). The "double glycine" leader motif of Type AII sequences and the 'FLNDL' motif of type AI sequences are mutually exclusive with the exception of lacticin 481 produced by *Lc. lactis*. The lacticin 481 peptide contains both the 'FNL' motif and the "double glycine" motif; both motifs are thought to play a role in bacteriocin maturation (152).

Modification enzymes

The complex series of modifications necessary to yield a mature, biologically active lantibiotic molecule enlist enzymes involved in processing and modification (Lan B/C, LanM, LanD) or cleavage and transport (LanP, LanT). Disruption of genes encoding

modification and processing proteins as well as cleavage and transport proteins has yielded insight to their functionality and their essential roles in lantibiotic biosynthesis. These roles are discussed in detail below.

Processing and Modification Enzymes. The proposed formation of lanthionines and β-methyllanthionines is a two step mechanism (209). The first step is catalyzed by the dehydratase, LanB. In this reaction, LanB dehydrates serine and threonine residues found within the prepeptide yield the unsaturated amino acids, 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb), respectively. LanB dehydratases are approximately 1000 amino acids in length, usually with a molecular weight of 120 kDa (27). These enzymes typically are not well conserved, reflecting the large variations in substrates and end-products. Although exceptions are present as in the ericin S and subtilin systems produced by different strains of *B. subtilis*, these proteins not only show a high degree of similarity (83%) and are structurally close, each containing identical ring locations and leader peptides (185). Functional genomic analysis of LanB dehydratase in the nisin system revealed that the overexpression of NisB resulted in an increased efficiency in dehydration leading to complete dehydration of serine residues that typically escape modification (113). In vivo experiments found that mutants deficient in NisB failed to produce nisin and accumulated unmodified nisin prepeptides. Conversely, mutants deficient in NisC, an alternative modification protein, failed to produce nisin, yet dehydrated peptides were recovered (113). Taken together, these data confirm that NisB is a dehydratase responsible for selective dehydration of serine and threonine found within the nisin precursor peptide. Such genomic evidence is also available in the Pep5 system, a class I bacteriocin produced by *Staphylococcus epidermidis* (18).

Following modification by dehydration, a portion of dehydrated residues undergo an additional modification to form thioether bridges. The cyclase, LanC, was found to catalyze the addition of cysteine thiol to dehydrated serine and threonine in a region-selective and stereo-selective manner (27). Most LanC cyclases are 400 amino acids in length containing alternating hydrophilic and hydrophobic regions (132). LanC proteins usually contain a number of conserved motifs and highly conserved glycine, cysteine, and histidine residues. Kopenen et al (113) found that mutants deficient in NisC produced dehydrated prepeptides yet did not yield any cyclization products. These results confirmed that NisC was responsible for cyclization of thioether rings, either directly or via induction of NisB.

The gene clusters of lacticin 481, lactocin S, lacticin 3147, and cytolysin do not contain genes encoding LanB or LanC proteins, instead these lantiobiotic systems contain a single modification enzyme, LanM, believed to play a role in post-translational modification of these bacteriocins (129, 167, 183). LanM enzymes require ATP and the divalent cation, Mg2+ for activity (27). These enzymes range between 900-1000 amino acids in length with the C-terminus of many LanM proteins showing similarity to LanC proteins including some conserved motifs (129). However, no sequence homology to LanB proteins is present; therefore, it is unlikely that LanM proteins arose from a fusion of *lanB* and *lanC* genes. In gene clusters containing *lanM*, no other modification proteins are encoded, indicating that LanM alone may be responsible for both dehydration and cyclization reactions. Disruption of the *lctM* gene in the lacticin 481 system resulted in a complete abolishment of lacticin 481 activity (200). Similarly, disruptions in the *mutM* gene of mutacin II produced by *S. mutans* and the *cylM* gene of cytolysin produced by *E.*

faecalis prevented the production of their respective bacteriocins, indicating that LanM is an essential enzyme in bacteriocin biosynthesis (29, 37). *In vitro* analysis revealed that *lctM* encodes a synthetase involved lacticin 481 biosynthesis. Xie et al (216) showed that LctM converts the prepeptide LctA into dehydrated species. The products were then analyzed and found to possess cyclic structures indicating that a single protein encoded by *lctM* is responsible for dehydration and cyclization of the prepeptide LctA. Interestingly, the two component lantibiotic, lacticin 3147 produced by *Lc. lactis* encodes two LanM proteins in its gene cluster, LtnM1 and LtnM2. Functional genomic analysis revealed that each prepeptide, LtnA1 and LtnA2, requires a dedicated LtnM for proper modification and processing (129).

Mutacin 1140 produced by *S. mutans* is the only LAB, to date, that possesses the oxidoreductase, LanD (88). LanD proteins are small enzymes approximately 200 amino acids in length that contain noncovalently bound coenzyme, flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). Bacteriocin gene clusters that encode LanD typically possess the unusual amino acid S-[(Z)-2-aminovinyl]-D-cysteine. LanD catalyzes the oxidation and decarboxylation of a C-terminal cysteine group prior to thioether formation. Functional analysis of the epidermin system suggested that EpiD is responsible for oxidation and decarboxylation of a terminal cysteine residue. Addition of this residue to the dehydrated amino acid, didehydroalanine, presumably catalyzed by LanC, yields S-[(Z)-2-aminovinyl]-D-cysteine (27).

Proteases and Transport Enzymes. A mature, biologically active lantibiotic can only be produced after sufficient modification by LanB/C or LanM enzymes, cleavage of the leader sequence, and translocation out of the cell. Lantibiotics of LAB in general do not

possess an N-terminal *sec*-dependent signal sequence and are not secreted from the cell via the general secretory pathway (106). Instead, lantibiotics are processed and exported by dedicated ABC transporters. Processing of the type AI leader sequence, characterized by the 'FLNDL' motif, occurs through the action of a serine protease, denoted as LanP. Bacteriocin systems containing LanP include lactocin S and nisin produced by *Lc. lactis*, and mutacin II and III produced by *S. mutans* (29, 158, 183, 223). Proteolytic cleavage typically occurs before or just after translocation by the ABC transporter complex, designated as LanT. LanP proteases vary in size between 270 and 680 amino acids in length and contain the C-terminal conserved motif 'LPTXG' that may act as a membrane anchor (132). van de Meer et al (204) found that in vitro analysis of intact *Lc. lactis* expressing NisP, a protease within the nisin gene cluster, was able to cleave modified nisin precursors while modified nisin precursors were not cleaved by either cell-free supernatants or membrane free fractions. These results support the model that the serine protease is likely attached to the cell wall. However, the location of LanP is known to vary based on the system. LasP, encoding a protease in the lactocin S system produced by *Lc. Lactis,* and EpiP produced by *Staphylococcus epidermidis* are both localized within the cytoplasm due to the absence of LPXTG motif (72, 183). The role of LanP was confirmed through gene disruption. Inactivation of the *nisP* gene resulted in the production of fully modified nisin with no antimicrobial activity due to the presence of an attached leader sequence (223). Furthermore, Kuipers et al (117) demonstrated *in vivo* that *nisP* encoded a protein catalyzing the removal of the leader sequence, but only after formation of the thioether rings, indicating cleavage of lantibiotics occurs after dehydration and cyclization reactions take place For many systems, LanP is not an essential enzyme required for

efficient lantibiotic biosythesis. Subtilin produced by *B. subtilis* is a type AI lantibiotic that relies on nonspecific intracellular proteases for subtilin maturation. Experiments involving a subtilin leader sequence attached to a nisin propeptide, expressed in both *Lc. lactis* and *B. subtilis*, found that only *B. subtilis* was able to cleave the leader region resulting in antimicrobial activity (26). *Lc. lactis* was unable to process the modified hybrid prepeptide. These data indicate that the substrate specificity of *B. subtilis* is more relaxed than *Lc. lactis*. Stein et al (186) observed that cleavage of the subtilin leader peptide may also be obtained through incubation with culture supernatants from non-producing strains of *B. subtilis* indicating that nonspecific proteases can process subtilin to maturation.

ABC transporters, designated as LanT, have been identified in all LAB lantibiotic systems to date. In Type AI lantibiotics, ABC transporters are approximately 600 amino acids in length and consist of two subunits containing an N-terminal transmembrane domain and a C-terminal ATP-binding domain (176). The ATP-binding domain located near the C-terminus is characterized by the highly conserved Walker A and B motifs. Many of these proteins are essential for translocation of the bacteriocin molecule from the cell (26). Functional analysis of *nisT* and *lasT* found that disruption of these transporters led to a loss of bacteriocin production, a build up of processed nisin in the cytoplasm, and a decrease in the level of immunity (164, 183). In contrast, type AII lantibiotics such as lacticin 3147, cytolysin mutacin II, and lacticin 481 contain genes encoding ABC transporter proteins typically 700 amino acids in length. These ABC transporters possess an N-terminal peptidase domain and a C-terminal ATP-binding domain, as well as a transmembrane domain. Gene clusters of type AII lantibiotics typically do not include a

serine protease protein responsible for cleavage of the leader peptide region, instead the ABC transporter possesses the dual function of cleavage of the leader peptide concomitant with export of the bacteriocin molecule (146). The 'double glycine' motif seen in type AII lantibiotics is thought to act as the proteolytic site for cleavage of the leader sequence. The double glycine leader motif is also present in class II bacteriocins plays a similar role in the maturation of these bacteriocins (203). Although the exact peptidase role of lantibiotic ABC transporters must still be elucidated, experiments with ABC transport systems for class II bacteriocins have shown peptidase activity. Overexpression of the N-terminal lactococcin A transporter LcnC in *Lc. lactis* led to the formation of mature lactococcin A in the cytoplasm (146). However, Uguen et al (199) found that the lactococcin A transporter (class II) from *Lc. lactis* is not interchangeable with the lacticin 481 (lantibiotic) transporter LctT, eventhough both transporters contain the double glycine leader motif and possess 47% sequence identity. These findings suggest that LanT proteins recognize processing sites other than the double glycine motif.

Immunity proteins

Immunity proteins provide protection against the antagonistic activity of the producer's cognate bacteriocin. The exact molecular mechanisms used to confer immunity remain largely unknown, yet two distinct systems of lantibiotic immunity have been identified. Immunity to lantibiotics involves the action of either a single immunity protein, LanI, or a series of transport protein domains, LanEFG. In the case of nisin *(Lc. lactis)* and epidermin (*S. epidermidis*),both LanI and LanEFG mechanisms contribute to immunity.

To date, three LanI immunity proteins have been identified in the LAB: lacticin 3147 and nisin produced by *Lc. Lactis;* and cytolysin produced by *E. faecalis* (176). These proteins do not share any significant sequence homology to one another or other predicted LanI proteins produced by gram-positive bacteria (27). LanI proteins are larger proteins of approximately 200 amino acids in length. They are suspected to be located in the membrane, suggesting these proteins may bind to the bacteriocin molecule, preventing entry or pore formation (129). The function of LtnI, a 116 amino acid protein of the lacticin 3147 gene cluster was confirmed through expression of *ltnI* in a lacticin 3147 sensitive strain. The gene, *ltnI* is found on the four gene cluster, *ltnRIFE*. *ltnR* encodes a Xre-family of transcriptional regulators and is thought to regulate transcription of downstream genes. Deletion analysis revealed that LtnI alone is capable of conferring the same level of immunity as the wildtype (130). Similar results were seen in the cytolysin system produced by *E. faecalis*. To confirm the function of CylI, the protein believed to confer immunity in the cytolysin system; Coburn et al (31) cloned *cylI* onto a recombinant plasmid that was transformed into a cytolysin-sensitive strain of *E. faecalis*. This transformant was immune to cytolysin, confirming that CylI alone can confer protection to this bacteriocin (36). Conversely, expression of the nisI gene in a nisin-sensitive strain produced discreet levels of immunity compared to the wildtype. Levels of immunity increased upon addition of mature nisin, yet full immunity was not recovered (164). These results indicated that additional gene products were necessary for full immunity to nisin.

The single immunity peptide, LanI, is not the only mechanism of defense lantibiotics employ for protection. Gene clusters encoding lacticin 481, mutacin II, nisin, and lacticin 3147 all include transporters that may also contribute to self-protection (27).

These proteins, LanFEG all show homology to ABC transporters but the individual domains are instead encoded by separate genes. *lanF* encodes the intracellular ATP-binding domain, while *lanE* and *lanG* encode membrane-spanning domains (198) . It is hypothesized that these proteins mediate immunity through intake and subsequent intracellular degradation or by active extrusion of the respective peptide, keeping the lantibiotic concentration in the membrane below a critical level (155). Work done by Rince et al (167) found that protection against lacticin 481 produced by *Lc. lactis* could be achieved by expressing the *lctEFG* genes and the absence of any one gene of this cluster resulted in a loss of immunity. Full protection against nisin was found to require the action of not only the NisI protein, but NisFEG proteins as well (187). Disruptions of any of these genes result in a modest level of immunity comparable to wildtype, that can be restored upon complementation (187). Interestingly, in the lacticin 3147 system, full immunity was conferred through the expression of *ltnI* gene only, despite the presence of two genes (*ltnFE)*, which show similarity to *lanFEG* involved in self protection. LtnFE are homologous to ABC transporters that are also capable of conferring immunity (130).

Class II bacteriocins of LAB

Class II bacteriocins represent the largest, most diverse group of LAB bacteriocins to date. Lactobacilli, streptococci, lactococci, enterococci, pediococci, and leuconostoc all produce at least one class II bacteriocin (43). Class II bacteriocins produced by LAB to date are listed in Table 2. Much like lantibiotics, class II bacteriocins are further organized into three distinct groups based on antagonistic activity, molecular weight, and their structural gene (58). Class IIa bacteriocins are composed of *Listeria*-active bacteriocins

characterized by the sequence motif YGNGV and the presence of one or more disulphide bridges. Due to their efficacy against the foodborne pathogen, *Listeria monocytogenes*, the class IIa bacteriocins are the largest and most extensively studied subgroup of class II antimicrobials. Bacteriocins of this class include pediocin produced by *Pediococcus acidilactici*, mesentericin produced by *Leuconostoc mesenteroides*, and carnobacteriocin produced by *Carnobacterium piscicola* (24, 69, 162). Members of the class IIb bacteriocins require the complementary action of two peptides to achieve full inhibitory activity. In some cases, each peptide may be inactive or confer very little antimicrobial activity, as in the case of lactococcin G produced by *Lactococcus lactis* and lactacin F produced by *Lactobacillus johnsonii* (68, 151). Though synergistic inhibition is observed when one-peptide bacteriocins are used in combination; two peptide bacteriocins of class IIb refer only to sets of peptides whose genetic determinants exist on the same operon. Antimicrobial peptides that belong to class IIc bacteriocins typically represent all bacteriocins that are not active against *Listeria* species or that do not require the complementary action of two peptides. Class IIc bacteriocins, consequently, represent a rather diverse group of antimicrobials whose members include *Lb. acidophilus* and *E. faecalis* (33, 35).

The biosynthesis of class II bacteriocins is summarized in Figure 4. Biosynthesis of lantibiotics and class II bacteriocins is very similar each beginning with the production of a prepeptide, a mature peptide with a leader sequence attached. Like lantibiotics, the leader sequence of all class II bacteriocins provides a site for proteolytic cleavage resulting in activation and transport, as well as rendering the bacteriocin biologically inactive while still within the cell (203). The leader peptide of class II bacteriocins is similar to the type

AII leader sequences of lantibiotics; both contain the conserved $Gly^{-2}Gly^{-1}Xaa^{+1}$ sequence motif (82). Once the preptide is formed, it is then exported from the cell via a dedicated export system composed of an ABC transporter and its accessory protein. These proteins cleave the prepeptide just downstream of the double glycine motif concomitant with export to yield a mature and biologically active antimicrobial peptide.

Many class II bacteriocin genetic clusters have been identified and their functions confirmed. These determinants are summarized in Figure 3. Class II bacteriocin gene clusters can exist on the chromosome, a plasmid or both as in the case of carnobacteriocin produced by *C. piscicola* (162) Class II bacteriocins require a minimum of four conserved genes for production (202). These consensus genetic elements include one or more prepeptides, proteins involved in regulation and sensory, typically a two component histidine protein kinase and response regulator, a dedicated export system encoding an ABC transporter and accessory protein, and a dedicated immunity protein. As is the case with lantibiotics, several putative genes with unknown functions are also present in these gene clusters.

Class II structural peptides and genes

All class II bacteriocins are synthesized as a prepeptide composed of the mature antimicrobial peptide with its leader sequence attached. The double glycine leader sequence is present in virtually all class II bacteriocins with the exceptions of divergicin produced by *C. divergens* and acidocin B produced by *Lb. acidophilus*. These bacteriocins contain a typical secretion leader sequence and are exported via the general secretory pathway. For most other class II peptides, the leader sequence render the bacteriocin biologically inactive while within the cell and serves as a recognition signal for the

transporter system (148). Cleavage of the leader sequence occurs upon export. The presence of the double glycine leader motif is essential for bacteriocin biosynthesis. Fremaux et al (68) found that mutations in the leader sequence resulted in the complete abrogation of bacteriocin secretion. Mature bacteriocin peptides typically range from 20-60 amino acids in length with a molecular weight less than 10 kDa (58). The bacteriocin structural gene or genes encode small cationic peptides with a high isoelectric point and contain a large number of small amino acids with hydrophobic or amphilic regions, that promote membrane interaction. Typically, bacteriocin structural genes are not highly conserved; the N-terminus of class II bacteriocins is thought to bind to the cellular membrane while the C-terminus is involved target specificity (97). Using hybrid bacteriocin peptides, Johnsen et al discovered that the C-terminal domain of class IIa bacteriocins was involved in both target specificity and immunity recognition as well (97). Regions of homology exist in the N-terminal leader sequence of primarily class IIa bacteriocins only. These mature peptides contain the YGNGV motif. The motif was once thought to act as a recognition site for receptors on the surface of sensitive cellular membranes (64). Recent studies have revealed that the presence of N-terminal positively charged amino acids, instead of the YGNGV motif, was responsible for membrane binding of pediocin PA-1 (30). Synthetic pediocin PA-1, produced by *P. acidilactici,* containing a patch of positively charged molecules was found to bind strongly to lipid vesicles. In contrast, synthetic pediocin containing the traditional YGNGV motif resulted in a 10-fold lower relative affinity to the lipid vesicles. The true function of this motif remains unknown.

Immunity proteins

A number of factors contribute to the relative sensitivity or resistance to a particular bacteriocin, including membrane composition or the physiological state of the cell. Yet, like lantibiotics, producers of class II bacteriocins have developed distinct mechanisms of self-protection. Immunity peptides of class II bacteriocins are typically located within the same operon, proximal to the structural gene (146). These proteins are small ranging from 51 to 150 amino acids in length, typically possessing low homology to other immunity peptides, despite strong similarity in the respective antimicrobial peptides (63). For instance sakacin A produced by *Lb. sakei* and curvacin A produced by *Lb. curvatus* are identical bacteriocin molecules whereas their putative immunity proteins are 90 and 51 amino acids in length respectively (10, 60). The exact molecular mechanism by which immunity proteins protect the producer remains unknown. Chikindas et al (31) found that membrane vesicles from sensitive cells became leaky after bacteriocin exposure whereas immune cells did not. Furthermore, work done by Nissen-Meyer et al (150) and Quadri et al (163) found that protection is not conferred to sensitive cells simply upon addition of immunity proteins.

Typically immunity proteins confer protection only to the cognate bacteriocin. However, Franz et al (66) found that one immunity gene may protect against multiple bacteriocins, as is the case with carnobacteriocin A and enterocin B produced by *C. piscicola* and *E. faecalis,* respectively. These immunity proteins are in fact interchangeable between the two each bacteriocin systems. Class IIb bacteriocins, those that require the complementary action of two peptides, do not possess separate immunity proteins for each peptide. LafI and AbpIM, the immunity proteins in the lactacin F

produced by *Lb. johnsonii* and ABP-118 produced by *Lb. salivarius* respectively, provides immunity to both peptides (4, 65). The single peptides possess no antimicrobial activity and antagonistic activity occurs only through the action of both peptides.

Transport Proteins

The processing and export of bacteriocins containing the double glycine leader motif depend on the joint action of two proteins, the ABC transporter and its accessory protein. ABC transporters have been implicated in the transport of a number of substrates including sugars, peptides, and amino acids found in both prokaryotes and eukaryotes (87). ABC transporters are one of the most highly conserved genetic determinants in class II bacteriocin gene clusters and are characterized by the presence of approximately six transmembrane domains, an N-terminal proteolytic domain and a C-terminal ATP binding domain. The proteolytic and ATP-binding domains are both located in the cytoplasm (82). The hydrolysis of ATP induces a conformational change in the transporter resulting in the removal of the leader sequence and export of the bacteriocin peptide across the cytoplasmic membrane (203). The transport machinery normally tolerates some degree of sequence variation. Therefore, one export system is capable of transporting a number of bacteriocin or peptide precursors with varying leader sequences, as evident in the carnobacteriocin and plantaricin systems produced by *C. piscicola* and *Lb. plantarum* respectively (48, 162). In class II bacteriocins the ABC transporter is typically located in the same operon or adjacent to the operon encoding the bacteriocin precursor and its immunity protein (58). Class II ABC transporters are typically 100-200 amino acids larger than their lantibiotic counterparts and positioned with an accessory protein. These accessory proteins are characterized by an N-terminal transmembrane domain and are

typically situated adjacent to the ABC transporter protein (202). Both the ABC transporter and its accessory protein are essential for bacteriocin biosynthesis (81, 205, 222). Havarstein et al (82) found that the first 150 amino acids of the N-terminal pediocin and lactococcin G transporters successfully cleaved their respective bacteriocin precursors, thus confirming the proteolytic functionality of ABC exporters. After cleavage, bacteriocin activity was detected intracellularly using colicin V deficient mutants of *Escherichia coli*. Taken together, these results confirm the ABC transporters role in both translocation of bacteriocin molecules, and cleavage of the leader sequence. However, the specific role of the accessory protein remains unknown.

Secretion dependent transport

Though most class II bacteriocins are exported from the cell via the ABC transporter complex, some bacteriocins rely on a *sec*-dependent secretion system. Enterocin P produced by *E. faecium*, divergicin A produced by *C. divergens*, and acidocin B produced by *Lb. acidophilus* are all exported from the cell via the general secretion pathway (34, 213). These gene clusters do not possess genes encoding a dedicated export system, nor do these peptides possess a double glycine leader sequence in their N-terminus. Instead, the general secretory pathway was found to export these bacteriocins from the cell. In the divergicin system, Worobo et al (213), successfully replaced the secretion signal of the gene encoding an alkaline phosphatase with that of divergicin, resulting in the complete production of alkaline phosphatase (213). These results demonstrated that a general peptidase was responsible for maturation of divergicin, instead of a dedicated ABC transport system.

Regulation of bacteriocin production by Lactic Acid Bacteria

The dogma that unicellulear microorganisms do not employ some type of communication system was put to the test approximately 30 years ago using the marine bacterial species *Vibrio harveyi* and *Vibrio fischeri* (188). These gram-negative microorganisms were found to produce light only at higher cell densities. Until this discovery, the exchange of signaling molecules was thought to be a characteristic of eukaryotes only. Research now indicates that individual species of bacteria, both gram-negative and gram-positive, utilize an assortment of signals to communicate (15). These communication molecules are used in a complex regulatory network involved in such cellular processes as bioluminescence, genetic competence, sporulation, and antibiotic production (136).

The method by which these organisms communicate has been referred to as quorum sensing (136). Quadri (160) defined quorum sensing as cell-to-cell communication that enables unicellular microorganisms to behave multicellularly resulting in a population wide synchronized behavioral response as a function of an environmental stimulus, such as cell density. Quorum sensing mechanisms essentially are composed of conventional signal transduction pathways conducted globally instead of individually where, like traditional two component systems, the ultimate response is the regulation of gene expression. In this manner, populations of microbes can efficiently couple gene expression to changes in their external environment. The "language" of quorum sensing differs based on the type of microorganism. Gram-negative microbes typically synthesize *N*-acyl homoserine lactones as communication signals (70). *N*-acyl homoserine lactones

are diffusible signal molecules that modulate physiological processes based on cell density or growth phase (111). Quorum sensing can be seen in such gram negative organisms as *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, and *Erwinia carotovor*a. In gram-positive bacteria, communication signals are synthesized as small peptides that are secreted and induce or modulate complex physiological processes based on cell density and growth phase (111). Quorum sensing mechanisms identified in such gram-positive organisms as *Stretpomyces* spp., *Staphylococcus* spp., *Bacillus* spp., and *Lactococcus* spp. (55). This diversity demonstrates the importance of communication in a number of biological processes including genetic competence, sporulation, growth, pathogenesis, and bacteriocin production (55).

Advances in the area of LAB functional genomics have not only elucidated a number of bacteriocin genetic loci but have also uncovered clues about the complex regulatory network involved in bacteriocin production. The exact molecular mechanisms that LAB use to regulate production are only just beginning to be understood. It is apparent that LAB use a number of different mechanisms to modulate this process which may occur at the level of transcription or translation and is a function of quorum sensing. The biological relevance of a quorum regulation mechanism could be to ensure that bacteriocin levels are sufficient to kill competitors and that the explosive increase in production limits survival in the target population (110). In the following sections, a number of bacteriocin regulatory mechanisms that follow the quorum sensing schematic are described. The bacteriocin system that best characterizes the method of molecular regulation is also discussed.

LAB bacteriocin regulation by induction

Nisin: the archetype for bacteriocin autoinduction

Virtually all mechanisms of bacteriocin control, transcription or translation, induction or repression, involve the traditional two component regulatory system consisting of a histidine protein kinase (HPK) and a response regulator (RR). The HPK serves as an environmental sensor typically on the cellular membrane that monitors specific signals (191). The RR is located in the cytoplasm and is activated by its cognate HPK in order to trigger an adaptive response. Most RR act as transcriptional factors that modulate bacteriocin operon expression (191). Two component systems are ubiquitious in bacteria that mediate cellular responses to chemical and physical signals (54). Systems that regulate bacteriocin production typically occur through the binding of a small secreted peptide to the HPK of the two component regulatory system. Induction by the bacteriocin molecule itself is referred to as an autoinduction loop and is found in many lantibiotic systems, including subtilin produced by *Bacillus subtilis* and nisin produced by *Lactococcus lactis* (107). The prototype for bacteriocin production and regulation, nisin, induces its own production. The production of nisin is cell-density dependent and regulated at the transcriptional level (116). The nisin gene cluster is composed of eleven genes encoding enzymes necessary for the complete regulation, modification, processing, export, and immunity of nisin from the cell. Initial genetic evidence of nisin regulation found that *nisK* and *nisR*, the histidine protein kinase and response regulator, respectively, of the two component signal transduction pathway was essential for complete nisin biosynthesis. Yet their roles in the induction of nisin remained unknown. The introduction

of a 4-bp deletion in the *nisA* gene (Δ*nisA*) provided the means in order to finally analyze the exact mechanisms of nisin induction and regulation (116). This mutation resulted in a complete loss of nisin production as well as a loss of Δ*nisA* transcription. Furthermore, upon addition of exogenous fully modified nisin to the culture medium, transcription was restored. These data indicated that nisin possessed dual functionality as an antimicrobial, as well as a signaling peptide. However, unmodified exogenous nisin failed to restore transcription, indicating that post-translational modifications of nisin are also required for induction. The authors further demonstrated that *nisK*, encoding a histidine protein kinase played a vital role in regulation when the ; *nisK* gene was insertionally inactivated, nisin production was not inducible, despite the addition of exogenous nisin or its derivatives. Taken together, Kuipers et al (116) found that fully modified nisin acted as an extracellular signal for the histidine protein kinase and induced the transcription of its own gene, as well as downstream.genes. The general model for HPK activity is that the kinase interacts with the inducing peptide within the HPK's input domain. This model assumes a direct relationship with the N-terminal domain of the HPK and the inducer. Using spaK, a histidine protein kinase from the subtilin system produced by *B. subtilis* and nisK, Kleerebezem et al (110) were able to produce a hybrid HPK composed of an N-terminal input domain from SpaK and a C-terminal transmitter domain from NisK. The hybrid protein could then be expressed in a non-lantibiotic producing strain of *Lc. lactis* containing *nisR* on its chromosome. After induction with subtilin, a fully functional sensor kinase could only be obtained when the entire SpaK input domain was present and fused to the NisK transmitter domain. These data support the current model of sensor-HPK interactions (107).

Additional analysis revealed that the nisin gene cluster is transcribed as a total of three transcriptional units; *nisA* gene is transcribed as a high level monocistronic unit as well as part of the polycistronic *nisABTCIP* unit (117). The polycistronic unit occurs at a much lower transcriptional level presumably due to the presence of a terminator sequence downstream of *nisA*. The control of these transcriptional units is derived from a promoter upstream of *nisA* (P_{nisA}) and the transcription of *nisBTCIP* is the result of limited readthrough at the terminator region (40). The other transcriptional units present consist of *nisRK* and *nisFEG* which are under the control of two promoters upstream of *nisR* (P_{nisR}) and *nisF* (P_{nisF}), respectively (40). The activity of the *nisA* and *nisF* promoters is nisin-mediated, while the *nisR* promoter appears to be nisin-independent and exhibits constitutive transcription levels (107). P_{nisA} activity (*nisA* alone and *nisBTCIP*) is most likely required to ensure proper equilibrium between the production level of nisin precursors and the capacity of modification, export, and processing machinery. The P_{nisF} inducible promoter activity provides higher immunity when cells produce higher nisin concentrations. The constitutive nature of the P_{nisR} promoter ensures the rapid response of the cell to changes in nisin concentration. Each promoter within the nisin gene cluster contains a pentanucleotide direct repeat in the -35 region. Response regulators typically possess an N-terminal receiver domain coupled with a C-terminal output domain involved in DNA binding, once the RR is phosphorylated or activated (189). The presence of the helix-turn-helix motif supports the DNA binding theory. It is predicted that the response regulator binds to the repeats found in the nisin promoters. Recently, direct evidence supporting this theory was performed using the subtilin system. Nisin and subtilin gene clusters show remarkable homology [reviewed by Kleeberezem et al (107) and Siezen et al

(181)]. Mutation analysis of repeats in thesubtilin promoter found that SpaR, the response regulator of this system, specifically binds to these repeats as a dimer. The inducible activity of promoters that were mutated was reduced. These data support the theory that the response regulator acts as a transcriptional activator that binds to DNA sequences in the promoter region (186). Similar autoinduction schemes have been seen in other lantibiotics including subtilin produced by *B. subtilis* and salivaricin A produced by *Streptococcus salivaricus* UB1309 (186, 201). In the *S. salivarius* system *salA*, the gene encoding the salivaricin A peptide, was found to induce transcription of its own gene, as well as genes involved in processing, modification, export, and immunity. Upton et al (201) constructed a mutant containing an insertionally inactivated modification gene, *salB*. The mutant failed to produce any salivaricin A and resulted in loss of *salA* transcription. Transcription could be restored, however, upon the addition of exogenous salivaricin A peptides. These results indicated that salivaricin A regulation is similar to nisin, in that the intact antimicrobial peptide functions as a signaling peptide, presumably through the two component regulatory system found within the gene cluster. The addition of salivaricin A1, a derivative of salivaricin A induced transcription of *salA* in *S. salivarius*. These results indicate that communication signals not only occur between members of the same species, but may also occur between members of different species.

Induction by inducing peptides

In each case of nisin, subtilin, and salivaricin A, transcription was regulated by autoinduction where the bacteriocin structural gene induced transcription of its own gene and downstream genes necessary for complete biosynthesis. In these systems, the HPK and RR played significant roles in sensing the concentration of the bacteriocin molecule

and initiating transcription via the action of the response regulator (189). Regulation of bacteriocins such as plantaricin produced by *Lb. plantarum*, carnobacteriocin produced by *C. piscicola*, and pediocin produced by *P. acidilactici*, occurs in much the same manner except induction occurs via a different signaling peptide (24, 48, 162). For these bacteriocins, regulation is mediated through the so-called three-component regulatory system (148). In this system, three genes are co-transcribed, a bacteriocin-like pheromone peptide and the traditional two component regulatory system encoding the sensor, HPK and transcription factor, RR. The secreted pheromone binds to the HPK resulting in autophosphorylation (46, 170). The phosphoryl group is then transferred to the RR inducing a conformational change. The resulting conformation permits the binding of RR to regulated promoter regions and triggers gene expression (58). This regulatory system was first discovered in cultures of *L. plantarum* that lost their ability to produce bacteriocin after extreme dilution (47). Bacteriocin production could only be restored upon the addition of sterile spent media. The inducing factors or pheromones were found to be proteinaceous in nature, smaller than bacteriocin peptides, and possess a typical double glycine leader peptide indicating that they are also processed from the cell via an ABC transporter complex (23). The pheromones were also cationic in nature, similar to bacteriocin peptides themselves, but typically exhibit no antagonistic activity (58). Inducing factors are highly strain specific and are able to only activate their cognate HPK, typically encoded immediately downstream of the pheromone gene itself. Brurberg et al (23) found that induction of plantarcin C11 by the sakacin-inducing factor was only possible after the introduction of the sakacin histidine protein kinase. These results

indicated the specific nature of inducing peptides as well as shed some light on their interaction with the cognate HPK (23).

Bacteriocin systems controlled by three component regulatory systems usually possess promoter elements characterized by the presence of direct or inverted repeats in the -40 and -80 regions (Figure 5) (147). These promoter elements contain a conserved -10 region and a poorly conserved -35 region. The -40 and -80 promoter regions contain direct or inverted repeats of approximately 10 residues with spacing consistent with two turns of DNA, suggesting an interaction with a transcriptional (147). It is hypothesized that this region is the precise location for binding of an activated (phosphorylated) RR (50, 169, 170) Risøen et al (168) confirmed using electron mobility shift assays that the RR binds to the direct repeats found in the regulated promoter region of the sakacin gene cluster produced by *Lb. sakei*. Additional studies analyzing the sakacin P locus revealed that promoters preceding bacteriocin structural genes and pheromones are tightly regulated and highly active when induced; whereas, promoters preceding regulatory and transport genes were less active when induced and less tightly regulated (23). Taken together, a general picture of three component regulation emerges where small amounts of inducing peptides are produced through low constitutive expression of regulatory and transport genes. Upon achieving a certain threshold level, the system is autoinduced, leading to an increase in transcription of bacteriocin structural genes and pheromones resulting in maximum activation of promoters and maximum production of pheromones and bacteriocins (58). Generally, most LAB bacteriocin systems containing three-component machinery are regulated in this manner. Plantaricin produced by *Lb. plantarum* offers a unique case in that it's three component regulatory system consists of two response regulator genes (169).

The plantaricin gene cluster is composed of a total of 16 genes and organized into five different operons (48). This system encodes two two-peptide bacteriocin systems and transcription of all five operons is induced by the pheromone, plantaricin A. Deip et al (50) found that once induced, transcription continues throughout the exponential growth phase and is abruptly halted when cells enter the stationary phase. The *plnC* and *plnD*, each encode for response regulators within the plantaricin cluster. These genes were each over-expressed in the producer strain, *Lb. plantarum* C11 and resulted in large amounts of plantaricin produced in both induced and uninduced conditions. In contrast, over-expression of *plnD*, resulted in low levels of transcription and poor plantaricin production. Moreover, although some induction was seen in cells that over-expressed *plnD*, production was only achieved upon addition of the pheromone at 100 times that of the wildtype. Additionally, induced cultures that over-expressed *plnD*, were able to produce only a fraction of the bacterocin seen in wildtype and cultures that over-expressed *plnC*. These results indicate that the plantaricin regulatory system is more highly complex where *plnC* functions as a strong activator, while *plnD* functions as a repressor that may result in the down regulation observed in the stationary phase (50).

Another extraordinary example of bacteriocin regulation occurs in carnobacteriocin produced by *Carnobacterium piscicola* (161). This genetic system is particularly complex, composed of three separate bacteriocin loci encoding three bacteriocin peptides. Carnobacteriocins B2 and A are located on two separate plasmids whereas carnobacteriocin BM1 is located on the chromosome (161). Collectively, the loci contain two-three component regulatory systems with all operons containing characteristic regulated promoters (147). These results imply possible co-regulation between the

bacteriocin systems. Two signaling peptides have been identified within this system, the carnobacteriocin B2 bacteriocin molecule and a pheromone peptide, CbnS. CbnS is a secreted peptide that regulates the production of carnobacteriocin BM1 and B2. These molecules were found to regulate transcription of all gene clusters (161). Both inducing peptides act through the two component regulatory system (109). Eijsink et al (50) speculated that this carnobacteriocin system may represent an 'intermediate stage' between the regulation observed in class I bacteriocins, such as nisin, and that of class II bacteriocins, such as plantaricin.

Induction by environmental signals

Genetic studies have recently begun to elucidate the possible roles that environmental conditions have on the induction and regulation of bacteriocin production. Environmental conditions such as pH and temperature have been found to effect the regulation of lacticin 481 produced by *Lc. lactis* and sakacin A produced by *Lb. sakei* respectively (46, 89).

Lacticin 481 is a lantibiotic with a group AII leader sequence (167). The lacticin 481 gene cluster comprises six genes involved in production, modification, transport, and immunity. Remarkably, unlike most other lantibiotics, lacticin 481 does not contain any specific regulatory genes (53, 167). Regulation of lacticin 481 occurs at the transcriptional level and is stimulated by the presence of low pH. During the growth of *Lc. lactis*, natural acidification occurs through the accumulation of lactic acid within the growth media from a pH of 7.0 to 5.8 (89). Production of lacticin 481 begins during mid exponential phase and continues into stationary phase, yet Hindre et al (89) found that early production can be obtained in an initially acidic medium that results in a higher level of production.

Further analysis confirmed that transcriptional control occurs mainly through the action of two promoters present within the lacticin 481 gene cluster. Both strong promoters precede the lacticin 481 structural gene. Control of immunity genes were found to be the result of a third much weaker promoter that is located within the gene encoding an ABC transporter. Interestingly, the effect of low pH, not lactic acid, was found to trigger promoter activity. Media that was acidified to pH 5.8 using acetic acid prior to bacteriocin production resulted in higher lacticin 481 levels (27). Since this gene cluster does not contain any specific regulatory genes, control of both promoters must occur through the action of a more general regulator than those typically associated with LAB bacteriocin systems.

Sakacin A is a class II bacteriocin produced by *Lb. sakei* (46). The sakacin A gene cluster is composed of seven genes with a traditional three component regulatory system. Diep et al (46) found that growth of *L. sakei* and sakacin A production was strongly effected by temperature. High amounts of sakacin A were produced from cultures grown at 25ºC, whereas production was reduced by cells grown at 33.5ºC and completely shut down when cells were grown at 34.5º C (46). Production could be restored if the temperature was lowered to 25-30ºC or upon the addition of the sakacin A pheromone at the nonpermissive temperature of 33.5ºC. The importance of the sakacin A pheromone in induction was demonstrated by the construction of a mutant containing an inactivated sakacin A pheromone gene, *sap-ph*. The mutant failed to produce sakacin A, even when grown at optimal temperatures. Production could be restored upon the addition of synthetic sakacin A pheromone.

In each of these cases, growth conditions had a profound effect on bacteriocin production through transcriptional regulation. Currently, it is unknown how growth

conditions specifically interfere with bacteriocin regulation (146). Environmental factors may affect pheromone secretion and processing or the affinity of the HPK to the pheromone. Interaction of the HPK with the pheromone may also be dependent on the high net charge of the pheromone indicating electrostatic interactions (146). As in competence development, bacteriocin production may be sensitive to a number of physiological changes within the cell.

Regulation by repression

Regulation of immunity by the action of a repressor

The first example of a repressor modulating bacteriocin activity was reported by McAuliffe et al (133) in the two-peptide lacticin 3147 system produced by *Lc. lactis*. The lacticin 3147 gene cluster includes 10 genes organized on two divergent transcriptional units. The first transcriptional unit encodes the two-component bacteriocin peptides, their cognate modification enzymes, and the processing and transport enzymes under the control of a constitutive promoter preceding the bacteriocin structural genes. The second transcriptional unit encodes one protein involved in regulation, and the immunity proteins, all of which is under the transcriptional control of a promoter preceding the immunity genes (131). Genes encoding a signal transduction mechanism have not been identified in this region. Instead LtnR, a repressor of the PBSX (Xre) family is responsible for the regulation of its own transcription and that of downstream immunity genes (130). McAuliffe et al (131) confirmed that modulation occurs through the binding of LtnR to intergenic regions of the constitutive promoter preceding lacticin 3147 structural genes and also to the regulated promoter preceding immunity genes.. Therefore, once a certain concentration of LtnR and immunity proteins has accumulated within the cell, expression

is then repressed. Consequently, derepression occurs once the cell becomes sensitive to lacticin 3147 due to the absence of proteins that confer immunity. In this manner, a constant level of immunity is obtained. In contrast, transcription of the structural and modification genes is constitutive, but an attenuator present downstream of the structural genes is thought to control transcription of the modification genes (131).

Regulation of production by repression

Cytolysin produced by *Enterococcus faecalis* is a two peptide, class I bacteriocin whose inhibitory spectrum includes most gram-positive bacteria (79). This gene cluster contains eight genes transcribed as two units; one transcriptional unit containing the structural cytolysin genes as well as modification, processing, export and immunity genes; the second unit is transcribed in the opposite direction and contains only the regulatory genes, *cylR1* and *cylR2* (79). Expression of the cytolysin gene cluster is controlled by two divergent, overlapping promoters. Interestingly, *cylR1* and *cylR2* do not show any homology to members of two component regulators. Mutational inactivation of these genes, individually or in combination resulted in constitutive cytolysin transcription (80). In the presence of both CylR1 and CyR2 1 no expression was observed. CylR1 and CyR2 are both required for repression of cytolysin expression and that repression occurs through the obstruction of the promoter via CylR2 binding to the promoter and preventing transcription of the cytolysin operon (80) . It is further hypothesized that CylR1 may interact with CylR2 to stabilize this obstruction (173). Transcriptional analyses further demonstrated that repression of biosynthetic cytolysin transcript could be alleviated through the action of one peptide, *cylL2*, of the two peptide bacteriocin system. Expression from the repressed cytolysin promoter was induced by the addition of

exogenous CylL2. However, neither CylL1, nor unmodified CylL2 were capable of de-repression. Therefore Haas et al (80) concluded that cytolysin regulation requires CylR1, CylR2, and CylL2 to effect expression of cytolysin in a quorum sensing dependent manner. CylL2 acts as signal that once it accumulates beyond a certain threshold triggers de-repression of cytolysin promoter resulting in high levels of cytolysin expression (173).

A third example of regulation by repression is seen in lactocin S produced by *Lb. sake* (183, 184). Transcription of the gene cluster occurs as two separate units under the control of divergent, overlapping promoters; one unit contains the biosynthetic machinery including the lactocin S structural genes, while the other contains regulatory genes. Lactocin S production is modulated by LasX, a protein that serves as an activator for transcription of lactocin S biosynthetic genes, while at the same time repressing its own transcription (165). Lactocin S is produced constitutively, therefore, it is hypothesized that LasX maintains this steady state (183).

Streptococcus mutans: a case of global regulation

The oral cavity typically contains a large number of microorganisms with over 500 different species (115). Furthermore, this environment is difficult with constant cycles of feast and famine with variations in pH and salt concentrations due to food intake from the host (114). As a result, many organisms have established an arsenal of survival mechanisms that not adapt to fluctuations in nutrients and pH, but also form biofilms and produce bacteriocins to kill competing organisms. *Streptococcus mutans* is a gram-positive organism commonly found as a member of the human oral microbiota (2). *S. mutans* was found to produce two bacteriocins, mutacin I and mutacin IV. Mutacin IV is a two-peptide class II bacteriocin with a narrow spectrum of inhibition against other

streptococcal species including *S. gordonii* and *S. sanguis*, typically those that inhabit the same environmental niche (114). Recently, mechanisms of regulation have been elucidated for each of these bacteriocins. Interestingly, mutacin I and IV are regulated by different mechanisms closely linked to other cellular processes of *S. mutans* (115, 134, 157). Two forms of regulation prevail in this organism, the ComCDE system and the LuxS system (115, 134).

ComCDE machinery is a three component regulatory system that serves as an intraspecies communication scheme in response to cell density. ComD and ComE encode a two-component regulatory system consisting of a histidine protein kinase and a response regulator. This system has been shown to control natural competence and biofilm formation (120). The LuxS system involves the production of autoinducer-2 (AI-2) signaling molecules in response to cell density and has been identified in both gram-negative and gram-positive microorganisms (179). The LuxS system has also been implicated in biofilm formation and stress tolerance (135, 210). These signaling molecules are believed to be responsible for interspecies communication and studies show that LuxS-positive bacteria are able to induce the AI-2 reporter species, *Vibrio harveyi* (214). Thus, it is possible that AI-2 is a universal signaling molecule that mediates interspecies interactions in such communities as the oral cavity or the gastrointestinal tract. The LuxS system in a novel regulatory pathway was found to control mutacin I, whereas the ComCDE system dictated mutacin IV production (115, 134).

Induction of mutacin IV transcription is initiated in response to *comC* activation. The ComCDE system was first observed as the regulation machinery involved in the modulation of natural genetic competence (5). The competence stimulating peptide

(ComC) binds to the sensor protein (ComD) and triggers autophosphorylation, which is then transferred to ComE, the response regulator resulting in gene expression. Mutations in *comC* and *comD* resulted in markedly reduced levels of mutacin IV production in pelleted and planktonic cells. Mutations in *comE*, the cognate response regulator, resulted in abrogation of mutacin IV production, indicating that the ComCDE signal transduction pathway regulates not only competence development but mutacin IV bacteriocin production as well. Furthermore Kreth et al (115) found that addition of ComC resulted in not only an increase in mutacin IV production, but in genetic competence as well. After addition of ComC, mutacin IV production peaked at three hours, while transformation efficiency as a function of competence peaked after two hours. These results indicated that mutacin production and competence are coordinated. It was then hypothesized that *S. mutans* produces mutacin IV in order to lyse sensitive cells, allowing opportunity for the uptake of exogenous DNA via competence induction (115). This was confirmed using *S. gordonii*, a mutacin IV sensitive strain that also inhabits the oral cavity. Using mutations in either mutacin I or mutacin IV or both, the authors demonstrated that transformation efficiency was greatest in strains producing mutacin IV, Mutacin I did not affect competence. Therefore, mutacin IV production plays an important role in competence via the same ComCDE regulatory pathway. To date, this is the first genetic evidence that the regulation of bacteriocin production may coordinately regulate other physiological processes.

Conclusions

The study of bacteriocins produced by LAB has not only yielded vital genetic information on these organisms but has unexpectedly provided insight into the manner in

which these microorganisms sense and respond to the environment around them and to each other. A model for class I and class II regulation has emerged based on autoinducing peptide mediated quorum sensing. Though the levels of complexity differ among classes, bacteriocin production seems to be regulated through the traditional two component regulatory system of a histidine protein kinase and response regulator. In this system, the message, either the bacteriocin molecule itself or an inducing peptide, binds to the histidine protein kinase ultimately resulting in gene activation by the response regulator. However, many aspects of this model remain poorly understood; the role of the environment, the detailed mechanism that result in DNA binding and activation by the response regulator, as well as any additional effects on other cellular processes that may result from inducing peptides. Further analysis is needed in order to fully understand all aspects of the regulatory network responsible for bacteriocin production.

Recently, genetic evidence suggests that bacteriocin production may be a small piece of an intricate, complex system regulating not only bacteriocin production, but perhaps competence, sporulation, or stress tolerance. The characterization of additional bacteriocin systems as well as other cell-density dependent behavioral responses will no doubt result in a more complete, comprehensive picture of the quorum sensing phenomena in LAB providing essential insights into not only individual cellular responses, but also to population behavior and adaptation. In this manner, the genome of *Lactobacillus acidophilus* NCFM was surveyed and a potential operon for a bacteriocin was identified and functionally characterized.

TABLE 1. Lantibiotics produced by LAB

Lantibiotic	Producer strain	Reference
	TypeA(I)	
Nisin A	*Lactococcus lactis* ATCC 11454	(23)
Nisin Z	*Lactococcus lactis* N8	(120)
Nisin Q	*Lactococcus lactis* 61-14	(190)
Streptin	*Streptococcus pyogenes* BL-T	(176)
Mutacin 1140	*Streptococcus mutans* JH1140	(74)
Mutacin I	*Streptococcus mutans* CH43	(131)
Mutacin III	*Streptococcus mutans* UA787	(132)
Mutacin B-Ny266	*Streptococcus mutans* Ny266	(119)
	Type A(II)	
Cytolysin	*Enterococcus faecalis*	(15)
Carnocin UI49	*Carnobacterium piscicola* UI49	(161)
Lacticin 3147	*Lactococcus lactis* DPC 3147	(112)
Lacticin 481	*Lactococcus lactis* CNRZ 481	(140)
Salivaricin A [Lys2,Phe7]	*Streptococcus pyogenes* T11 (M type 4)	(152)
Salivaricin A	*Streptococcus salivarius* 20P3	(144)
Streptococcin A-FF22	*Streptococcus pyogenes* FF22	(81)
Mutacin II	*Streptococcus mutans* T8	(27)
Smb	*Streptococcus mutans* GS5	(189)
Macedocin	*Streptococcus macedonicus* ACA-DC198	(62)
BHT-A	*Streptococcus rattus* BHT	(80)
Bovicin HJ50	*Streptococcus bovis* HJ50	(180)
Lactocin S	*Lactobacillus sakei* L45	(118)
Plantaricin W	*Lactobacillus plantarum* LMG 2379	(77)
Plantaricin C	*Lactobacillus plantarum* LL441	(64)

TABLE 2. Class II bacteriocins produced by LAB

Class II bacteriocin	Producer strain	Reference
	Subclass IIA	
Acidocin A	*Lactobacillus acidophilus* TK9201	(100)
Acidocin 8912	*Lactobacillus acidophilus* TK8912	(101)
Bavaricin A	*Lactobacillus bavaricus*	(118)
Bavaricin MN	*Lactobacillus sake*	(99)
Curvacin A	*Lactobacillus curvatus*	(197)
Sakacin A	*Lactobacillus sake* Lb706	(90)
Sakacin P	*Lactobacillus sake* LTH673/674	(93)
Plantaricin UG1	*Lactobacillus plantarum* UG1	(61)
Divergicin A	*Carnobacterium divergens*	(213)
Divergicin M35	*Carnobacterium divergens* M35	(195)
Carnobacteriocin BM1	*Carnobacterium piscicola* LV17	(162)
Carnobacteriocin B2	*Carnobacterium piscicola* LV17	(162)
Carnocin H	*Carnobacterium* 377	(19)
Carnocin KZ213	*Carnobacterium piscicola* 213	(104)
Carnocin CP5	*Carnobacterium piscicola* CP5	(86)
Piscicocin CS526	*Carnobacteriocin piscicola* CS526	(219)
Enterocin A	*Enterococcus faecium*	(11)
Enterocin P	*Enterococcus faecium*	(78)
Leucocin B-Ta11a	*Leuconstoc gelidum* UAL187	(62)
Leucocin A-TA33a	*Leuconstoc carnosum*	(154)
Leucocin B-TA33a	*Leuconstoc mesenteroides*	(153)
Leucocin C-TA33a	*Leuconstoc mesenteroides*	(153)
Mesentericin B105	*Leuconstoc mesenteroides*	(83)
Mesentericin Y105	*Leuconstoc mesenteroides* Y105	(69)
Pediocin AcH/PA-1	*Pediococcus acidilactici* H/PAC 1.0	(205)
	Subclass IIB	
Acidocin J1132	*Lactobacillus acidophilus* JCM1132	(194)
Acidocin LF221	*Lactobacillus gasseri* LF221	(124)
Gassericin T	*Lactobacillus gasseri*	(102)
Lactacin F	*Lactobacillus johnsonii* VPI11088	(68)
Lactobin A/ amylovorin L471	*Lactobacillus amylovorus*	(42)
Plantaricin EF	*Lactobacillus plantarum* C11	(48)
Plantaricin JK	*Lactobacillus plantarum* C11	(48)
Plantaricin S	*Lactobacillus plantarum* LPC010	(125)
Plantaricin NC8	*Lactobacillus plantarum* NC8	(126)
Lactococcin G	*Lactococcus lactis*	(151)
Lactococcin MN	*Lactococcus lactis*	(139)
Lactococcin MMT24	*Lactococcus lactis* MMT24	(74)
Thermophilin 13	*Streptococcus thermophilus*	(208)
Enterocin 1071	*Enterococcus faecalis*	(12)

TABLE 2. (cont.)

Subclass IIC		
Acidocin CH5	*Lactobacillus acidophilus* CH5	(33)
Lactacin B	*Lactobacillus acidophilus* NCFM	(13)
Plantaricin 1.25β	*Lactobacillus plantarum*	(57)
Curvaticin FS47	*Lactococcus curvatus*	(71)
Lactococcin A	*Lactococcus lactis*	(92)
Lactococcin 972	*Lactococcus lactis*	(128)
Enterocin B	*Enterococcus faecium*	(25)
Enterocin L50	*Enterococcus faecalis*	(35)
Carnobacteriocin A/ Piscicolin 61	*Carnobacterium piscicola*	(66)
Structures/sequences not yet determined		
Enterocin AS-48	*Enterococcus faecalis* subsp. *liquefaciens* A-48-32	(45)
Enterocin RJ-11	*Enterococcus faecium*	(218)
Durancin L28-1A	*Enterococcus durans* L28-1	(221)
Bacteriocin ST15	*Enterococcus mundtii* ST15	(39)
Pediocin PD-1	*Pediococcus damnosus* NCFB 1832	(77)

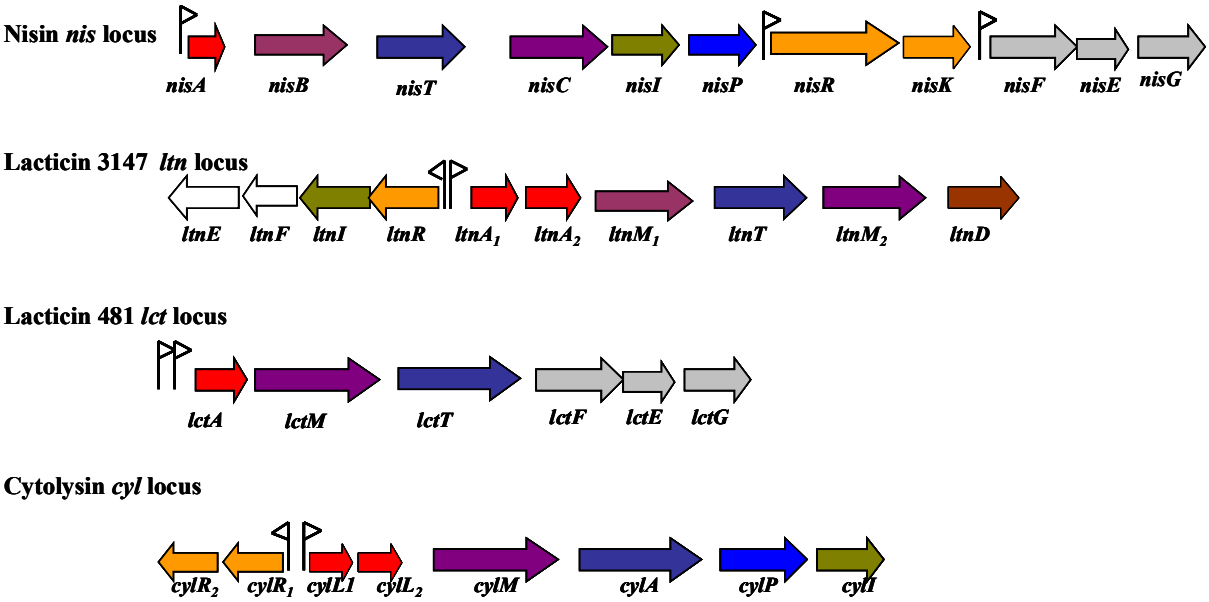

Figure 1. Schematic representation of known gene clusters involved lantibiotic biosynthesis in various lactic acid bacteria (adapted from McAuliffe et al. 2001). Not drawn to scale. Genes are represented by an arrow. Genes with similar functions are highlighted in the same color; red for structural genes; purple for modification; dark blue for transport; light blue for proteolytic processing; orange for regulation; green for immunity. Genes with unknown function are highlighted in white. Promoters ( ) are indicated.

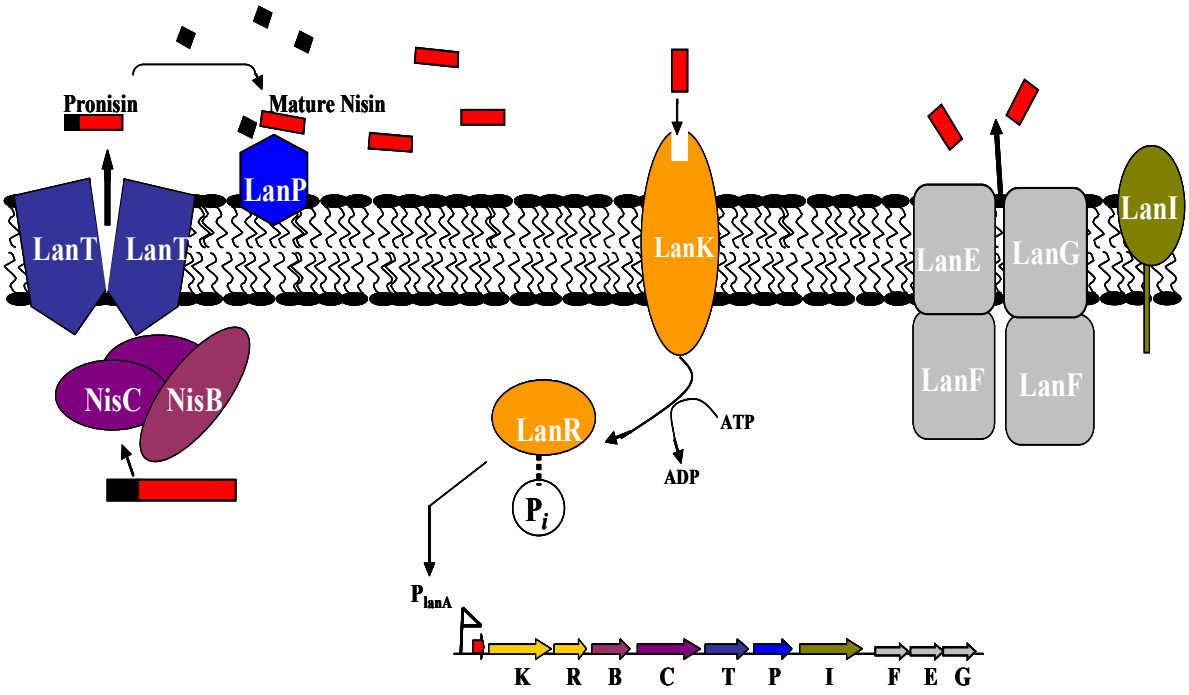

Figure 2. General schematic representation showing the biosynthesis, regulation, and immunity machinery responsible for lantibiotic production. Extracellular changes in lantibiotic concentration result in the autophosphorylation of LanK and transfer of a phosphoryl group from LanK to LanR. This results in transcription of all biosynthetic genes necessary for production. LanA, the bacteriocin prepeptide, is processed by the LanB dehydratase, and the LanC cyclase to generate a modified lantibiotic peptide. The prepeptide is exported from the cell by LanT and the leader sequence is removed by the action of a serine protease, LanP. Both LanI and LanFEG can be involved in the protection of the producer from the lantibiotic. LanI may shield or sequester the active lantibiotic molecule, while LanFEG may assist in the transport of the bacteriocin from the membrane to the extracellular environment.

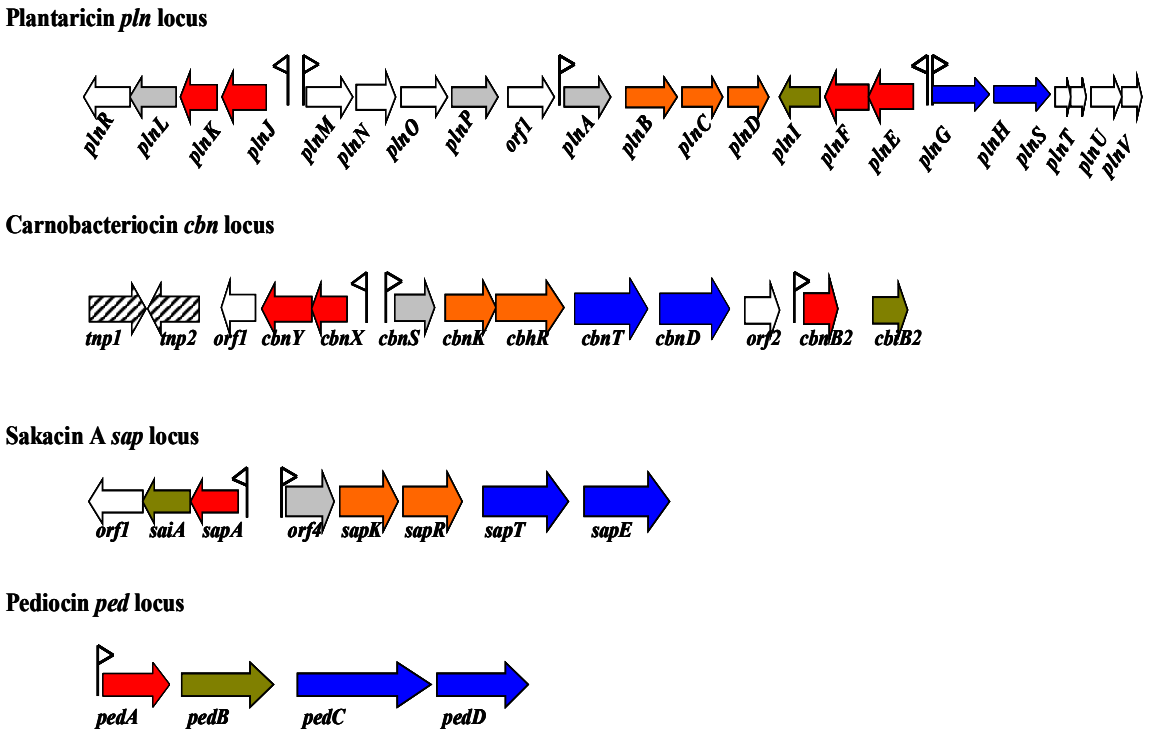

Figure 3. Schematic representation of known gene clusters involved in class II bacteriocin biosynthesis in various lactic acid bacteria (adapted from van Belkum et al. 2000). Not drawn to scale. Genes are represented by an arrow. Genes with similar functions are highlighted in the same color; red for structural genes; gray for inducing peptides; blue for transport; orange for regulation; green for immunity. Genes with unknown function are highlighted in white. Transposon elements are shaded. Promoters ( ) are indicated.

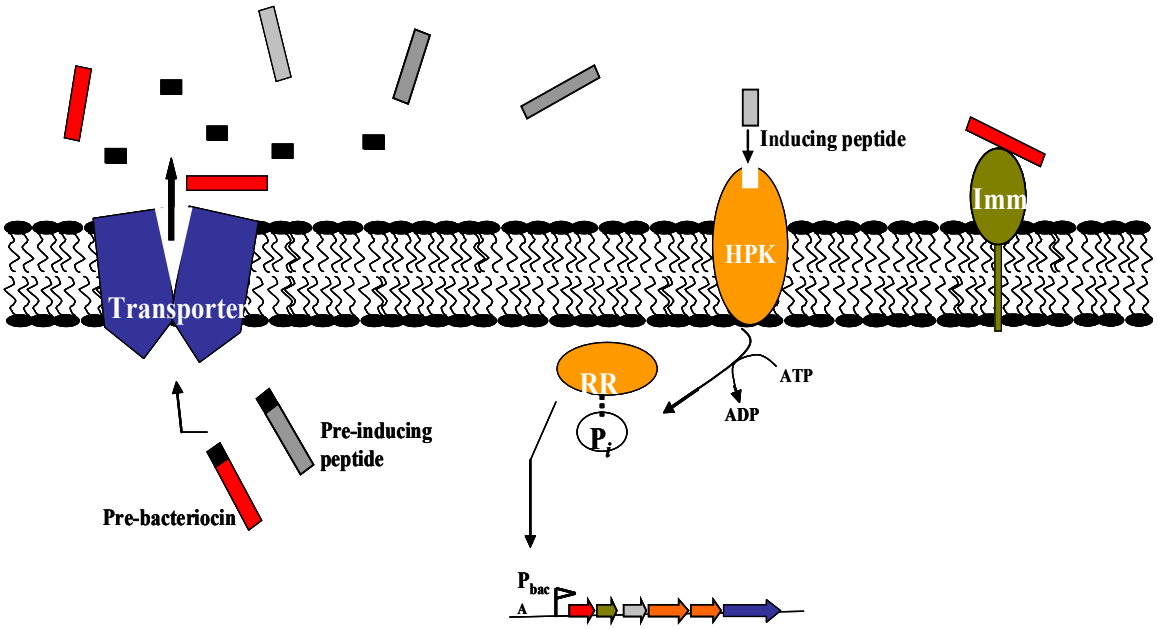

Figure 4. General schematic representation showing the biosynthesis, regulation, and immunity machinery responsible for class II bacteriocin production. Extracellular changes as a result of inducer concentration result in the autophosphorylation of hisitidine kinase HPK and transfer of a phosphoryl group from HPK to the response regulator RR. This results in transcription of all biosynthetic genes necessary for production including genes coding for the bacteriocin peptide and inducing peptides. Cleavage of the leader sequence occurs concomitant with export by the transporter complex. Immunity proteins (Imm) protect the producer from its own bacteriocin peptide.

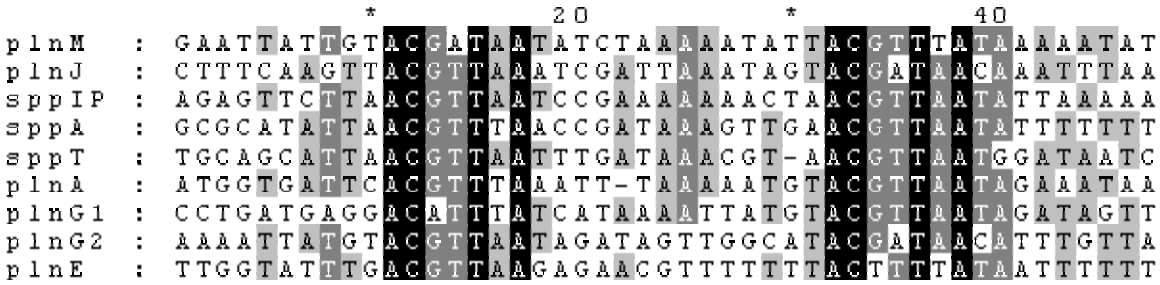

Figure 5. Bacteriocin promoters regulated by a three component regulatory system (Kotelnikova et al 2002). Shown are the promoter elements found in front of *spp* genes *sppIP*, *sppA*, *sppT*) from *Lb. sake* LTH673 and *pln* genes (*plnM*, *plnJ*, *plnA*, *plnG1*, *plnG2*, *plnE*) from *Lb. plantarum* C11. Residues that are conserved in all nine sequences are shaded in black; residues that are conserved in at least seven sequences are shaded in gray.

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CHAPTER II

Identification of an operon involved in the production of Lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*

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INTRODUCTION

The human gastrointestinal tract is home to anywhere between 500-1000 different species of bacteria reaching populations as high as 10^{11} organisms per gram of contents (217). With populations this diverse, individual species must acquire ways to compete and communicate in an environment where nutrients are limited. In response, many bacteria have evolved mechanisms against competing microorganisms. Such mechanisms include competitive exclusion, production of organic acids, hydrogen peroxide, and bacteriocins. Bacteriocins are small, ribosomally synthesized antimicrobial peptides produced by both gram-positive and gram-negative bacteria (166). Lactic acid bacteria (LAB) in particular produce a wide array of these peptides active against spoilage and pathogenic microorganisms, as well as closely-related species that inhabit the same environmental niches (17, 56). In general, bacteriocins range in size from 20-60 amino acids, possess a net positive charge, and are typically inhibitory to closely-related species with notable exceptions including pediocin, nisin, and lacticin 3147 (107, 121, 133). LAB bacteriocins are classified into three distinct categories based on genetic and biochemical analysis (106): class I bacteriocins known as lantibiotics, require extensive post-translational modifications for biological activity; class II bacteriocins are small, heat stable proteins with a high isoelectric point that undergo minimal modification for bioactivity; and class III bacteriocins consist of larger heat labile proteins of which there are only a few examples, helveticin J produced by *Lactobacillus helveticus*, enterolysin A and enterocin produced by *Enterococcus faecalis* LMG2333 and *E. faecalis* MMT05, respectively, are members of this class (75, 96, 149).

Recent advances in LAB functional genomics coupled with the public availability of many LAB genomic sequences (7, 108, 156) have uncovered the genetic loci for a number of known and potentially new bacteriocins. The most prevalent, class II bacteriocins (148) consists of a large number of heterologous peptides produced by numerous species of LAB including *Lactobacillus* spp., *Lactococcus* spp., *Pediococcus* spp., *Leuconostoc* spp., and *Carnobacterium* spp. (175). Despite such extraordinary diversity, consensus genetic elements necessary for bacteriocin production have been elucidated, including (i) one or more structural genes encoding a prepeptide, characterized by the presence of a conserved N-terminal extension containing a so-called double glycine leader sequence (Gly^{-2}-Gly^{-1}-Xaa); (ii.) a dedicated immunity gene typically located within the same operon; (iii) a gene encoding a dedicated ATP-binding cassette (ABC) transporter; and, (iv) a gene encoding an ABC transporter accessory protein. The ABC transporter and its accessory protein work to cleave the pre-bacteriocin at its double glycine leader sequence concomitant with export to yield a mature and biologically active bacteriocin molecule (146).

Many LAB bacteriocins are regulated by an inducing peptide (49, 65). These signaling peptides activate the two component regulatory system usually composed of a histidine protein kinase and a response regulator. This pathway ultimately results in the transcription of all components necessary for the production and processing of the bacteriocin peptide. The exact mechanism that triggers bacteriocin induction remains unknown, yet cell density and *quorum* sensing appear to play important roles. Beyond a certain threshold level, the concentration of inducing peptide accumulates to a sufficient level to induce bacteriocin production, sensed by the histidine protein kinase. It is then

possible to induce neighboring cells resulting in concerted bacteriocin production by the bacterial population. Such cell-to-cell signaling is apparent during competence development by *Streptococcus pneumoniae* and nisin production by *Lactococcus lactis* (107, 112, 122).

Lactacin B is a class II, chromosomally-encoded bacteriocin produced by the lactic acid bacterium, *Lactobacillus acidophilus* NCFM (13). Previous biochemical analysis revealed that lactacin B is a heat stable, catalase-insensitive protein, with a molecular weight of approximately 6.5 kDa (13, 14). Its inhibitory spectrum includes *Enterococcus faecalis* but is limited to closely-related species including *Lactobacillus delbrueckii*, *Lactobacillus bulgaricus*, and *L. helveticus*. Genome sequence analysis of *L. acidophilus* NCFM revealed a region of interest possibly responsible for lactacin B production (7). This region is composed of putative genes that show homology to the regulatory and export systems of the class II bacteriocin, lactacin F produced by *Lactobacillus johnsonii* NCC533 (3). We therefore investigated this region in order to determine its role in lactacin B production, processing, and export.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultivated in Luria-Bertani (Difco, Detroit, MI) broth at 37ºC with shaking, when appropriate, *E. coli* cultures were selected or propagated on Brain Heart Infusion agar (Difco) supplemented with 150 µg/ml erythromycin (Em) or 10 µg/ml chloramphenicol (Cm). Lactobacilli were propagated in MRS broth (Difco) at 37ºC without agitation. Agar media was prepared by adding 1.5% (w/v) agar to liquid broth, overlay agar was prepared by adding 0.75% (w/v) agar to liquid

broth media. When appropriate, 5 μg/ml Cm and/or Em were added to MRS broth or agar plates.

Bacteriocin production assays. Lactacin B activity was assayed using an adaptation of the direct method for bacteriocin detection (103). Briefly, stationary phase cultures of *L. acidophilus* NCFM were spotted (5 μl) onto an MRS agar plate. The plate was incubated at 37ºC overnight and then 100 μl of an overnight culture of the indicator, *L. delbrueckii* ATCC 4797 was added to 10 ml of molten MRS overlay agar (0.75% w/v) and then poured evenly onto the surface of the agar plate. After 19-24 hour incubation, any zones of inhibition indicating antagonistic activity of lactacin B, were noted and recorded.

Molecular cloning. Plasmid DNA from *E. coli* was isolated using the Qiaprep Spin Miniprep kit according to manufacturer's instructions (Qiagen Inc., Valencia, CA). Genomic DNA from *L. acidophilus* NCFM was isolated using the UltraClean™ Microbial DNA Isolation Kit according to manufacturer's instructions (Mo-Bio Laboratories, Solana Beach, CA) with the following modification: 2 ml of an overnight culture of *L. acidophilus* cells were inoculated into 10 ml of fresh MRS media and incubated at 37º C for 2 hours. Plasmid DNA from *L. acidophilus* was isolated using the modified protocol outlined by Walker et al (207). Standard protocols were used for endonuclease restriction and ligation reactions as described by Sambrook et al (178). DNA ligase, DNA polymerase I, and restriction enzymes were purchased from Roche (Roche Applied Science, Indianapolis, IN). Digested DNA was eluted from 1 % agarose gels using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Orange, CA). The PCR Core Kit using standard Taq DNA polymerase I (Roche, Indianopolis, IN) was used to generate PCR products for gene disruption. Roche's Expand Long Template PCR System was used

to generate PCR products for cloning purposes. PCR products and *Lactobacillus* plasmid DNA was further purified using Qiaquick® PCR Purification Kit (Qiagen, Valencia, CA). Electrocompetent *E. coli* cells were prepared using the method outlined by Ausebel et al (9) and electroporated using the Gene Pulser unit (Biorad Laboratories, Richmond, CA). Electrocompetent *L. acidophilus* cells were prepared using the protocol outlined by Walker et al (206). Primers used in this study are listed in Table 2.

Insertional inactivation of the ABC Transporter Gene. A 745-bp internal fragment of the ABC transporter was PCR amplified using the primers outlined in Table 2. This internal fragment was then cloned onto the integrative plasmid pORI28 via BglII/XbaI sites, respectively. The resultant 2.3 kb plasmid, pTRK852, was then introduced by electroporation into *L. acidophilus* NCFM containing the helper plasmid, pTRK669 (174). Subsequent steps were carried out according to the protocol described by Russell and Klaenhammer (174). Plasmid integration and junction fragments within the mutant NCK1758 were confirmed by PCR using primers external to the region of homology and Southern hybridization analyses using standard procedures (9).

Plasmid construction and gene expression. To identify and analyze genes suspected to be involved in lactacin B production, a 933-bp fragment containing the first four putative genes in the lactacin B gene cluster was amplified by PCR using the primers listed in Table 2. The amplicon was then cloned onto the high-copy-number plasmid, pTRK696 using the restriction enzyme sites EcoRI/SalI respectively. All manipulations were performed in *E. coli* MC1061. The resulting plasmid, pTRK853, was transformed into *L. acidophilus* NCFM, clones were evaluated for production of lactacin B.

Bacteriocin induction and activity assays using synthetic peptides. Customized synthetic peptides were prepared by Genscript (www.genscript.com). The mature amino acid sequences of LBA1801 and LBA1800 were chemically synthesized using Genscript's proprietary method. Attempts to synthesize the mature amino acid sequence of LBA1803 and LBA1802 were unsuccessful. The antagonistic activity of synthetic peptides encoded by ORFs LBA1801 (*LBA1801'*) and LBA1800 (*LBA1800'*), at concentrations ranging from 1 mg/ml to 100 pg/ml, were analyzed using *L. delbrueckii* ATCC 4797 as the sensitive indicator strain. Antagonistic activity of synthetic bacteriocin peptides are typically analyzed using micromolar to picomolar concentrations (51). Approximately 1µg/ml of each synthetic peptide was added to 10 mls of MRS broth inoculated with *L. acidophilus* NCFM. Once the cultures reached an optical density (OD_{600}) of 1.0 the cells were centrifuged and the supernatants were collected. The cell-free supernatants (CFS) were filter-sterilized and portions (3 µl) of twofold serial dilutions of each supernatant were then aliquotted onto an MRS agar plate containing *L. delbrueckii* ATCC 4797 as an indicator. After 19-24 hour incubation, zones of inhibition, indicating antagonistic activity of lactacin B were noted and recorded. The exact titer was defined by Barefoot et al (13) as the reciprocal of the highest dilution showing definite inhibition of the indicator lawn expressed as arbitrary units (AU) per milliliter.

RNA isolation and RT-PCR analysis. Cells from an overnight culture of *L. acidophilus* NCFM cells were harvested by centrifugation, resuspended in TE containing 1mg/ml lysozyme (Sigma, St. Louis MO) and incubated for 5 minutes at 55ºC. Total RNA was isolated from *L. acidophilus* NCFM using Ambion's RNAqueous® system (Austin, TX) according to manufacturer's instructions. Contaminating DNA was removed from RNA

samples using Ambion's DNA-free system. First strand cDNA was synthesized using Invitrogen's RT-PCR kit with the following modifications: a total of 5 µg of total RNA was used to synthesize first strand cDNA; RNA, RT-primer, and 10 mM dNTPs were combined and incubated at 70ºC for 15 minutes to denature RNA. Each reaction was then incubated on ice for 5 minutes, all remaining reagents were then added and the mixture was incubated for 2 hours at 42º C. The resulting cDNA products were amplified by PCR using primer oligonucleotides designed to amplify intergenic regions within the lactacin B gene locus. RT-PCR products were separated on a 1.5% agarose (w/v) gel stained with ethidium bromide. Primers used for RT-PCR analysis are listed in Table 2.

Computational analysis. ClustalX was used to align predicted protein sequences (196). TMHMM (http://www.cbs.dtu.dk/services/TMHMM) was used to predict transmembrane helices in putative proteins. DNA and amino acid sequences were analyzed for dyad symmetry indicative of intrinsic terminators using CloneManager Professional Suite 7 software package (SciEd Software, Cary, NC). Similarity searches using National Center Biotechnology Information (NCBI) database were performed using Basic Local Alignment Search Tool (Blastx) (8). Amino acid sequences were analyzed using the Swiss Institute of Bioinformatics' ExPaSy proteomics server to compute theoretical isoelectric points (http://us.expasy.org/tools).

RESULTS

Sequence analysis of Lactacin B Gene Locus. The *L. acidophilus* NCFM genome was recently sequenced (7). Bioinformatic analysis revealed a 9.4 kb polycistronic region of interest that could potentially encode the genetic determinants required for lactacin B production, processing, and export (Figure 1). The region was organized into three

clusters: a production and regulation cluster encoding a histidine kinase and response regulator protein, an export cluster containing the ABC transporter and its accessory protein, and a final cluster composed three proteins of unknown functionality. Each cluster was separated by an intrinsic terminator, the strongest terminators flanked the entire lactacin B region (ΔG = -13.4 kcal/mol and ΔG = -17.0 kcal/mol respectively). The region encompassed a total of 12 open reading frames (ORFs) (Table 3), each preceded by a plausible ribosomal binding site (RBS). Putative promoter elements were identified upstream of LBA1803 that resembled the consensus sequences of gram-positive promoters (138). Direct repeats 8 bp in length were also detected upstream of LBA1803. These repeats may potentially act as additional regulatory elements.

(i) ***Production/regulation cluster***. The lactacin B region begins with four putative genes (LBA1803-LBA1800) ranging from 38 to 53 amino acids in length. The 3'end of LBA1802 overlapped the first 13 amino acids of LBA1801. The deduced gene products encoded molecules characteristic of class II bacteriocin prepeptides; each protein is small, cationic, and contained an N-terminal double glycine leader sequence with a high isoelectric point. The amino acid sequence and relevant physicochemical properties are listed in Table 4. Homology searches for these putative genes showed no similarity to any known proteins in the NCBI database.

Both LBA1799 and LBA1798 showed strong similarity (43% and 55% identity, respectively) to lactacin F's (156) histidine protein kinase and response regulator (Table 3). Therefore, LBA1799 was designated LabK (*la*ctacin *B k*inase) and LBA1798 was designated LabR (*la*ctacin *B r*egulator). LabK was predicted to encode a 50 kDa protein containing six transmembrane domains. Bacteriocin histidine kinases are often predicted

to contain between five to seven transmembrane sequences (189). LabR encodes for a response regulator with a molecular weight of 31 kDa containing a conserved N-terminal regulatory domain and a C-terminal DNA-binding domain (190). An intrinsic terminator (ΔG –10.9 kcal/mol) rests 5 bp downstream of LabR.

(ii) ***Export Cluster***. LBA1797, located 190 bp downstream of LabR encoded an 83 amino acid cationic peptide with a double glycine leader motif. This putative gene displays no homology to any known protein in the database. Located downstream, LBA1796 (LabT) was predicted to encode an ATP binding cassette (ABC) transporter involved in the export of lactacin B from the cell. LBA1796 encodes a 720 amino acid protein belonging to the drug, peptide, and lipid (DPL) family of ABC transporters (38). The DPL family of proteins is typically composed of highly conserved ABC transporters involved in the export of both post-translationally modified and unmodified bacteriocin peptides as well as competence peptides. This protein showed strong similarity (84% identity) to the ABC transporter found within the lactacin F bacteriocin system produced by *L. johnsonii* NCC 533 (68). Typical to many ABC transporters (82, 87), LBA1796 contains an N-terminal peptidase domain, a total of five transmembrane spanning domains, and a C-terminal ATPase domain characterized by Walker motifs (Figure 2). The N-terminal hydrophobic domain recognizes and translocates the bacteriocin across the membrane, while the C-terminal domain binds to and hydrolyzes ATP, providing the energy required for translocation. LBA1794 was located 13 bp downstream of LBA1796. This protein was 22 kDa in size and was predicted to contain one transmembrane domain and showed similarity (58% identity) to the accessory protein also described within the lactacin F system (68).

(iii) ***Unknown cluster***. LBA1793 encodes a 438 amino acid protein downstream of LBA1794 with a molecular weight of 49 kDa and appears to contain one transmembrane domain. Overlapping the 5'end of LBA1796 rests an intrinsic terminator (ΔG=-10.9 kcal/mol). This predicted terminator began approximately 14 amino acids upstream of LBA1793 and extended 6 amino acids into LBA1793. Interestingly, this terminator was preceded by the presence of two sets of imperfect inverted repeats; a 7 bp inverted repeat located 13 bp upstream of the terminator, and a 5 bp inverted repeat located 75 bp upstream of the intrinsic terminator. LBA1793 also contained a disulfide bridge, a feature common to bacteriocins of the pediocin family (171). Disulfide bridges are thought to stabilize the structure of the peptide (52). LBA1793 showed similarity (29% identity) to an immunity protein found within *Streptococcus thermophilus* LMG18311. Located immediately downstream of LBA1793, LBA1792 and LBA1791 each encoded small, cationic peptides (63 and 67 amino acids respectively), with a double glycine leader motif. Both putative genes possessed no similarity to any known protein in the NCBI database.

LabT (LBA1796) is required for lactacin B production. Due to the high level of conservation seen amongst ABC transporters of class II bacteriocins, the identification of bacteriocin regions based on the presence of ABC transporters is a common, reliable method used to identify possible bacteriocin regions (81, 167, 222). Therefore, to confirm the role of this region in lactacin B production, specifically, the gene predicted to encode the ATP-binding cassette (ABC) transporter protein was targeted for inactivation. Using a directed integration system (174), LBA1796 was insertionally inactivated via homologous recombination. A 745 bp internal fragment of the ABC transporter gene was PCR amplified using the 1796-IntF/1796-IntR primers listed in table 2 and cloned on to pORI28

plasmid. Plasmid pTRK852 was then introduced into NCK1392, using selective pressure, and integrants were recovered. Integration events were confirmed through Southern hybridization analyses (data not shown) and PCR (Figure 3). Left and right junction fragments were confirmed through PCR using the primers 1796-ExtF/1796-ExtR listed in Table 2.

Overnight cultures of wild-type NCFM (NCK56) and the integrant (NCK1758), harboring a disrupted LBA1796 gene, were then analyzed for lactacin B activity using *L. delbrueckii* ATCC 4797 as the indicator strain (Figure 4). Whereas clear bacteriocin activity was observed in *L. acidophilus* NCFM, lactacin B activity of the integrant (NCK1758) was completely abolished. These results demonstrate that LBA1796, designated *labT* (*la*ctacin *B t*ransporter) plays a vital role in the production of lactacin B by *L. acidophilus*.

Lactacin B transcriptional analyses. Genes required for bacteriocin production are typically organized in operon structures (146). In order to determine if the lactacin B region was transcribed as a single unit or as individual genes, reverse transcriptase-PCR was performed over the intergenic regions between all 12 genes in the predicted operon. PCR amplicons of the predicted sizes were amplified from RNA extracted from wild-type *L. acidophilus* NCFM cells (data not shown). Evidence for the expression of transcripts between LBA1803-LBA1791 is shown in Figure 5. Amplicons were observed over the two regions encoding weak hairpins (between LBA1798-LBA1797 and LBA1794-LBA1793). RT-PCR reactions amplifying intergenic regions between LBA1804-LBA1803 and LBA1791-LBA1790 did not yield amplicons. These regions flank the lactacin B region and contain strong predicted intrinsic terminators (ΔG=-13.7 kcal/mol

and ΔG=-17.0 kcal/mol respectively). Taken together, these results suggested that the genetic determinants believed to be responsible for lactacin B production are located on a single transcript that includes the 12 genes between LBA1803-LBA1791.

Overexpression lactacin B. In order to investigate the structural genes potentially responsible for lactacin B production, the first four genes within the lactacin B operon were cloned onto the high expression plasmid, pTRK696. The resulting plasmid, pTRK853 was transformed into *L. acidophilus* NCFM. When compared to the parental control, *L. acidophilus* NCFM containing the plasmid pTRK853 exhibited distinctly larger zones of inhibition, indicating an increase in lactacin B activity (Figure 6A). Bacteriocin assays were conducted on MRS agar supplemented with 5 μg/ml chloramphenicol. *L. helveticus* NCK253 harboring pGK12 (96) encoding a chloramphenicol resistance gene was used as an indicator.

Analysis of synthetic peptides, *LBA1800* and *LBA1801* In an effort to investigate the properties of the first four peptides encoded within the lactacin B operon, attempts were made to synthesize the mature amino acid sequences of LBA1803, LBA1802, LBA1801, and LBA1800. Peptides LBA1800 and LBA1801 were successfully synthesized, but repeated attempts to synthesize LBA1803 and LBA1804 were unsuccessful. Peptides LBA1800 and LBA1801 were spotted directly onto a lawn of the sensitive indicator strain, *L. delbrueckii* ATCC 4797. No inhibitory activity was observed for either peptide, alone or in combination, using the highest concentration tested, 1μg/μl (data not shown). Previous reports have demonstrated that lactacin B is not detectable in broth cultures of *L. acidophilus* NCFM and activity was only observed using solid media (14). To determine if *LBA1801'* or *LBA1800'* could induce lactacin B in broth cultures, 1μg/ml of each peptide

was added to 10 ml of MRS broth containing a 1% inoculum of *L. acidophilus* NCFM. Once an OD_{600} of 1.0 was reached, the supernatants were collected and analyzed for lactacin B activity. Cell-free supernatants from cultures propagaged with *LBA1800'* produced inhibitory zones on the lawn of *L. delbrueckii* ATCC 4797,demonstrating lactacin B production (Figure 6B). Concentrations of *LBA1800* as low as 10 ng/ml were sufficient to induce lactacin B production in broth cultures and the amount of the bacteriocin produced increased with increasing concentrations of inducing peptide (Figure 6C). Cell-free supernatants from MRS containing *LBA1801'* and spent MRS did not display any antagonistic activity towards the indicator (Figure 6B).

DISCUSSION

Previously, lactacin B was biochemically identified as a class II bacteriocin produced by *L. acidophilus* NCFM. Lactacin B was proteinaceous in nature and inhibited such closely-related species as *Lactobacillus bulgaricus*, *L. helveticus*, and *L. delbrueckii* (13) . Genetic analysis revealed a region on the *L. acidophilus* chromosome likely to be involved in bacteriocin production. To confirm the role of this region in lactacin B production, the gene predicted to encode the ABC transporter (LabT, LBA1796)was insertionally inactivated. The complete abrogation of bacteriocin activity in the integrant confirmed that this region comprised the genetic determinants responsible for lactacin B processing and export. The *lab (la*ctacin *B)* operon consisted of twelve putative open reading frames located on a 9.4 kb region of *L. acidophilus* NCFM chromosome. A search within the NCBI database revealed a total of five genes with similarities to proteins found within other class II bacteriocin systems (Table 3); the remaining seven putative genes share a double glycine leader motif typified by bacteriocin prepeptides and pheromone-like

peptides. The double glycine motif acts as a proteolytic site for the ABC transporter and accessory protein. Consequently, the remaining seven putative genes are likely to encode proteins that are exported from the cell via this ABC transporter complex. The organization of the lactacin B operon is characteristic to many bacteriocin systems. The production complex consisting of bacteriocin structural and immunity genes precede the regulation and export machinery which are both present in tandem. No additional genes likely to encode for post-translational modification proteins were found within the operon.

To begin to elucidate the functionality of the first four genes within the lactacin B operon, the cluster was cloned onto a high copy number plasmid. When introduced back into *L. acidophilus* NCFM, overexpression of lactacin B was observed, indicated by larger, wider zones of inhibition when compared to the control. The first four genes within the lactacin B operon (LBA1803, LBA1802, LBA1801, and LBA1800) encode small, cationic peptides, each with an N-terminal double glycine leader motif. Therefore, we suspect that this region encodes the lactacin B structural gene or genes alongside inducing peptides. Inducing peptides or pheromone-like peptides are often present within bacteriocin loci and function to induce bacteriocin expression as a response to cell density, stress, or other external factors (46). Pheromone-like peptides usually possess characteristics similar to that of the bacteriocin peptides, but in most cases they do not possess any antimicrobial activity. Synthetic peptides *LBA1801'* and *LBA1800*' failed to exhibit any antagonistic activity individually or in combination against the indicator, *L. delbrueckii* ATCC 4797. Only *LBA1800*' was able to induce lactacin B production in broth cultures, indicating that it functions as an inducing peptide (*labIP*) involved in the regulation of bacteriocin production. *LBA1801'*, however, was unable to induce lactacin B production or exert any

antimicrobial activity against the indicator. Taken together, these data suggest that LBA1801 may encode one structural component of a two-component bacteriocin system. In this system, bacteriocin activity requires the complementary action of two peptides. This is a scenario similar to the closely related organism, *L. johnsonii,* producing the two component bacteriocin, lactacin F, where alone, each structural peptide fails to exhibit antimicrobial activity (3).

LBA1800 encodes a 25 amino acid peptide located 7 bp upstream of LabK and LabR. LBA1800 encodes for a small, cationic peptide with a high isoelectric point, and a conserved N-terminal double glycine leader motif (Figure 6). The N-terminal conservation suggests that this peptide is exported from the cell via an ABC transporter complex similar to the bacteriocin peptide itself. LBA1800, LabK, and LabR are suggested to comprise the so-called three component regulatory system of class II bacteriocins. In this system, a pheromone-like peptide is thought to bind to the sensor domain of the histidine kinase triggering activation of the kinase domain. A series of phosphorylation reactions from the histidine kinase to the response regulator results in bacteriocin expression (123). The response regulator acts to initiate or repress transcription of all other bacteriocin determinants. This type of interaction is common to many bacteriocin systems such as carnobacteriocin system of *Carnobacteriocin piscicola* and the plantaricin system of *Lactobacillus plantarum* (49, 161). Further analysis is needed to elucidate the exact function of LBA1792 and LBA1791, putative genes also containing conventional bacteriocin characteristics with unknown functionality. Based on comparisons with other bacteriocin operons, these determinants may encode immunity features.

The current model states that the three-component regulatory system exerts control at the transcription level. The presence of direct/inverted repeats and intrinsic terminators within the *lab* operon also suggest that additional regulatory elements may also work to control lactacin B production, as well. Many transcription regulators use direct and/or inverted repeats to control their target operons, stimulating or inhibiting the binding of RNA polymerase (168, 170). Both the carnobacteriocin B2 system produced by *Carnobacterium piscicola* and plantaricin C11 system produced by *Lactobacillus plantarum* exhibit the presence of direct repeats that are known to function as binding sites for cognate response regulators (48, 161). The presence of direct repeats upstream of LBA1803 may function as binding sites for a transcriptional regulator possibly LabR. Further transcriptional analysis of the *lab* promoter region is needed to elucidate the function of these repeats and their potential effects upon lactacin B production (67, 161).

Reverse transcriptase PCR confirmed that the determinants required for lactacin B production are situated on a single transcription unit, despite the presence of two predicted intrinsic terminators (ΔG= -10.9 kcal/mol) located downstream of LabR and overlapping LBA1793. These terminators may function to aid in the regulation of expression of each gene cluster. Phosphorylation has the ability to change the conformation of response regulators, in some cases, promoting dimerization, oligomerization, or interactions with other proteins (189). Induction as a function of phosphorylation has been implicated in the β-glucoside and sucrose operons of *E. coli* and *Bacillus subtilis*, respectively (84). Phosphorylation or dephosphorylation in response to the presence of a substrate induces conformational changes in cognate transcriptional regulators resulting in either termination or readthrough of intrinsic terminators (85). The response regulator predicted within the

lactacin B operon may function to control readthrough of the intrinsic terminator located just downstream of LabR, as a consequence of phosphorylation by the cognate histidine kinase. Additional undetermined proteins may also bind to the inverted repeats upstream of LBA1793 also working to control transcription. Further analysis is required to examine the function of these terminators in the overall regulation of lactacin B production.

Though lactacin B exhibits a narrow spectrum of inhibition, its significance may lie in its regulation. Qi et al (159) recently found that the inactivation of a gene encoding a histidine protein kinase in *Streptococcus mutans* resulted in the reduction of competence development, bacteriocin production, acid tolerance, as well as biofilm formation. These data suggest that bacteriocin regulation may be a component of a larger global regulatory network involved in communal survival under various environmental conditions. Further analysis identifying the functionality of each putative gene within the *lab* operon, and any regulation over this region will provide important insights into the mechanisms by which *L. acidophilus NCFM* competes and communicates within various ecological niches such as the gastronintestinal tract.

ACKNOWLEDGEMENTS

This work was partially supported by Danisco USA, Inc. (Madison, WI), the Southeast Dairy Foods Research Center, and by Dairy Management, Inc. We are grateful to Dr. M. A. Azcarate-Peril for reviewing the manuscript and to L.B. Buck, Dr. M. Miller, and E. Durmaz for their technical assistance and advice.

TABLE 1. Bacterial strains and plasmids

Bacterial strain or Plasmid	Relevant characteristic(s)[a]	Source or Reference
Strains		
Escherichia coli		
EC1000	RepA+ MC1000; host for pORI28-based plasmids	(119)
MC1061	Transformation host, plasmid free	
Lactobacillus acidophilus		
NCFM	NCK56; Plasmid free, parental strain; Lab^{+}, Lab^{R}	(13)
NCK1392	NCFM containing pTRK669, Cm^{R}	(174)
NCK1758	NCFM integrant LBA1796::pTRK852; Lab^{-}, Lab^{R}	This study
NCFM1760	NCFM containing pTRK853; Lab^{+}, Lab^{R}	This study
L. johnsonii		
NCK468	lactacin F producer, Laf^{+}, Laf^{R}	(68)
NCK64	Laf^{-}, Laf^{R}; frameshift in *lafA*	(143, 144)
NCK65	Laf^{-}, Laf^{S}; deletion derivative	(143, 144)
L. delbrueckii NCK235	Lab^{S}; lactacin B indicator	ATCC[b] 4797
L. helveticus NCK 253	Lab^{S}, Cm^{R}; lactacin B indicator	(3)
Plasmids		
pORI28	Em^{r}, ori (pWV01), replicates only with *repA* provided in *trans*	(119)
pTRK852	745-bp internal region of LBA1796 cloned via BglII/Xba sites of pORI28	This study
pTRK696	3 kb; High copy number shuttle vector, replicates in *E. coli* and *Lactobacillus* species; Cm^{R}	(193)
pTRK853	3.78 kb; pTRK696 containing LBA1803-LBA1800; Lab^{+}, Lab^{R}, Cm^{R}	This study

[a]Lab+, lactacin B producer; Lab-, lactacin B negative; LabR, lactacin B resistant; LabS, lactacin B sensitive; Laf+, lactacin F producer; Laf-, lactacin F negative; LafR, lactacin F resistant; LafS, lactacin F sensitive; CmR, chloramphenicol resistant; EmR, erythromycin resistant.

[b] ATCC, American Type Culture Collection.

TABLE 2. Primer sets used in this study

Primer Name	Primer Sequence
Insertional inactivation	
1796-IntF[a]	GATCAGATCTTTCATCATGGCTTCCTTG
1796-IntR[a]	GATCTCTAGATGGTTAAGAGCGAGTTTC
1796-ExtF[b]	GCCATAGGAATGATAATCATAG
1796-ExtR[b]	TAATCTTCAGCCAACCTTAC
EmF[b]	AATCGTGGAATACGGGTTTGC
EmR[b]	GCCTTTTCCTGAGCCGATTTC
Over-expression	
aed-69F	GACTGAATTCATATGCAGGAATGGAAG
aed-70R	GACTGTCGACAAGCCACCATATTCATC
RT-PCR analysis	
1804F	GCTCTTAGCGTTATGGCAGCTGTTATG
1802R	TATCTCTTCGGCCACCAATAACCTGAC
1802F	ATGAGAGCCATTGGCAGTATTC
1799R	AGTAAGCCACCATATTCATC
1799F	TGGCTGGTTTGACTTCTATATGAC
1798R	TACGCCATTCTTAGCTTTAGACTC
1798F	AGATGGTAACTTATTCTAGAGCGTACA
1797R	TGATCTACCAATTTGACTAGCACAATG
1797F	ATCAAAGGCTCAGCAGCATGTG
1796R	GCATAGCTCTCCCTACCTTTACG
1796F	TTGAAAGCTTGCCAGATAG
1794R	CCAGTAGATGTAACTGTACTC
1794F	GGCAATGCACCAATTATTAC
1793R	TTTATCCGCATGCACAATAG
1793F	AAAGTACACGGTACATCTC
1792R	AGGTAGCATATGTTCAGTATC
1792F	AGTAAGAAAGGGAGCAATC
1791R	TAGTCCAATTACCGAGACTG
1791F	ATTATATGCAGCGGTGAGGG
1790R	CACTCCATGTTCCACCTAAG

[a] Primer sets used to amplify the internal region for gene inactivation.
[b] Primer sets used to amplify junction fragments.

TABLE 3. Similarities of proteins in lactacin B operon to proteins within the NCBI database

Putative Open Reading Frame	Size (aa)	Homology (accession number)	Proposed function
LBA1803	53	None	Unknown
LBA1802	63	None	Unknown
LBA1801	38	None	Unknown
LBA1800	47	None	Unknown
LBA1799 *(labK)*	440	Two-component system protein histidine kinase (NP_964617)	Regulation of lactacin B
LBA1798 *(labR)*	270	Two-component system response regulator (NP_964619)	Regulation of lactacin B
LBA1797	83	None	Unknown
LBA1796 (*labT)*	720	ABC transporter permease component (NP_964620)	lactacin B export
LBA1794 *(labE)*	196	Transporter auxillary protein (NP_964629)	lactacin B export
LBA1793	438	Immunity protein [*Streptococcus thermophilus* LMG18311]	Unknown
LBA1792	63	None	Unknown
LBA1791	67	None	Unknown

TABLE 4. Pre-peptides of *lab* operon containing N-terminal double glycine leader motif

ORF	Amino Acid Sequence		Pre-peptide			Mature peptide		
	⬇		Size (aa)	pI	MW (Da)	Size (aa)	pI	MW (Da)
LBA1803	MQEWKKTTLSDNELIDVIGG	SAKSYIRRLGPDGGYGGRESKLIAMADMIRRRI	53	9.69	5955	33	10.9	3695
LBA1802	MKLRQEQLNRKELSQVIGG	RRDMILVALPHAVGPDGMPGSGRGGGAQMRAIGSIP PWRPNWWK	63	11.62	6960	44	11.83	4751
LBA1801	LAVFLHGVQIGG	SRIKQDARSVRKYDRIGIFFYSFKSA	38	10.53	4332	26	10.53	3140
LBA1800	MKKKVVKKTVLKEKELTKVVGG	KKAPISGYVGRGLWENLSNIFKHHK	47	10.35	5333	25	10.17	2880
LBA1797	MEKLMVLNEEKLSYVIGG	GNPKVAHCASQIGRSTAWGAVSGAATGTAVGQAVG ALGGALFGGSMGVIKGSAACVSYLTRHRHH	83	9.51	8311	64	10.32	6218
LBA1792	MKQIIITENKVILSKILGG	SSSIDDIGLNDTEHMLPLYSKKGSNHKRDVYLENPRY QTHFKFM	63	9.05	7294	44	6.79	5214
LBA1791	MNKFKDLNELELSNIAGG	SNNIFWTRVGVGWAAEARCMIKPSLGNWTTKAVSC GAKGLYAAVRG	67	9.51	6875	49	10.06	4900

Arrow indicates proteolytic cleavage site

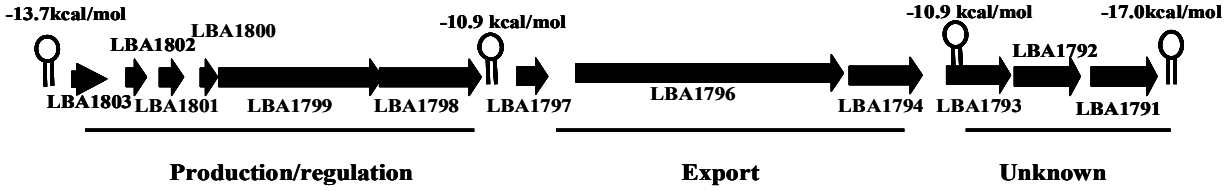

FIGURE 1. Gene organization of the lactacin B operon in *L. acidophilus* NCFM. Figure not drawn to scale. Putative intrinsic terminators ( ) and their calculated free energy are indicated.

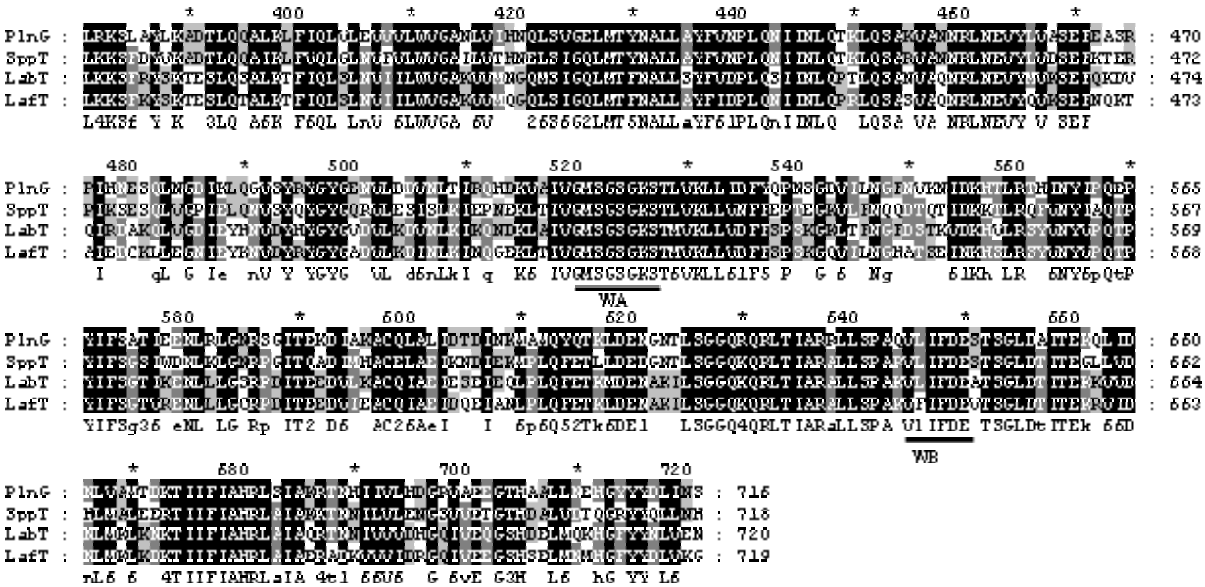

FIGURE 2. Alignment of the ABC transporter gene products of plantaricin (PlnG), sakacin (SppT), lactacin B (LabT), and lactacin F (LafT), (46, 93, 156). Residues that are conserved in all four sequences are shaded in black; residues that are conserved in at least two sequences are shaded in gray. WA, walker motif A; WB, walker motif B, are indicated.

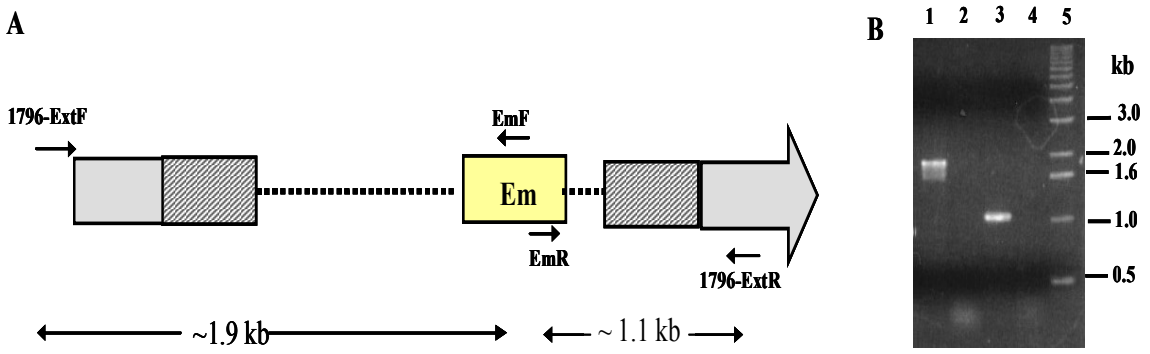

FIGURE 3. Organization of the insertion of pTRK852 into the predicted ABC transporter, LBA1796 in *L. acidophilus* NCK1758. (A) Diagram of LBA1796 in the NCK1758 chromosome. LBA1796 designated by a block arrow. The internal fragment targeted for homologous recombination is represented by a shaded box. Lengths of the left and right PCR generated junction amplicons are denoted. Em^R, erythromycin resistance determinant in pTRK852. Diagram is not drawn to scale. (B) Agarose gel resolution of the PCR amplicons at the left and right junctions of NCK1758. Lanes 1 and 3, NCK1758; lanes 2 and 4, NCFM; lane 5, 1-kb ladder.

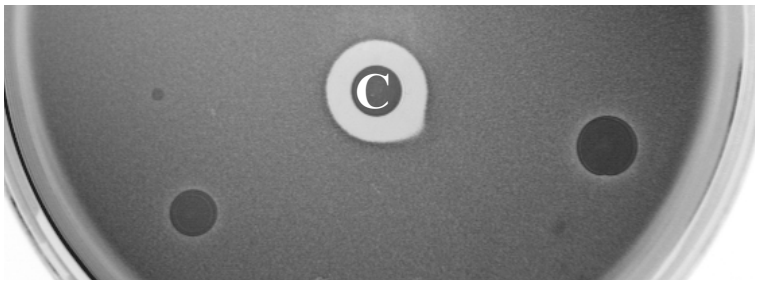

FIGURE 4. Agar well diffusion assay for bacteriocin production by NCFM (center well, C) and two isolates of NCK1758 (integrant LBA1796::pTRK852), surrounding wells. *L. delbrueckii* ATCC 4797 was used as the indicator for lactacin B production.

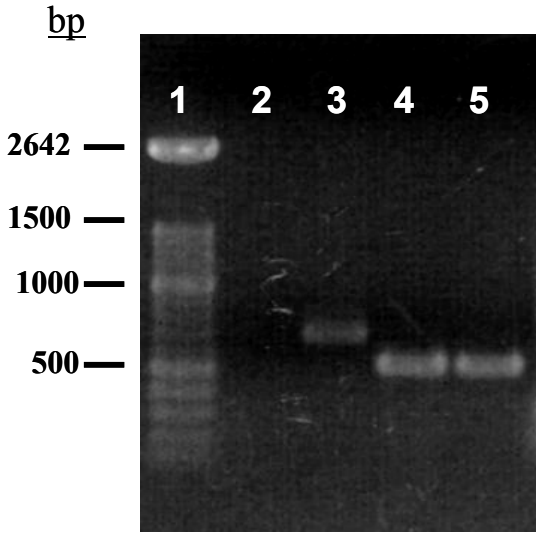

FIGURE 5. Representative RT-PCR analysis from *L. acidophilus* NCFM RNA over the intergenic regions of LBA1804 to LBA1802 and LBA1802 to LBA1799. Lane 1, Molecular weight marker, bp, base pair lengths are indicated; lanes 2 and 3, amplification using 1804F/1802R primers using *L. acidophilus* NCFM cDNA and genomic DNA, respectively; lanes 4 and 5, amplification using 1802F/1799R primers using *L. acidophilus* NCFM cDNA and genomic DNA, respectively. Subsequent RT-PCR reactions were performed for all intergenic regions in the same manner.

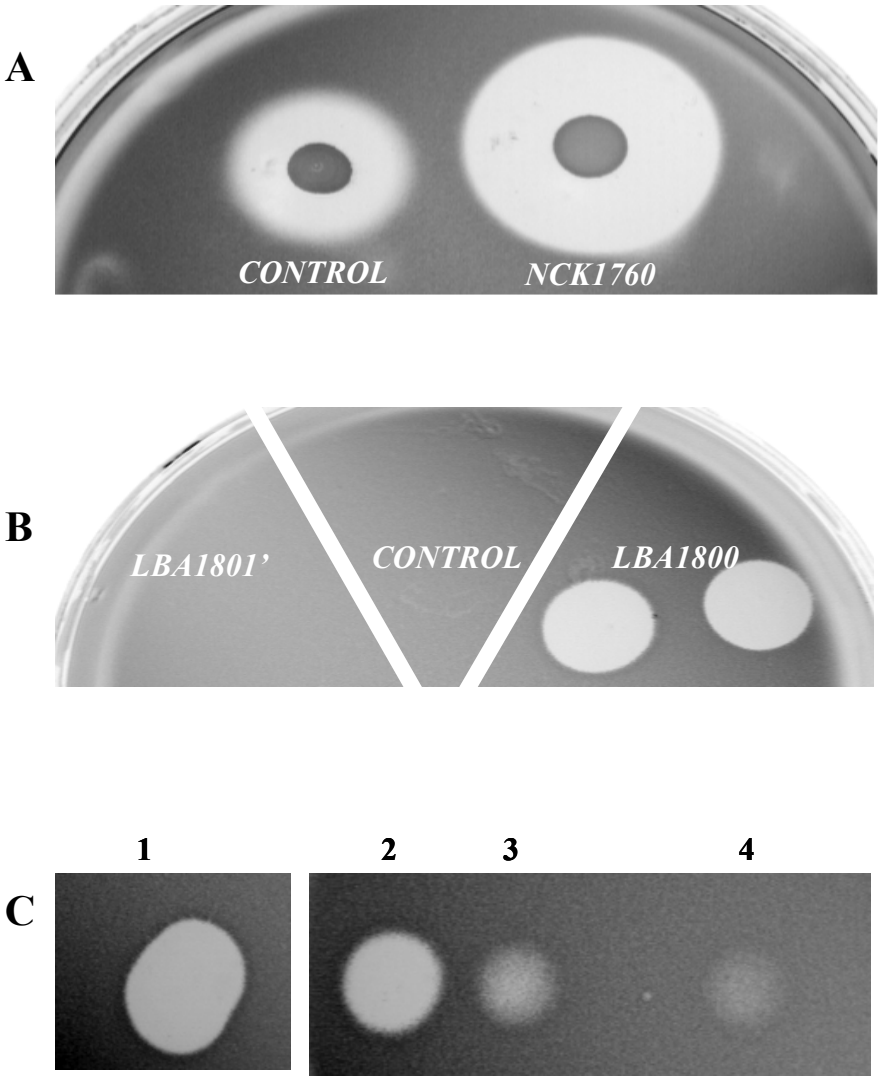

FIGURE 6. Bacteriocin assays for lactacin B production from cultures, in each case lactacin B was either overexpressed or induced. Panel A: Comparison of bacteriocin production by NCFM (pTRK696 base cloning vector) versus NCK1760 (pTRK852 encoding LBA1803, LBA1802, LBA1801, and LBA1800) from the lactacin B operon. *L. helveticus* NCK253 was used as an indicator. Panel B: Spot on lawn bacteriocin assays from cell free culture supernatants of *L. acidophilus* NCFM propagated with 1 μg/ml of *LBA1801'* , 1 μg/ml of *LBA1800*', or without any added synthetic peptides (control). *L. delbrueckii* ATCC4797 was used as an indicator. Panel C: Spot on lawn bacteriocin assays of cell free culture supernatants of *L. acidophilus* NCFM propagated with varying concentrations of *LBA1800'* . Lane 1, 1μg/ml; lane 2, 100ng/ml; lane 3, 20ng/ml; lane 4, 10ng/ml.

FIGURE 7. Alignment of inducing peptide gene products of LBA1800, sakacin 674 (Sap-Ph), ABP-113 (Abp-IP), and plantaricin A (PlnA) (48, 59, 65) Residues that are conserved in all four sequences are shaded in black; residues that are conserved in at least two sequences are shaded in gray.

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