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CHARACTERIZATION OF TWO BACTERIOCINS AND A FOOD GRADE PLASMID FROM CARNOBACTERIA

by

Randy W. Worobo

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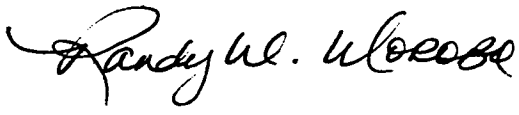

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Characterization of Two Bacteriocins and a Food Grade Plasmid From Carnobacteria** submitted by **Randy William Worobo** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Food Microbiology.**

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To Mom, Dad and Rod

ABSTRACT

Carnobacterium piscicola LV17A and *Carnobacterium divergens* LV13 are lactic acid bacteria that were isolated from vacuum packaged meat. *C. piscicola* LV17 was studied for its ability to produce plasmid-mediated bacteriocins, and *C. divergens* LV13 was used as an indicator strain. The plasmids for bacteriocin production were separated into different strains, and this study was done on *C. piscicola* LV17A, the producer organism for carnobacteriocin A. The structural gene responsible for carnobacteriocin A (*cbnA*) was identified on a 2-kb *Eco*RI fragment of pCP49, the plasmid containing the genetic information for carnobacteriocin A production. The *cbnA* gene encodes a 71 amino acid preprotein that is comprised of a 53 amino acid mature bacteriocin and an 18 amino acid N-terminal extension. Comparison of the molecular mass of carnobacteriocin A (5050.8 Da) determined by mass spectrum analysis to that predicted from the nucleotide sequence (5052.8 Da) indicated that the bacteriocin contains a disulfide bridge between the cysteine residues at positions 22 and 51. Downstream of *cbnA* there are 6 additional genes (*cbaI*, *cbaX*, *cbaK*, *cbaR*, *cbaT*, *cbaC*) that, based on homology studies, encode proteins similar to bacterial two-component regulatory systems and ATP binding cassette transporters. *C. divergens* contains a small (3470 bp) plasmid that encodes a narrow spectrum bacteriocin, divergicin A. The bacteriocin gene (*dvnA*) was identified and contains a classical signal peptide that is responsible for secreting the 46 amino acid divergicin A. A 514-bp *Eco*RV-*Acc*I fragment containing *dvnA* and *dviA* was cloned behind a P59 promotor and was shown to be capable of producing divergicin A in heterologous hosts, that including *Carnobacterium* spp., *Lactococcus lactis* and *Escherichia coli*. Divergicin A signal peptide was fused in front of the mature portion of alkaline phosphatase and produced active and mature alkaline phosphatase in *E. coli*.

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1. INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 GENERAL INTRODUCTION

Lactic acid bacteria (LAB) are a diverse group of Gram-positive, catalase positive, coccus- and rod-shaped bacteria that have in common the functional characteristic of converting carbohydrates into lactic acid as a major metabolic end product. This is achieved by homo- or heterofermentative pathways. They have a <50 mol% G+C content and belong to the so-called *Clostridium* branch of the Gram-positive bacteria. This has resulted in the genus *Bifidobacterium* no longer being classified with the LAB, because they have >50 mol% G+C and belong to the Actinomycetes branch (Schleifer and Ludwig, 1995). The generic organization of the lactic acid bacteria has undergone considerable change in recent years (Pot *et al.*, 1994). The LAB comprise a heterogeneous group of pathogenic and nonpathogenic bacteria that include the following genera: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus* and *Vagococcus*. The production of lactic acid by LAB has been associated with an inhibitory effect on other microorganisms and, because of this attribute, many LAB are used in the preservation of dairy, meat and vegetable products, beverages and animal feeds. Fresh meat and poultry provide nutritionally rich media, with high water activity (a_w 0.96-0.97) and acidic to close to neutral pH (pH 5.0 - 6.5) that under aerobic conditions are an ideal environment for the growth of spoilage bacteria, referred to as the Gram-negative, nonspore-forming *Pseudomonas*-like organisms. These bacteria cause spoilage due to off-odours, discoloration and slime development on meat. Under anaerobic conditions of vacuum or modified atmosphere packaging with elevated levels of carbon dioxide, the aerobic spoilage bacteria are inhibited and they are outgrown by LAB. The LAB microflora

Table 1.1. A summary of Class I bacteriocins produced by Gram-positive bacteria.

Nisin A	Buchman *et al.*, 1988.
Nisin Z	Mulders *et al.*, 1991.
Subtilin	Kaletta *et al.*, 1991.
Pep5	Kellner *et al.*, 1988a.
Epidermin/Staphylococcin 1580	Allgaier *et al.*, 1985; Sahl, 1994.
Gallidermin	Kellner *et al.*, 1988b.
Salivaricin A	Ross *et al.*, 1993.
SA-FF22	Tagg and Wannamaker, 1976.
Lacticin 481	Piard *et al.*, 1993.
Carnocin UI49	Stoffels *et al.*, 1993.

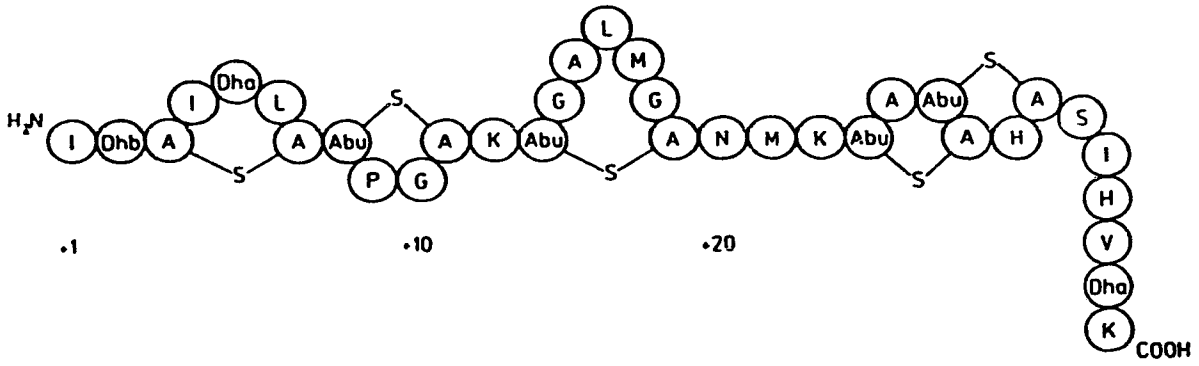

Figure 1.1. Diagrammatic representation of the nisin A molecule.

generally contributes to a markedly extended storage life of chill-stored fresh and processed meats (Enfors *et al.*, 1979).

Traditionally, preservation of meat relied on drying, curing, salting, fermentation, cooling and sterilization. Vacuum and modified atmosphere packaging of meats in conjunction with refrigeration extends their storage life because the undesireable end products of fermentation by adventitious and dominant LAB are only detected at some time after maximum population has been achieved (McMullen and Stiles, 1994). This occurs because the meat contains only a small amount of carbohydrate. The adventitious LAB microflora also varies. This makes it difficult to predict with appropriate accuracy the storage life of the meat. Some strains of *Lactobacillus sake* cause overt spoilage of the meat within three weeks of storage because of the production of sulfur compounds (Shay and Egan, 1981). The LAB associated with vacuum packaged meats include typical and atypical lactobacilli, and leuconostocs (Shaw and Harding, 1984). The lactobacilli include *Lactobacillus sake* and *Lactobacillus curvatus*. The "atypical" lactobacilli were subsequently classified in a new genus, *Carnobacterium* (Collins *et al.*, 1987), including *Carnobacterium divergens*, *Carnobacterium piscicola*, *Carnobacterium gallinarum* and *Carnobacterium mobile*. Subsequently, two additional species of *Carnobacterium* have been described, *C. funditum* and *C. alterfunditum* (Franzmann *et al.*, 1991). These species were isolated from anoxic waters in Antarctica, indicating that the genus *Carnobacterium* might be quite widely distributed in nature.

The carnobacteria share many morphological and physiological features with the lactobacilli, but they can readily be differentiated from the lactobacilli by their inability to grow on acetate agar, lack of gas production from glucose, the production of L(+) lactate isomer and oleic acid as its major $C_{18:1}$ fatty acid compared with *cis*-vaccenic acid which is common in other lactobacilli (Shaw and Harding, 1985). The LAB produce several inhibitory substances that include: organic acids, carbon dioxide, diacetyl,

hydrogen peroxide and bacteriocins. These inhibitory substances may contribute to the dominance of LAB in mixed fermentations. Bacteriocins of LAB were described by Klaenhammer (1988) as "proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium."

Bacteriocins provide the potential for an alternative to undesirable chemical additives as preservatives for foods, such as nitrates and nitrites, propionic and sorbic acids, and sulfur dioxide. Nisin is one of the bacteriocins produced by *Lactococcus lactis*. It has been used as a preservative agent in a variety of foods in more than 45 countries for over 20 years (Delves-Broughton, 1990). However, nisin has not proven useful for application in fresh or processed meats because of its physical properties such as low solubility at slightly acid to neutral pH and it is inactivated probably as a result of its strong affinity for phospholipids (Henning *et al.*, 1986; ICMSF, 1980). For these reasons, alternative bacteriocins that are more suitable for use in meats and that have a spectrum of antibacterial activity equivalent to or better than nisin are being studied. Possible alternatives to nisin include pediocin PA-1 produced by *Pediococcus acidilactici* that is used as a starter organism in fermented sausage production (Bhunia *et al.*, 1988) and plantaricin A that is produced by *Lactobacillus plantarum* isolated from olive fermentations (Ruiz-Barba *et al.*, 1994). Because of their relatively narrow activity spectra, single bacteriocins may have limited value for food preservation. A possible solution is to use multiple bacteriocins to achieve a synergistic or multiple hurdle effect. Adding more than one bacteriocin to meat or producing multiple bacteriocins in meat may not only provide a broader spectrum of activity but it may also decrease the chance of developing resistance to individual bacteriocins. Production of multiple bacteriocins by single producer strains requires extensive knowledge of their regulation, export and processing and their mode(s) of action.

1.2 OBJECTIVES

The principal focus of this study was to characterize the bacteriocin(s) and genes involved in the inhibitory activity of *Carnobacterium piscicola* LV17A and to investigate the possibility of developing a food grade vector for production of multiple bacteriocins. The review of the literature that follows (section 1.3) was prepared with emphasis on the class I and class II bacteriocins produced by the lactic acid bacteria. The study was divided into four main areas of research as outlined below:

1. The initial research study (Chapter 2) describes the characterization of carnobacteriocin A produced by *C. piscicola* LV17A, in particular, the structural gene responsible for carnobacteriocin A production.

2. In the second stage of the research (Chapter 3) the genes involved in immunity, secretion and regulation of carnobacteriocin A are described.

3. With the goal to develop a food grade cloning vector for multiple bacteriocin production, the sequence and analysis of a small, high-copy number plasmid from the meat isolate *C. divergens* LV13 was studied (Chapter 4). This part of the work also focused on the characterization of a bacteriocin that utilizes the general protein export (*sec*-) pathway for its secretion rather than the dedicated secretion systems associated with other class I and class II bacteriocins to date. The potential for use of this plasmid as a food grade vector and the bacteriocin genes for production of multiple bacteriocins will be discussed.

1.3 REVIEW OF THE LITERATURE

1.3.1 Bacteriocins of lactic acid bacteria

Although the first LAB bacteriocin, nisin A, was identified over 70 years ago, information about nisin and its complex regulation, immunity and secretion genes is incomplete and its genetic control is still being studied. From the time of its discovery the potential of nisin for its application in various foods was recognized and its use as a preservative in foods is permitted in over 45 countries around the world (Delves-Broughton, 1990). The applications for nisin range from its use as a sporicidal agent in processed cheeses, to use as an antilisterial agent in raw eggs and as a preservative in beer and wine. It has been this broad range of applications and nisin's limited applicability in certain foods such as meats has stimulated interest in other bacteriocins produced by LAB. Presently, the information about other bacteriocins is overwhelming and in some cases the level of information about bacteriocins and their complex secretory and regulatory genes is approaching that of nisin.

Based on the physiological and genetic information on the bacteriocins, Klaenhammer (1993) grouped them into 4 main classes:

Class I lantibiotic bacteriocins

Class II small, heat stable, nonlantibiotic bacteriocins

Class III heat labile bacteriocins

Class IV complex bacteriocins with carbohydrate or lipid moieties associated with the active bacteriocin.

Interest in alternatives to existing food additives has dictated the focus of research on bacteriocins. The class III and IV bacteriocins have limited potential for application in food systems because of their physical properties, especially their lack of heat stability and the size of the molecules. For this reason, interest in bacteriocins has focused on the Class I and II bacteriocins. The class I bacteriocins are referred to as lantibiotics, a name derived from the unusual amino acids (lanthionine and β–methyllanthionine) that are characteristic of these bacteriocins. Thioether ring structures are produced by posttranslational modifications of the amino acids serine, threonine and cysteine. The best described bacteriocins of the class I bacteriocins are epidermidin produced by *Staphylococcus epidermidis* and nisins A and Z produced by strains of *Lactococcus lactis* subsp. *lactis*. The lantibiotic bacteriocins are listed in Table 1.1. Nisin A is a small hydrophobic peptide (3488 Da) consisting of 34 amino acids, containing five thioether ring structures and three dehydrated amino acid residues of dehydroalanine and dehydrobutyrine (Figure 1.1).

Because nisin is produced by a LAB that is important in food technology, this review will focus on nisin as a representative of the class I bacteriocins. Interest in nisin was stimulated by its antimicrobial activity. Nisin is not only active against closely related LAB but it is also active against a broad range of Gram-positive bacteria that include pathogens such as *Enterococcus faecalis*, *Listeria monocytogenes* and *Staphylococcus aureus*. Nisin also inhibits the outgrowth of spores in *Bacillus* and *Clostridium* spp. For this reason, applications for nisin have been primarily for heat processed foods where the residual microflora are most often sporeforming bacteria. The mechanism of action of nisin is known for both vegetative cells and bacterial endospores. The bactericidal activity of nisin occurs through its incorporation into the cytoplasmic membrane and the formation of pores. These pores lead to depletion of the pH gradient and subsequent loss of the proton motive force. These pores are hypothesized to be formed as a result of the hydrophobic nature of nisin that gives it an affinity for the cell

membrane or they are attracted to a target receptor site on the surface of the cell. Nisin molecules may form internal cross bridges and combine to form a rigid, thermostable pore in the membrane of vegetative cells. The activity of nisin against bacterial spores is believed to be associated with the unusual dehydrated amino acid residues found in the nisin molecule. These unusual amino acids react with free sulfhydryl groups (Lui and Hansen, 1990). In spores, the inactivation of the sulfhydryl groups prevents the outgrowth of the spore.

The production of mature nisin requires a cascade of coordinated events that are controlled by several genes. Nisin A is encoded by the *nisA* structural gene. It encodes prepronisin consisting of 57 amino acids, which includes a N-terminal leader sequence of 23 amino acids that is cleaved at a Pro-Arg- site. The propeptide then undergoes posttranslational modifications to form dehydro residues and thioether bridges. The genes required to produce and secrete nisin, as well as to protect the producer from the active peptide, are located on a 15-kb DNA fragment of the nisin-sucrose transposon Tn5276 (Figure 1.2). Numerous genes have been identified and their hypothesized or proven functions have been described (Buchman *et al.*, 1988; Dodd *et al.*, 1990; Kaletta and Entian, 1989).

The nisin producer organism must have a protective mechanism against nisin to prevent suicidal production. This is done in part by the "immunity" protein of the *nisI* gene. The *nisI* gene is under common regulation with the *nisA* structural gene (Kuipers *et al.*, 1993). The immunity protein is believed to contain a lipoprotein signal sequence in the N-terminus, a hydrophobic core and a polar region after the consensus cleavage site. *NisI* alone produces only 1 to 4% of the wild type immunity (Kuipers *et al.*, 1993).

The *nisR* gene has been shown to be involved in the regulation of the nisin gene cluster. NisR has homology to other two-component regulation systems such as ComA from *Streptococcus pneumoniae* (Hui and Morrison, 1991), SpaR of subtilin (Klein and

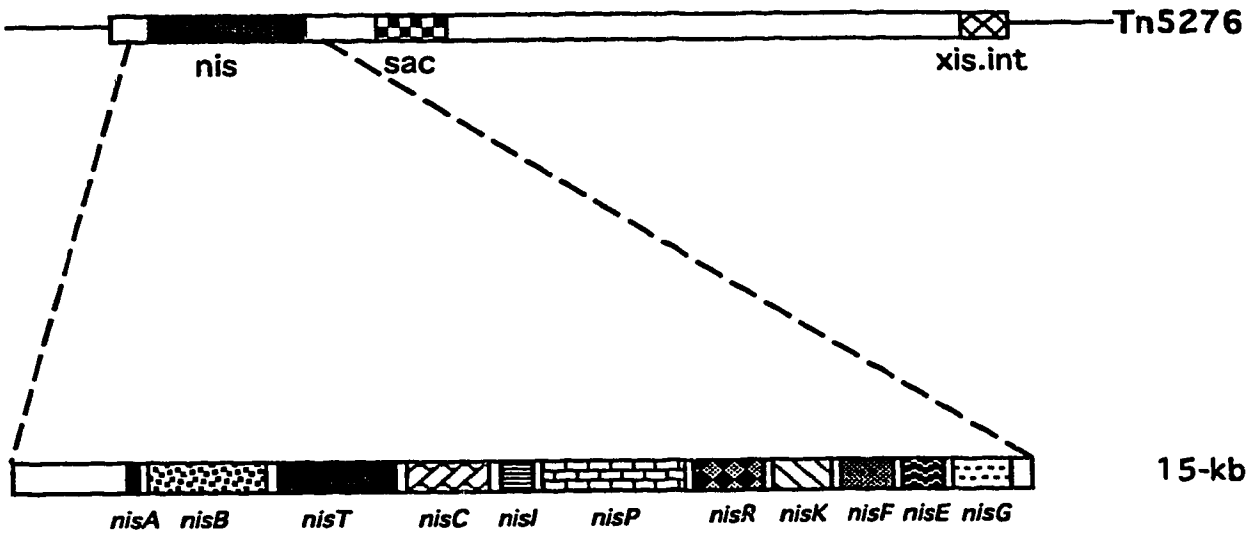

Figure 1.2. Schematic diagram of the 70-kb Tn5276 containing the nisin operon. nis is the nisin polycistronic operon that is comprised of *nisA* the structural gene for nisin; *nisB*, *T*, *C* and *P* are involved in the processing and secretion of nisin; *nisI*, *F*, *E* and *G* are involved in the immunity and regulation of nisin immunity; *nisR* and *K* are response regualtor and histidine protein kinase, respectively. sac is the sucrose metabolic operon.

Entian, 1983), PhoP from *Bacillus subtilis* (Seki *et al.*, 1987) and OmpR from *Escherichia coli* (Comeau *et al.*, 1985). This two-component regulatory system provides tight control of the function of certain genes. The presence of *nisR* is required for production of nisin and it also regulates nisin immunity. This regulation system is dependent on a response regulator and a histidine protein kinase (NisK). There is a sensor component that is phosphorylated by the histidine protein kinase. Phosphorylation and dephosphorylation of the response regulator controls the biosynthesis of the nisin molecule and the other proteins, such as the immunity protein, that are associated with nisin. The sensor component for NisR has not been identified but it may be phosphorylated by a chromosomally encoded sensor protein kinase or by the protein kinase of the sucrose phosphotransferase uptake system (van der Meer *et al.*, 1993). Inactivation of either *nisR* or *nisK* of these genes results in a dramatic decrease in production of nisin and immunity to nisin.

Additional gene products such as NisB, NisT, NisC, NisE, NisF and NisG are required for the maturation and secretion of mature nisin as well as regulation of nisin immunity. The *nisP* gene product is a putative protease that is responsible for cleavage of the 23 amino acid N-terminal extension of prepronisin. NisP has homology with the subtilases, a family of subtilisin-like serine proteases. NisP protease has an autocatalytic site at residues 192 to 195 (van der Meer *et al.*, 1993). This feature of autocatalysis implies that the protein is synthesized as a precursor protein and subsequently exported across the cell envelop. The NisP protein contains a putative C-terminal anchor that is believed to allow the protein to remain intact on the exterior of the cell surface and cleave off the exiting prenisin molecules. After export, maturation of the NisP is achieved by autocatalytic processing. Alternatively, the NisP may utilize a helper protein that cleaves and activates NisP to yield an active protease (van der Meer *et al.*, 1993).

Recently, Siegers and Entian (1995) identified three additional genes contained in the nisin gene cluster. These three genes were identified as *nisE*, *nisF* and *nisG*. Homology studies of proteins NisE and NisF show high homologies to ATP-binding cassette (ABC) transporter proteins, whereas NisG encodes a hydrophobic protein that may act in a similar manner to the immunity proteins described for several colicins (Garrido *et al.*, 1988). When the respective genes (*nisF*, *E* and *G*) were inactivated, all strains were capable of producing nisin. However, all three mutants were more sensitive to nisin than the wild type strain (Siegers and Entian, 1995). These results suggest the involvement of NisI, E, F and G in nisin immunity. Similar collective groups of genes for immunity have been identified for *mcbF/mcbE* of microcin B17 (Garrido *et al.*, 1988) as well as the *spaF/spaG* ABC transporter of subtilin immunity (Klein and Entian, 1994).

Nisin has been used extensively in certain foods such as heat processed cheeses, yogurts, cheeses and canned vegetables. Most of the applications have been in mildly acidic foods because nisin is poorly soluble and inactivated at pH >5.0. For these reasons, nisin application to other foods such as low acid canned foods and fresh meats has little utility. Investigation of alternative bacteriocins for use in other foods with a greater physical adaptability has prompted the study of Class II bacteriocins. These bacteriocins are small, heat stable, nonlanthionine containing, membrane-active peptides. Class II bacteriocins are in general similar to nisin but they do not undergo the extensive posttranslational modification. Another notable difference is the amino acid sequence at the cleavage site. For nisin, the cleavage occurs after a -2 Pro -1 Arg sequence, whereas in class II bacteriocins cleavage occurs after a -2 Gly -1 Gly motif. The class II bacteriocins produced by LAB that have been reported to date are listed in Table 1.2. Unlike nisin, the spectrum of activity of class two bacteriocins is more limited. Their spectrum includes similar organisms such as closely related LAB as well as the Gram-positive pathogens *Listeria*, *Enterococcus* and some *Clostridium* spp. The classification by Klaenhammer (1993) of class II bacteriocins is actually based on their mode of action

Table 1.2. Subgroups of the Class II bacteriocins from lactic acid bacteria.

Class IIa	
Pediocin PA-1	Marugg *et al.*, 1992.
Pediocin AcH	Bhunia *et al.*, 1991.
Sakacin A/Curvacin A	Holck *et al.*, 1992; Tichaczek *et al.*, 1992.
Sakacin P	Tichaczek *et al.*, 1992.
Leucocin A	Hastings *et al.*, 1991.
Leucocin B-Ta11a	Felix *et al.*, 1994.
Mesentericin Y105	Maftah *et al.*, 1993.
Carnobacteriocin B2/BM1	Quadri *et al.*, 1994.
Sakacin 674	Holck *et al.*, 1994a.
Acidocin A	Kanatani *et al.*, 1995.
Bavaricin A	Larsen and Norrung, 1993.
Divercin V41	Pilet *et al.*, 1995.
Piscicocin V1	Pilet *et al.*, 1995.
Class IIb	
Lactococcin G	Nissen-Meyer *et al.*, 1992.
Lactococcin M	van Belkum *et al.*, 1991; Stoddard *et al.*, 1992
Lactacin F	Allison *et al.*, 1994.
Plantaricin A	Nissen-Meyer *et al.*, 1993.
Class IIc	
Lactococcin B	Venema *et al.*, 1993.
Class IId	
Carnobacteriocin A/Piscicolin LV61	Worobo *et al.*, 1994; Holck *et al.*, 1994b.
Lactococcin A	van Belkum *et al.*, 1991.
Colicin V	Gilson *et al.*, 1990.

and (or) spectrum of activity. The subgroup IIa are antilisterial peptides that seem to share a common consensus sequence of Tyr-Gly-Asn-Gly-Val-Xaa-Cys (YGNGVXC) and that is usually contained in the N-terminal region of the mature peptide. Subgroup IIb bacteriocins share the unique feature of requiring two different peptides for activity. Subgroup IIc bacteriocins are thiol activated peptides that require a reduced cysteine for their activity.

Initial categorization and grouping of bacteriocins was based on physical properties. With preliminary genetic information, they were further classified according to both physical and genetic characteristics. Additional information regarding their immunity, regulation, maturation and secretion is progressing to the level of knowledge about nisin. Thus far it is apparent that there are two types of operons/cistrons involved in yielding the different mature bacteriocins. The first group has genes similar to the polycistronic operon for nisin. It has a two-component regulation system and an ATP binding cassette (ABC) translocator and an accessory protein. The class II bacteriocins identified to date that contain these regulation and translocator genes include sakacin A, plantaricin A, carnobacteriocin B2 and lactococcins A, B and M. Immediately following the structural gene, this group of bacteriocins has the common feature of a confirmed or putative immunity gene that encodes immunity proteins consisting of 69 to 154 amino acids. There is no homology among the immunity proteins at either the amino acid or the nucleotide sequence level. The only common feature between the immunity proteins is the fact that they are lipoproteins. The regulatory genes of these bacteriocins show marked homology to each other, as well as to the family of transcriptional regulatory proteins of two-component regulatory systems (Allbright *et al.*, 1989; Stock *et al.*, 1989). Normally, the two component regulatory system contains a response regulator and a histidine protein kinase. This is the case for nisR and nisK in nisin (Engelke *et al.*, 1994), subtilin (spaR and spaK), sakacin A (sapR and sapK), sakacin P (sppR and sppK),

plantaricin A (plnB and plnD/C) and carnobacteriocin B2 (cbnR and cbnK). This group of regulated bacteriocins is similar to other well established two-component regulatory systems, such as the agr (accessory gene regulator) -regulatory system in *Staphylococcus aureus* that controls the expression of extracellular and cell wall associated proteins (Vandenesch *et al.*, 1991). This system relies on the sensor to regulate transcription of the bacteriocin operon by phosphorylating the response regulator according to the external environment of the cell i.e. its nutrient status. The sensor components for some of the two-component regulatory systems have yet to be identified. For bacteriocins, preliminary evidence indicates that the mature bacteriocin induces phosphorylation of the response regulator (Saucier *et al.*, 1995). The second set of genes encode the translocator and accessory proteins. This set of genes is common to all of the bacteriocin secretion systems that have been identified to date, including: Mesentericin Y105 (Héchard *et al.*, 1992); Carnobacteriocin BM1 and B2 (Quadri *et al.*, 1996); Pediocin PA-1 (Marugg *et al.*, 1992); Leucocin A (van Belkum *et al.*, 1995); Sakacin A (Holck *et al.*, 1992); Lactococcins A, B and M (Stoddard *et al.*, 1992); and Colicin V (Gilson *et al.*, 1990).

The second set of class II bacteriocins are those that only contain the translocator and accessory genes. This group includes leucocin A, mesentericin Y105, Pediocin PA-1 and the lactococcins. For pediocin and the lactococcins, the production of the mature bacteriocin requires only four genes, the structural, immunity, translocator and accessory protein. Lactococcin A is the same but there is a different arrangement of the genes. Leucocin A and mesentericin Y105 are similar bacteriocins differing by only two amino acids (Hastings *et al.*, 1991; Héchard *et al.*, 1992). There are five genes associated with production of these bacteriocins and exceptional homology exists between all of the genes (Fremaux *et al.*, 1995; van Belkum and Stiles, 1995). Leucocin A has been produced in heterologous hosts with only these five genes cloned into the host cell (van Belkum and Stiles, 1995).

The translocators of all of the bacteriocins are highly homologous not only in size but in sequence and conservation of specific amino acid residues. The translocator genes have homology to genes encoding proteins of signal sequence independent, bacterial translocation systems. The translocator has an ATP binding domain near the C-terminus that is located approximately 200 amino acids on the cytoplasmic side of the membrane. The translocator also contains an N-terminal hydrophobic membrane domain, that generally consists of six membrane spanning regions, and resembles ABC transporter proteins. All of the ABC transporter genes encode proteins of approximately 700 amino acids. To date, most of the ABC export proteins for bacteriocins contain two conserved amino acids (cysteine and histidine) at residues (approximately) 9 and 86. These conserved amino acids have been shown for lactococcin G, pediocin PA-1/AcH and carnobacteriocin B2. These conserved residues are believed to be the active site for the peptidase function of the ABC transporter protein located at the N-terminus of the translocator. For lactococcin G and pediocin PA-1/AcH it has been shown that the N-terminus of the translocator is responsible for, and independently capable of, cleavage of the prebacteriocin peptide at the Gly-Gly recognition site (Håvarstein *et al.*, 1995). The ABC translocator is also responsible for the export of the bacteriocin across the cell membrane (Håvarstein *et al.*, 1995). This peptidase function in the N-terminus appears to be specific for proteins that contain a Gly-Gly cleavage/processing site. The peptidase appears to be fused directly in front of the transporter gene. This is common for many protein exporter systems with a size that is similar to the ABC translocator, excluding the N-terminus peptidase region (approximately 550 amino acids). It is postulated that both the ATP binding and the proteolytic domains are located inside the bacterial cell. Because most of the functional ABC transporters contain two domains of each, it is believed that the ABC transporters exist as homodimers in the cell.

Integral to both the cleavage and the export of the protein is the Gly residue at the -2 position in the N-terminal extension. Previous studies with colicin V (Gilson *et al.*,

1990) and lactacin F (Fremaux *et al.*, 1993) showed that if the Gly- at the -1 position was changed to Val- and at the -2 position Arg-, or Ser- there was no detectable bacteriocin outside of the cell. Håvarstein *et al.* (1995) postulated a conformational change after cleavage that is necessary for the export across the cell membrane. A similar translocator system exists for *Streptococcus pneumoniae* and the secretion of a competence factor. This small basic extracellular peptide coordinates competence induction among cells in *S. pneumoniae*. The presence of a double glycine processing site has not yet been established. Closely associated with the ABC transport protein is an accessory protein. The accessory protein is approximately the same size for all bacteriocin transport systems, i.e. 475 amino acids. It has a common feature of being largely a hydrophilic protein with a potential membrane spanning region in the N-terminus of the protein. The exact role of this hydrophilic protein is not fully established but it is integral to the transport of the protein/peptide with the ABC transporter protein. Inactivation of the accessory gene results in complete loss of bacteriocin production.

Recently, a new group of bacteriocins has been identified. They have similar physical characteristics to the class II bacteriocins but they differ in their unique processing site and N-terminal extension. These bacteriocins have an Ala-Xaa-Ala sequence at the processing site. The N-terminal extensions of class II bacteriocins have a highly homologous amino acid sequence and the typical length of the N-terminal extension is 18 to 21 amino acids. This differs from most secretion signals because they are typically longer (28 to 31 amino acids) for Gram-positive bacteria and they have a markedly different amino acid composition in the separate regions of the signal sequence. The Ala-Xaa-Ala cleavage site is a typical processing sequence for signal peptides that utilize the *sec*-dependent pathway for the export from the cell. Numerous proteins utilize the *sec*- or general export pathway, these include: proteases, fimbriae, phosphatases, pili and general maintenance proteins for the cell. The peptide antibiotic AS-48 produced by *Enterococcus faecalis* S-48 is a ring structure comprised of 70 amino acids that is

produced as a linear peptide and then the N- and the C- termini are connected by posttranslational modifications (Martinez-Bueno *et al.*, 1994). The second bacteriocin of this type is acidocin B, that has a typical signal peptide cleavage site as well but it is not posttranslationally modified (van der Vossen *et al.*, 1995). It has not been demonstrated that either of these bacteriocins can be produced with only their structural and immunity genes, while utilizing the chromosomal *sec*-pathway for secretion of the bacteriocins. In this thesis (chapter 4) the production of divergicin using only the structural and immunity genes in a heterologous hosts has been demonstrated (Worobo *et al.*, 1995). Conclusive evidence that divergicin or the other compounds are exported by accessing the *sec*-pathway will require the results of pulse chase experiments.

The *sec*- or general secretory pathway that is proposed for the export of these unique bacteriocins relies on six Sec proteins (Pugsley, 1993). SecB is found in the cytosol and it is believed to be a tetramer or part of a protein complex that binds to presecretory proteins. The SecB protein acts as a chaperone for preproteins in the cytosol and takes them to the SecA protein. The SecA protein is also a cytosolic protein but it is associated with the cell membrane. SecA binds with and hydrolyzes the ATP which is necessary for protein translocation. The four remaining proteins SecY, SecD, SecE and SecF all contain membrane spanning regions and they are believed to span the cytoplasmic membrane. SecD and SecF have similar amino acid sequences and they are postulated as having related functions. SecY and SecE proteins form a complex of two integral cytoplasmic membrane proteins that are involved in transfer of preproteins across the cytoplasmic membrane. The function of the SecD and SecF proteins is not clearly understood but their role in the export of proteins across the membrane is integral. It has been proposed that their function may be in clearing the translocation channel and in protein folding.

The application of these *sec*-dependent bacteriocins provides an alternative means for the use of bacteriocins as biopreservatives in meats and other foods. The class II bacteriocins have limited use because of their relatively narrow spectrum of activity and the class I bacteriocins have limitations because of their insolubility and inactivation at neutral pH. An innovative solution to this problem would be the incorporation of the genes of several class II bacteriocins into one organism to provide a broad range of activity and suitable physical characteristics for their application to food systems. The problem with this alternative is the amount of DNA that is required for the production of each bacteriocin. Complementation of heterologous bacteriocins with regulation and maturation genes is not common or in most cases it is not possible. The discovery of bacteriocins that are capable of utilizing an existing secretion pathway, the general protein export pathway, may provide a means for multiple bacteriocin production. By fusing the signal sequence of divergicin in front of several different structural genes for mature bacteriocins, along with their respective immunity genes it may be possible to condense the information required for bacteriocin production from 10-kb to 0.5-kb. The construction of "multiple bacteriocin cassettes" will facilitate the direct application lactic acid bacteria as starter cultures with a broad spectrum of inhibitory activity.

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2. CHARACTERISTICS AND GENETIC DETERMINANT OF A HYDROPHOBIC PEPTIDE BACTERIOCIN, CARNOBACTERIOCIN A, PRODUCED BY *CARNOBACTERIUM PISCICOLA* LV17A.[1]

2.1 INTRODUCTION

The lactic acid bacterium, *Carnobacterium piscicola* LV17, isolated from the adventitious microflora of vacuum packaged meat as an "atypical *Lactobacillus*- type" organism (Shaw and Harding, 1984) produces heat stable bacteriocins that are detected early in the growth cycle (Ahn and Stiles, 1990a,b). The parental strain contains three plasmids. Separation of the plasmids revealed that "early" bacteriocin production is mediated by the 49 MDa plasmid (Ahn and Stiles, 1992). Bacteriocins are proteinaceous, antimicrobial compounds that typically inhibit closely related bacteria (Klaenhammer, 1988). Nisin A, produced by *Lactococcus lactis* subsp. *lactis*, has been extensively studied and is used commercially as a food preservative (Delves-Broughton, 1990), but its application is limited by its insolubility and instability at pH > 5. Interest in the use of bacteriocins for food preservation has increased with the discovery of many new active compounds produced by lactic acid bacteria (Klaenhammer, 1988). Some of these have been extensively characterized to reveal new "classes" of bacteriocins, including heat stable, low molecular weight, hydrophobic nonlantibiotic compounds: lactacin F from *Lactobacillus acidophilus* (Muriana and Klaenhammer, 1991); lactococcin A from *L. lactis*

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subsp. *cremoris* (Holo *et al.*, 1991); leucocin A-UAL187 from *Leuconostoc gelidum* (Hastings *et al.*, 1991); mesentericin Y105 from *Leuconostoc mesenteroides* subsp. *mesenteroides* (Héchard *et al.*, 1992); pediocin PA-1 from *Pediococcus acidilactici* (Marugg *et al.*, 1992; Lozano *et al.*, 1992) and sakacin A from *Lactobacillus sake* (Holck *et al.*, 1992). All of these compounds, except for mesentericin Y105, have been shown to be ribosomally synthesized with an N-terminal extension to the structural peptide that is cleaved at a peptide bond adjacent to a common Gly-Gly (-2, -1) site.

Elucidation of the amino acid sequences of the widely reported pediocins PA-1 and AcH (Motlagh *et al.*, 1992; Henderson *et al.*, 1992; Lozano *et al.*, 1992) revealed that these bacteriocins are the same compound. Exceptional similarity exists between leucocin A and mesentericin Y105, despite the different sources of the producer strains. They consist of 37 and 36 amino acids, respectively, with variation only in amino acid residues 22, 26 and the terminal amino acid of leucocin A, yet the activity spectra are strikingly different (Hastings *et al.*, 1991; Héchard *et al.*, 1992). Similarly, there is considerable homology between the first 20 amino acids of leucocin A, pediocin PA-1, sakacin A, sakacin P, and curvacin A (Hastings *et al.*, 1991; Marugg *et al.*, 1992; Holck *et al.*, 1992; Tichaczek *et al.*, 1992). Many other bacteriocins may possess analogous sequences but lack structural characterization. Complete elucidation of the amino acid sequence and the genetic determinants of bacteriocin activity can provide insight into structure and function relationships and modes of action of these compounds. It is also a prerequisite for determination of three dimensional structure by NMR spectrometry (Henkel *et al.*, 1992; Sailer *et al.*, 1993). In this study we describe the characteristics of carnobacteriocin A produced by *Carnobacterium piscicola* LV17A, including the complete amino acid sequence and the nucleotide sequence for the structural gene involved in synthesis of this bacteriocin.

2.2 METHODS

2.2.1 Bacteriology

The bacteriocin-producing organism *C. piscicola* LV17 was identified as a nonaciduric *"Lactobacillus"* isolated from vacuum packaged meat by B. G. Shaw (Institute for Food Research, Bristol, U.K.). The parent strain contains three plasmids (Ahn and Stiles, 1990b). Strains LV17A and LV17A.17 are bacteriocinogenic (Bac^+) and contain the 49 MDa plasmid or a 17 MDa religated *Bgl*II fragment of pCP49, respectively (Ahn and Stiles, 1992). Production of bacteriocin was monitored against *C. divergens* LV13 (Shaw) by a spot-on-lawn technique (Ahn and Stiles, 1990a). Cultures were stored in Cooked Meat Medium (Difco Laboratories Inc., Detroit, MI) at 4 °C and at -70 °C in APT broth (Difco) at pH 6.5 containing 20 % glycerol. Stock cultures were subcultured in APT at 25 °C on two successive days before use in an experiment. Growth of *C. piscicola* LV17A for bacteriocin purification was done in semi-defined medium (CAA) as described by Hastings *et al.* (1991). *Escherichia coli* strains were grown in Luria-Bertani (LB; Sambrook *et al.*, 1989) broth or agar at 37 °C, and stored at -70 °C in LB broth with 20 % glycerol. The bacterial strains and plasmids used in this study are listed in Table 2.1.

2.2.2 Purification of carnobacteriocin A

The procedure was adapted from Sailer *et al.* (1993). The producer strain was grown at 25 °C in 2 liters of CAA adjusted to and maintained at pH 6.2 with sterile 1 M NaOH using a pH stat (Chem-Cadet®, Cole Palmer, Niles, IL) while being stirred gently under a steady flow of nitrogen (40 ml min^{-1}). After 16 h (NaOH consumption 150 ml) the supernatant fluids were collected by centrifugation at 8000 x *g* for 20 min at 4 °C. The culture supernatant (2150 ml) was applied directly to an Amberlite XAD-8 column (4.5 x 45 cm; BDH Chemicals Ltd., Poole, U.K.) washed with 2 litres of 0.1% trifluoroacetic acid (TFA), 1L 20 % EtOH, 1L 30 % EtOH, and eluted with successive 1L volumes of 45

Table 2.1. Bacteria and plasmids used to study bacteriocin production by *C. piscicola* LV17A.

Bacterial Strain or Plasmid/phage Type	Description [a]	Source or Reference
Carnobacterium piscicola		
LV17A	Bac$^+$ Imm$^+$ containing pCP49	Ahn and Stiles, 1990b
LV17A.17	Bac$^+$ Imm$^+$ containing 17 MDa *Bgl*II religated fragment of pCP49	Ahn and Stiles, 1992
LV17B	Bac$^+$ Imm$^+$ containing pCP40	Ahn and Stiles, 1990b
Carnobacterium divergens		
LV13	Bac^s	B. G. Shaw, Bristol, U.K.
Escherichia coli		
MV 1193	Δ (*lac-pro*AB), *rps*L, *thr*, *end* A, *spc*B15, *hsd* R4, Δ (*srl-rec*A) 306:: Tn 10 (Tet^r) F' [*tra* D36, *pro* AB$^+$, *lac* I^q *lac* Z Δ M15]	Sambrook *et al.*, 1989
PLASMIDS		
pUC118	lacZ', Amp^r, 3.2 kb	Vieira and Messing, 1987
pK194	lacZ', Km^r, 2.4 kb	Jobling and Holmes, 1990
pRW5.9U	Amp^r, *Cbn*A, 5.9 kb	This study
pRW4.4K	Km^r, *Cbn*A, 4.4 kb	This study
PHAGE		
M13K07	Helper phage for pUC118	Sambrook *et al.*, 1989

[a] Bac$^+$ represents the production of carnobacteriocin A.

Imm$^+$ represents the immunity to carnobacteriocin A

% and 80 % EtOH in 0.1 % TFA. Active fractions were combined and concentrated to 30 ml using a rotary evaporator at 30 °C, mixed with the same volume of acetonitrile (MeCN) and loaded onto a Sephadex LH-60 column (5 x 25 cm) pre-equilibrated with 50 % MeCN in 0.1 % aqueous TFA. The active fractions were pooled and concentrated by rotary evaporation to 5 ml. The concentrate was applied in 0.5 ml portions to a C_8 HPLC column (VYDAC, 10 x 250 mm, 10 μm, 300 Å, flow rate 3 ml min^{-1}, mobile phase: A, 0.1 % (v/v) TFA in water; B, 70 % acetonitrile in 0.1 % TFA) and eluted using a gradient method: first 43 % to 54 % B solvent in 4 min and then 54 % to 61 % B in 7 min. Fractions were monitored for absorbance at 218 nm and activity against the indicator strain. Purification of separated active peptides was completed by isocratic elution on the same column (flow rate 3.5 ml min^{-1}, in 46 % , 55 % and 57 % of B solvent for A1, A2, and A3, respectively).

2.2.3 Stability of carnobacteriocins A

The effects of pH, heat treatment, and selected organic solvents on the activity of purified carnobacteriocins A1 (1600 arbitrary units (AU ml^{-1}), A2 (200 AU ml^{-1}) and A3 (400 AU ml^{-1}) were determined. Samples were suspended in the following buffers: pH 1.0 and 3.0 glycine-HCl (50 mM and 30 mM, respectively), pH 5.0 in citric acid-HCl (50 mM), pH 7.0 Tris-HCl (50 mM), and pH 9.0 Tris-NaOH (50 mM). Samples at each pH were heated in a boiling water bath for 30 min. Residual activity was determined after 2 and 24 h at each pH level and immediately after heat treatment. To test effects of organic solvents, the carnobacteriocins were suspended in 50 % (v/v) solutions of aqueous 0.1 % TFA with acetone, acetonitrile, ethanol, ethyl acetate, methanol, and tetrahydrofuran. Lyophilized samples were also dissolved in 0.1 % TFA, stored at 5 °C for 14 days, and checked by HPLC. Degradation was estimated from the appearance of decomposed products and residual activity.

2.2.4 Determination of N-terminal amino acid sequence of carnobacteriocin A

Purified carnobacteriocins from *C. piscicola* LV17A were subjected to Edman degradation analysis on an automated gas phase sequencer (Applied Biosystems model 470A, Foster City, CA) with on-line phenylthiohydantoin derivative identification by reversed-phase HPLC (Applied Biosystems model 120A).

2.2.5 Mass spectrometry of carnobacteriocins

All mass spectra were kindly acquired by Dr. Robert B. Cody at Jeol USA (Peabody, MA) on a Jeol SX102A instrument using an electrospray interface for fast atom bombardment mass spectrometry (FAB MS) in the positive ion mode. Samples of A1, A2, and A3 obtained by HPLC purification were dissolved in 1:1 (v/v) aqueous methanol containing 2 % (v/v) glacial acetic acid and were analyzed by direct infusion through a sample loop. Jeol software was employed to determine molecular weights from the envelops of multiply charged peaks in the *m/z spectra*.

2.2.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Purified carnobacteriocin preparations were examined using 20 % polyacrylamide gels and 3 M Tris-HCl, pH 8.8, as described by Laemmli (1970). Electrophoresis was done at constant current of 20 mA. Gels were fixed in 5x (v/v) formaldehyde for 30 min and stained with Coomassie Blue (Bio-Rad Laboratories Ltd., Mississauga, ON).

2.2.7 DNA isolation, manipulation, and hybridization

Large scale preparation of plasmid DNA from *C. piscicola* LV17A was done by a modification of the Klaenhammer (1984) method. The method was scaled up 10x using cells from 1 liter of culture grown in APT broth. The resuspended cell solution was carefully adjusted to pH 8.0 for lysozyme treatment, and to pH 7.0 after alkaline lysis. Plasmids were purified by CsCl density gradient ultracentrifugation (Sambrook *et al.*,

1989). Small and large scale plasmid isolation from *E. coli* was done according to Sambrook *et al.* (1989). Restriction endonucleases and T4 DNA ligase (Boehringer-Mannheim, Dorval, PQ or Bethesda Research Laboratories, Burlington, ON) were used as recommended by the suppliers. DNA cloning and hybridization were done by methods described by Sambrook *et al.* (1989). Southern and colony blot hybridizations were done on Hybond N nylon membranes (Amersham Corp., Arlington Heights, IL), and DNA probes were labeled with [γ^{32}]P-ATP (Amersham) using T4 polynucleotide kinase (Pharmacia) or with digoxigenin UTP using terminal transferase (Boehringer-Mannheim). Hybridization and washes were done at 30 °C. A degenerate 23-mer oligonucleotide, RWO1 (5'GAXGGXGTXAA(CX)TAXGGXAA(AG)GG3', X=bromouracil) was used to detect carnobacteriocin A gene(s) in all Southern and colony blot hybridizations. Oligonucleotides prepared as sequencing primers were synthesized on an Applied Biosystems 391 PCR Mate synthesizer.

2.2.8 Molecular cloning of carnobacteriocin A gene(s)

Plasmid DNA from *C. piscicola* LV17A was digested with a range of restriction enzymes that have unique restriction sites within the multiple cloning site (MCS) of pUC118. A 2.4-kb *Eco*RI fragment that hybridized with the carnobacteriocin probe (RWO1) was cloned into pUC118 and screened by colony blot hybridization for successful clones. Plasmid DNA from Bac$^+$ clones was digested with *Eco*RI and the 2.4-kb *Eco*RI insert was eluted from the gel using a one step method for isolation of DNA fragments (Heery *et al.*, 1990) and cloned into the MCS of pK194. Positive pUC118 clones were used to produce single stranded DNA for sequencing.

2.2.9 Nucleotide sequencing of plasmid DNA

Single and double stranded DNA was sequenced by *Taq* DyeDeoxy Cycle sequencing on an Applied Biosystems 373A sequencer. Single stranded DNA was

sequenced from the pUC118 clone (pRW5.9U) using specific sequence oligonucleotides and the universal primer of pUC118. The complementary strand was sequenced in the double stranded form using the pK194 clone (pRW4.4K) as the sequencing template. Single stranded DNA was produced by infecting the *E. coli* clone containing the pUC118 clone with M13K07 helper phage and incubating at 37 °C with rotary shaking (200 rpm) for 75 min. Kanamycin (Km) was then added to give a final concentration of 75 $\mu g\ ml^{-1}$ and incubation was continued for 18 h. Supernatant containing the progeny phage was harvested by polyethylene glycol precipitation (Applied Biosystems Instruments recommended procedures). Large scale preparation of single stranded DNA was done by 50x scaling up of the same procedure. Opposite orientation nucleotide sequencing of the 2.4 kb *Eco*RI fragment was tested on existing positive clones. Opposite orientations were attempted by cutting the clone of pRW5.9U and religating the pool of fragments.

2.2.10 Nucleotide sequence accession number

The DNA sequence was submitted to Genbank (Los Alamos, NM) and was given the accession number L23112.

2.3 RESULTS

2.3.1 Purification and stability of A carnobacteriocins

Carnobacteria do not grow well below pH 5.0 and do not produce bacteriocin below pH 5.5 (Ahn and Stiles, 1990a). From the data in Figure 2.1 it can be seen that with the pH held constant at 6.5 the greatest amount of bacteriocin activity was detected between 9 - 18 h of incubation at 25 °C. The bacteriocin is unstable and activity decreases rapidly during further incubation after 18 h. Using the culture supernatant of *C. piscicola* LV17A and the purification steps shown in Table 2.2, a total yield of 4.6×10^6 arbitrary activity units (AU) determined against *C. divergens* LV13 was obtained. A total of 1.9×10^6 AU (41 %) was recovered after purification by reversed-phase HPLC. Following this isolation

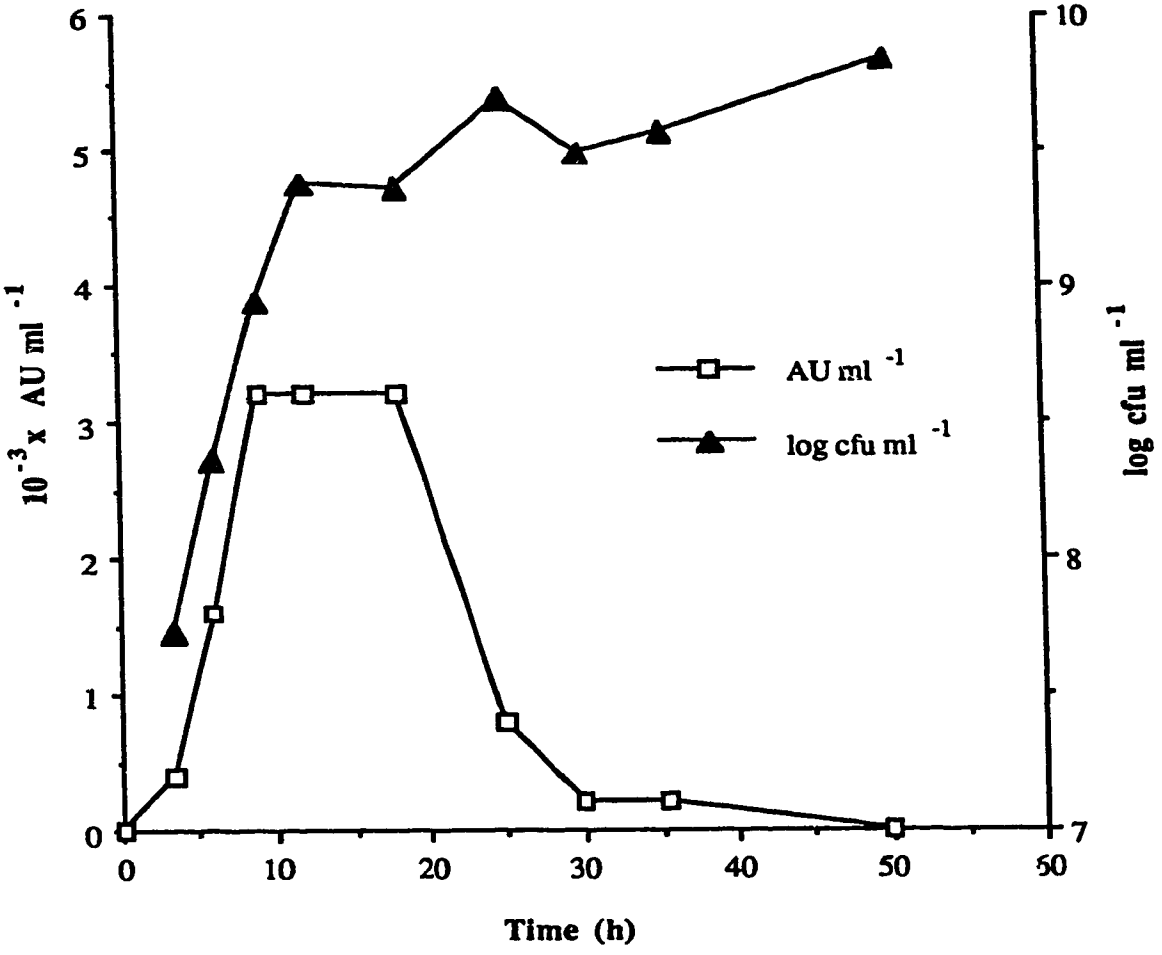


Figure 2.1. Bacteriocin production and activity [in arbitrary units (AU)] of carnobacteriocin A produced by *Carnobacterium piscicola* LV17A with time at constant pH of 6.5 at 25 °C.

Table 2.2. Purification of carnobacteriocins A produced by *C. piscicola* LV17A at pH 6.5

Purification stage	Volume (ml)	Activity (AU ml^{-1})[a]	Total activity (AU)	Amount of protein[d] (mg ml^{-1})	Specific activity (AU mg^{-1})	Activity recovery (%)	Fold purification
Supernatant	5800	0.8×10^3	4.64×10^6	3.3	2.43×10^2	100	1
XAD-8 fractions[b]	130	3.2×10^4	4.16×10^6	1.7	1.94×10^4	90	80
Sephadex LH-60 fractions[b]	220	1.28×10^5	2.88×10^6	0.17	7.9×10^4	62	325
C-8 column fractions[b]	300	0.64×10^5	1.92×10^6	0.08	8.24×10^4	41	339
RP-HPLC[c]							
A1	0.15	0.64×10^5	9.6×10^3	0.7	9.15×10^4		
A2	0.92	1.28×10^5	1.18×10^5	0.5	2.56×10^5	8.6	
A3	0.40	6.83×10^5	2.73×10^5	1.0	6.83×10^5		

a) AU, activity units determined against *Carnobacterium divergens* LV 13.

b) Pooled active fractions were concentrated to the given volumes.

c) Active fractions were lyophilized and resuspended in 0.1 % TFA.

d) Determined by method of Lowry as modified by Markwell using BSA as a standard.

protocol three extracellular, active peptides were observed (Figure 2.2) and separated by HPLC. The major product, carnobacteriocin A3, was separated with a yield of about 800 μg from 2L of culture broth. Carnobacteriocin A2 was obtained with a lower yield of 500 μg, whereas A1 proved to be a mixture of degradation products. Carnobacteriocins A1 and A2 also displayed lower specific activities. Gel electrophoresis indicated molecular weights of approximately 4000 Da (Figure 2.3). Although partially separated carnobacteriocins A decomposed rapidly (possibly due to proteolysis), pure lyophilized samples of carnobacteriocins A2 and A3 showed no decrease in biological activity and gave clean mass spectra after storage for several months at 4 °C (Figure 2.4). All three bacteriocins were unstable on storage for an extended period of time. During storage at 5 °C for 14 days in 0.1 % TFA, A1 was completely degraded, A2 was the most stable with 80 % of its activity remaining, and A3 retained only 10 % of its activity. None of the degradation products isolated by HPLC showed biological activity against *C. divergens* LV13 (data not shown). Data in Table 2.3 show that carnobacteriocin A1 is relatively heat stable (100 °C for 30 min) compared with A2 and A3. The carnobacteriocins were most stable at pH 1.0 and stability generally decreased with increase in pH. They were stable in the organic solvents tested; in fact enhanced activity was noted in some cases after 24 h of storage in the solvents.

The ratios between the different bacteriocins during growth of the producer strain at 25 °C at pH 6.5 are shown in Table 2.4. At the end of the log phase of growth the ratios of A1:A2:A3 were 4:1:1; this changed to 8:1:1 after another 18 h incubation; and after a further 26 h, only A1 could be detected. This is unlike the stability observed with the purified bacteriocins suspended in 0.1 % TFA.

2.3.2 Characterization of carnobacteriocins A

Mass spectral analysis using positive ion fast atom bombardment with an electrospray interface gave multiply charged molecular ions (Figure 2.4) which allo[illegible]

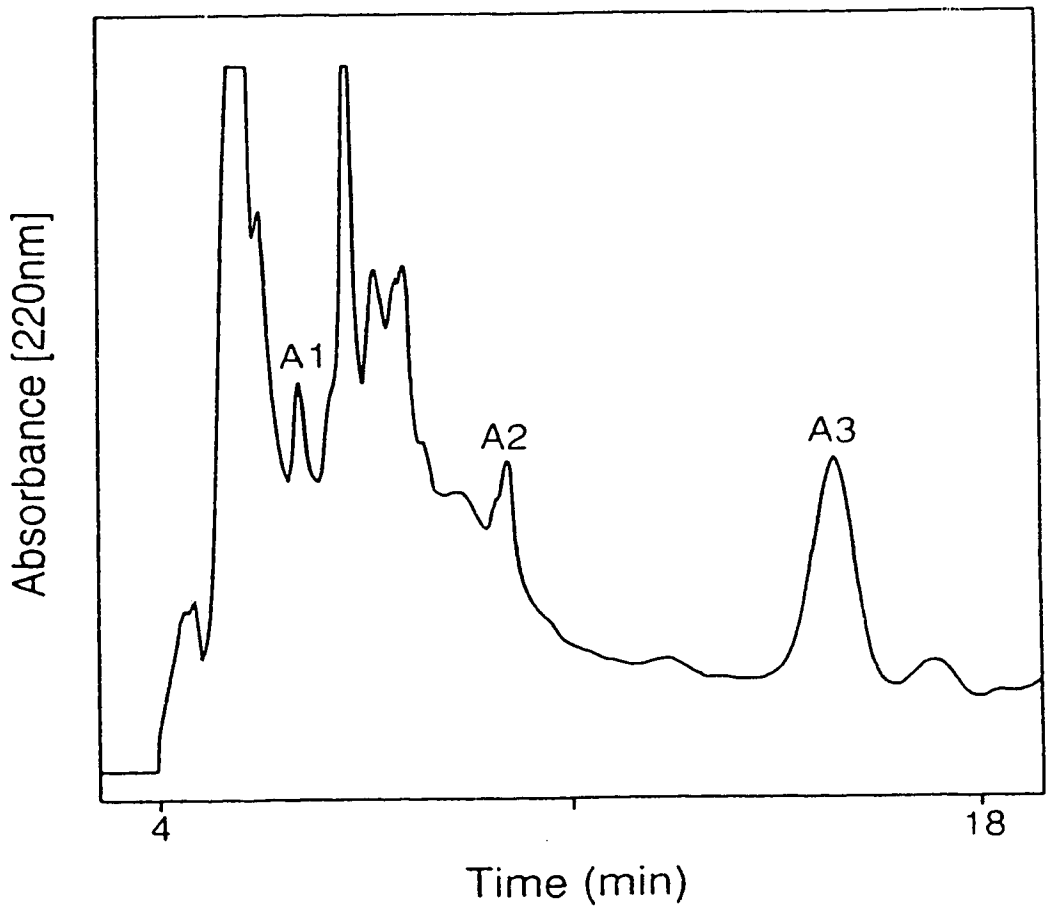


Figure 2.2. Elution pattern of carnobacteriocins A on a reversed-phase HPLC C-8 column (VYDAC) using 0.1% TFA/MeCN gradient. Peaks A1, A2 and A3 represent the active peaks obtained.

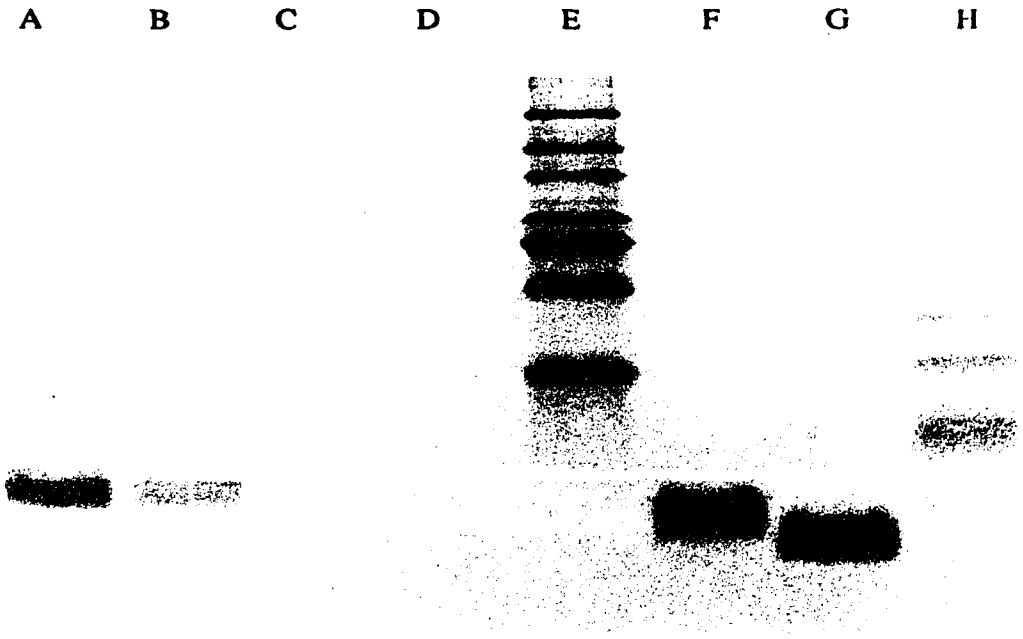


Figure 2.3. Polyacrylamide gel (20%) electrophoresis of carnobacteriocin A fixed with 5% formaldehyde and stained with Coomassie blue. Lane A, carnobacteriocin A3 (1µg); Lane B, carnobacteriocin A3 (0.6µg); Lane C, carnobacteriocin A3 (0.2µg); Lane D, Blank; Lane E, low molecular weight protein standards: 66,000, 45,000, 34,700, 24,000, 18,400, 14,300 daltons (Sigma); Lane F, Nisin; Lane G, Insulin; Lane H, low molecular weight protein standards: 16,949, 14,404, 8,159, 6,124, and 2,512 Daltons (Fluka, Biochemika).

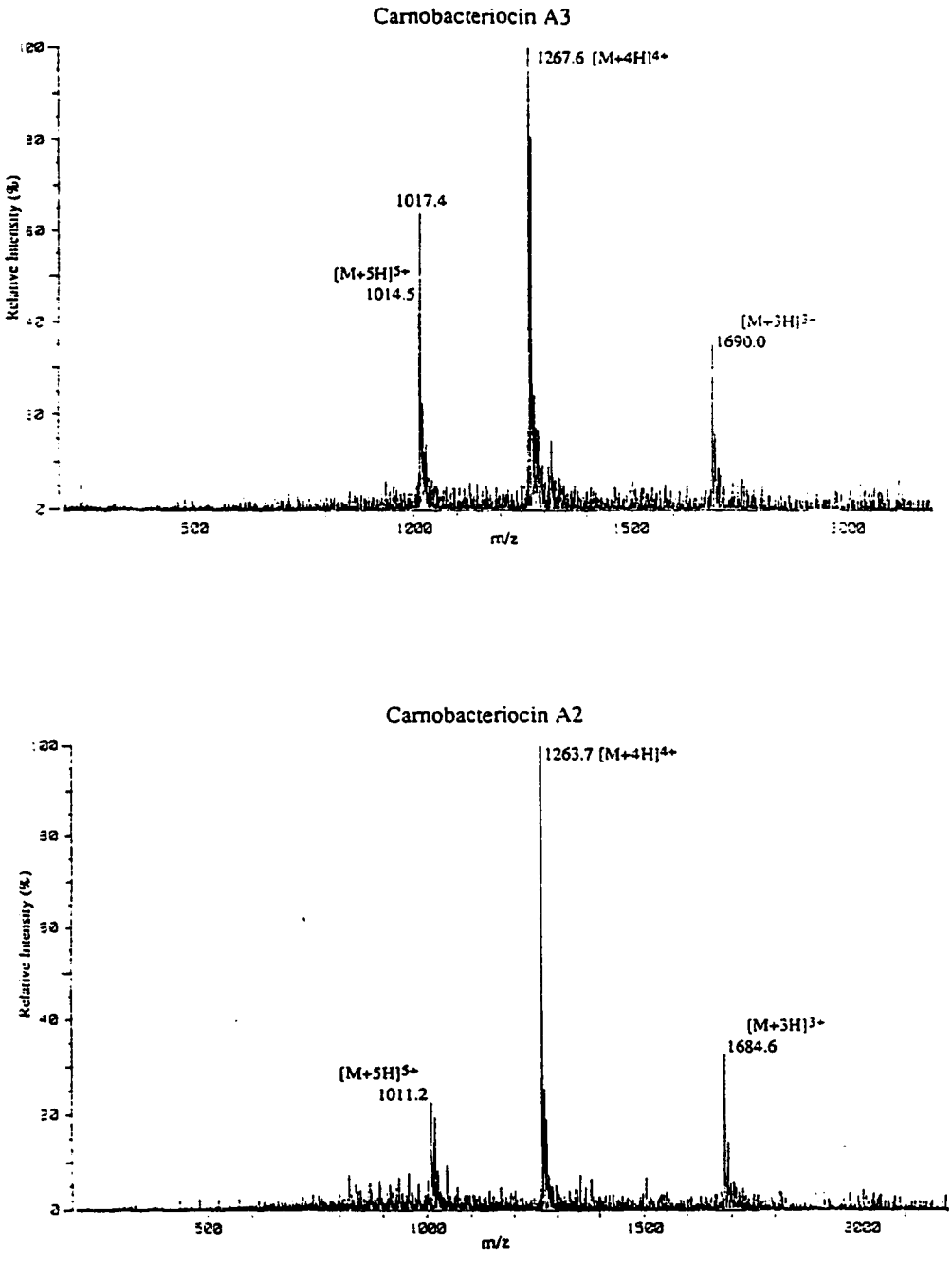


Figure 2.4. Electrospray FAB mass spectra (positive ion mode) of carnobacteriocins A2 and A3 showing multiply charged molecular ions from which atomic mass was calculated.

Table 2.3. The effect of pH, temperature and organic solvents (1:1 mixtures with 0.1% TFA) on the antimicrobial activity (AU ml^{-1}) of the purified carnobacteriocins A1, A2 and A3.

	A1			A2			A3		
Temperature °C	25		100	25		100	25		100
Time (h)	2	24	0.5	2	24	0.5	2	24	0.5
0.1% TFA (control)	1600	1600	1600	200	200	-	400	400	-
pH 1	800	400	400	200	200	200	400	400	0
pH 3	800	0	800	200	200	0	400	100	100
pH 5	800	100	200	<100	0	0	400	100	0
pH 7	200	<100	200	<100	0	<100	200	100	0
pH 9	100	0	0	100	100	100	400	<100	0
Ethyl acetate	-	-	-	200	200	-	400	400	-
Acetonitrile	1600	1600	-	200	800	-	400	400	-
Tetrahydrofuran	-	-	-	200	400	-	400	400	-
Ethanol	1600	1600	-	200	800	-	400	1600	-
Methanol	1600	1600	-	200	400	-	400	1600	-
Acetone	-	-	-	200	400	-	400	200	-

-, not tested

Table 2.4. Carnobacteriocins produced by *C. piscicola* LV17A during fermentation at 25 °C in APT broth at pH 6.5.

Carnobacteriocin	A1	A2	A3
HPLC retention time (min)	4	19	23
Incubation time (h)	Activity (AU ml^{-1})		
9	800	200	200
18	800	100	100
26	200	nd	nd
33	nd	nd	nd

nd, not detected

calculation of the average molecular masses as follows: for carnobacteriocin A3, 5,050.8 ± 0.3 Da; for A2, 5,067.0 ± 0.3 Da ; and for the major component of A1, 4,524.7 ± 0.4 Da. Edman degradation of all samples revealed up to 28 amino acids in the N-terminal sequences and that the only difference detected between the three compounds was at amino acid residue 16 (Table 2.5). After sequencing of 20 to 28 amino acids, the accuracy of identification decreased due to background peaks that resulted in a lower confidence level for the sequence. The amino acid analyses of the A2 and A3 bacteriocins are given in Table 2.6. These amino acid analyses agree with the subsequent results derived from the nucleotide sequence data, except for the failure to detect proline for A2.

2.3.3 Isolation of carnobacteriocin A structural gene

Plasmid DNA (pCP49 and pCP17) was isolated from *C. piscicola* LV17A and LV17A.17 and digested with restriction enzymes compatible with the multiple cloning site of pUC118. A degenerate 23-mer oligonucleotide (RWO1) probe was derived from the identical sequence of amino acid residues 5 to 12 of the N-terminus of all three bacteriocins. Hybridization was observed with a 2.4-kb *Eco*RI fragment. No other hybridization signal was observed in the remaining *Eco*RI fragments of pCP49 or when the probe was hybridized with pCP40, another native plasmid contained in the wild type strain of *C. piscicola* LV17 (Ahn and Stiles, 1990b). Shotgun cloning of a *Eco*RI restriction digest of pCP17 into the *Eco*RI site of pUC118 was performed and colonies were screened using alpha complementation. Colony blots were done to screen the white colonies for the correct insert. The positive clones were digested, Southern transferred and hybridized with the degenerate probe RWO1 to confirm the presence of the carnobacteriocin gene(s).

2.3.4 Structure of the carnobacteriocin A gene

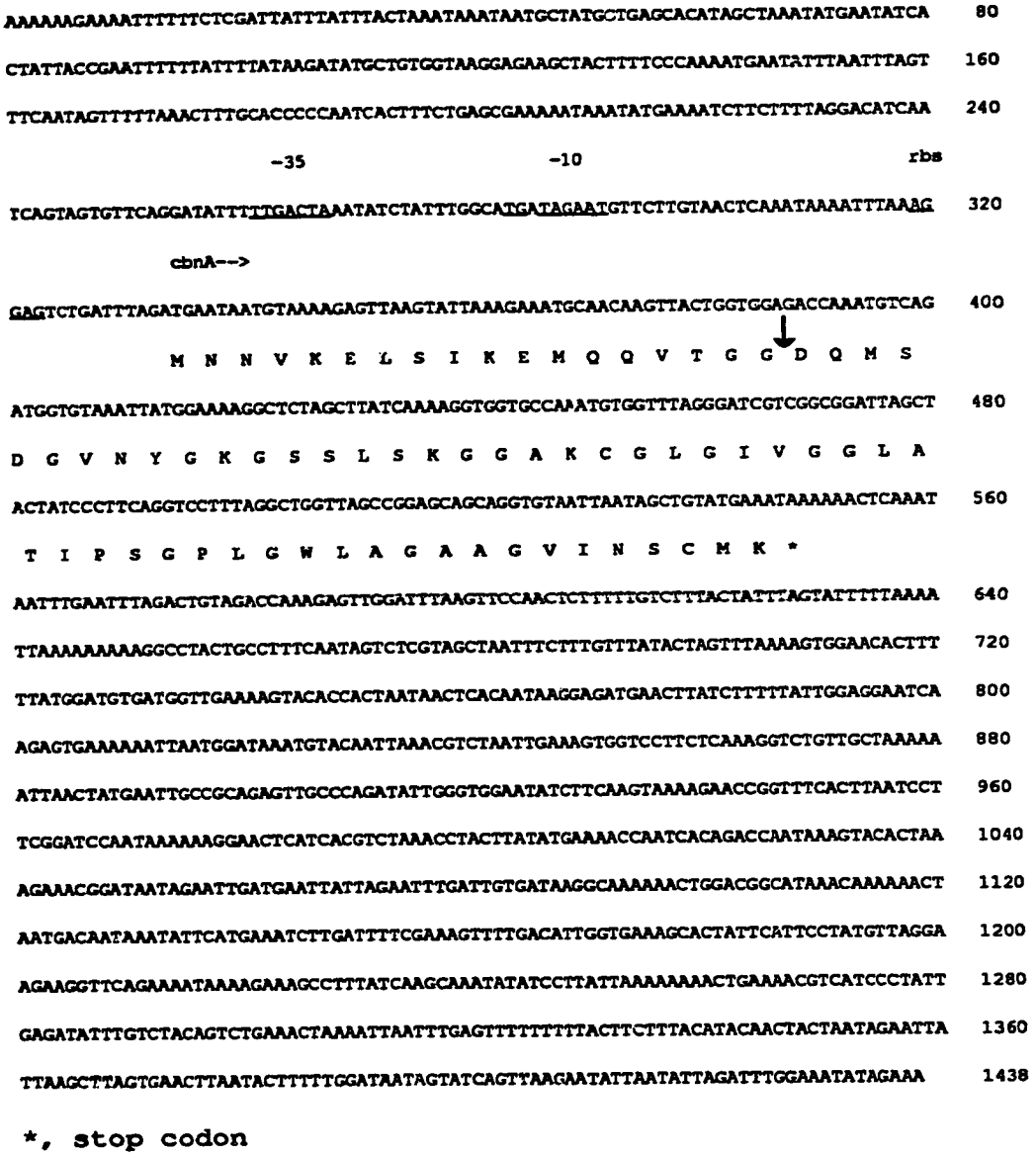
The nucleotide sequence of the *Eco*RI fragment is shown in Figure 2.5. Analysis of this sequence revealed the presence of only one open reading frame (ORF) with a putative

Table 2.5. N-terminal amino acid sequences of A carnobacteriocins purified by reversed-phase HPLC.

Bacteriocin	Amino Acid Sequence
	5 10 15 20 25
A1	D Q M S D G V N Y G K G S S L V K G G A . . . . .
A2	D Q M S D G V N Y G K G S S L S K G G A K G G L G I . . . . (C?)
A3	D Q M S D G V N Y G K G S S L S K G G A K G G L G I V G . . (C?)

Table 2.6. Amino acid analysis of carnobacteriocins.

	A2		A3	
Amino acids	pmol	Number of residues in sequence	pmol	Number of residues in sequence
Asn/Asp	929	5	3028	5
Ala	1065	6	3120	6
Gly	2864	13	19911	?
Gln/Glu	361	2	1100	2
His	127	1	377	1
Ile	430	2	1280	2
Leu	1138	6	3231	6
Lys	846	4	2473	4
Met	195	1	293	1
Pro	-	-	1279	2
Ser	1027	6	3332	6
Thr	232	2	734	2
Tyr	238	2	686	2
Val	424	2	1314	2



*, stop codon

Figure 2.5. Single stranded nucleotide sequence of a 1.4-kb *Eco*RI fragment of pCP17, a religated 25.5-kb *Bgl*II fragment of pCP49 from *C. piscicola* LV17A. The sequence shows one open reading frame commencing at nucleotide residue 334 encoding the structural gene for carnobacteriocin A (cbnA). No other open reading frame was detected. The potential ribosome binding site (rbs) and promotor regions (-10 and -35) are illustrated on the figure and underlined.

promoter site. The N-terminal amino acid sequences of the three carnobacteriocins A match the sequence derived from nucleotide sequencing, except for the valine residue at position 16 of carnobacteriocin A1. These data indicate that the residue is actually serine, as determined by Edman degradation analyses for carnobacteriocins A2 and A3. No other gene encoding other carnobacteriocins was found in the 2.4-kb fragment or in the *Eco*RI digest of genomic DNA isolated from *C. piscicola* LV17B that contains pCP40 (another native plasmid contained in the wild type *C. piscicola* LV17 strain) when hybridized with the carnobacteriocin A probe. The first amino acid of the N-terminal sequence matches with the nineteenth amino acid derived from the nucleic acid sequence, so that the prepeptide contains 71 amino acids and is comprised of an N-terminal extension consisting of 18 amino acids and the bacteriocin consisting of 53 amino acids. The putative ribosomal binding site (AGGAG) for the carnobacteriocin A (*cbnA*) structural gene has the optimal spacing of 9 bases upstream of the initiation codon (coordinate 319). A likely -10 promoter region (TAGAAT) has been identified at position 287 along with a TG sequence one nucleotide ahead of the -10 region. Upstream of the -10 region is a potential -35 region located at base pair 262. No significant second ORF was observed in the remainder of the 2.4-kb fragment. The ends of the 2.4-kb *Eco*RI fragment were identified by the *Eco*RI sites at each end of the fragment, along with the remainder of the M13/pUC multiple cloning site.

2.4 DISCUSSION

The technique for isolation of leucocin A-UAL 187 reported by Sailer *et al.* (1993) worked well for the purification of bacteriocins from *C. piscicola* LV17A. Three peaks (A1, A2, A3) were separated by HPLC which had activity against the indicator strain. This suggests either that three different bacteriocins are independently produced by *de novo* protein synthesis, or that subsequent posttranslational or chemical modifications of one or more bacteriocins occurs. N-terminal amino acid sequencing of the active substances

revealed that at least the first 15 amino acids were identical in each of the three peptides. This result indicates that there is only one parent antibacterial compound (carnobacteriocin A) directly generated from a single structural gene, and that this peptide is subsequently chemically transformed to other active derivatives. This was subsequently confirmed by experiments with the 23-mer oligonucleotide probe which hybridized with a single restriction fragment. Initial mass spectra for the three compounds isolated by HPLC were ambiguous (not reported) because of relatively rapid decomposition of the samples to complex mixtures. Subsequently, more rigorous HPLC purification gave stable samples that afforded clean fast atom bombardment (FAB) electrospray mass spectra for compounds A2 and A3, and showed that A1 was still an apparently inseparable mixture resulting from degradation at the C-terminal end of the parent bacteriocin. Comparison of the molecular mass of carnobacteriocin A3 (5050.8 ± 0.3) calculated from the mass spectrum to that predicted from the nucleotide sequence (5052.8) indicated that this parent bacteriocin contains a disulfide bridge between the two cysteines at positions 22 and 51 but is not otherwise modified. The difference of +16.2 Da in the molecular mass of A2 relative to A3 and the identical results from amino acid sequencing for the first 15 residues of both compounds suggests that carnobacteriocin A2 has the sulfur in methionine 52 oxidized to a sulfoxide. Similar oxidations have been confirmed by detailed structural analyses of carnobacteriocins BM1 and B1 produced by *C. piscicola* (unpublished data). Thus the actual amino acid sequences of A3 and A2 are identical except for the presence of a methionine sulfoxide at position 52 in the latter. Based on the mass spectra for A1, it is assumed that this is a breakdown product of A2 and A3.

Activity of carnobacteriocins A was rapidly lost during storage in buffered solutions at pH > 3, and the compounds are relatively unstable until completely purified. However, pure samples of A2 and A3 are completely stable for months in lyophilized form. The antimicrobial activity of carnobacteriocins A is relatively stable to organic solvents and heat, especially the mixture A1. Although a number of low molecular weight, heat stable,

nonlantibiotic, hydrophobic peptide bacteriocins have been purified and characterized from various lactic acid bacteria, carnobacteriocins A3 and A2 have a slightly larger molecular mass and show little amino acid sequence homology with other bacteriocins (Héchard *et al.*, 1992; Henderson *et al.*, 1992; Holck *et al.*, 1992; Lozano *et al.*, 1992; Marugg *et al.*, 1992; Motlagh *et al.*, 1992; Tichaczek *et al.*, 1992), including carnobacteriocins B1 and B2 produced by the same *C. piscicola* organism (unpublished data).

Despite difficulties with stability of purified carnobacteriocin A, as many as 20 to 28 amino acids were determined in the N-terminal sequence for all three compounds. Detection of a single structural gene *cbn*A on the plasmid pCP49 coding for a serine residue at position 16 of the N-terminal amino acid sequence confirmed that carnobacteriocins A2 and A3 are products of the same gene. The possibility of the structural gene for carnobacteriocin A1 being on the chromosome was excluded by hybridizing the carnobacteriocin probe with the chromosomal DNA. It was concluded that carnobacteriocins A1, A2 and A3 have only one structural gene and that initial tentative identification of valine at position 5 of carnobacteriocin A1 resulted from difficulty in interpretation of the Edman degradation data of this apparently inseparable peptide mixture.

Production of more than one bacteriocin by a single organism is not unique. In the case of *C. piscicola* LV17, Ahn and Stiles (1992) demonstrated that bacteriocin production was mediated by different plasmids. In the case of lactococcins A, B, and M (van Belkum *et al.*, 1991, 1992) all three bacteriocins were shown to be produced by *Lactococcus lactis* subsp. *cremoris* 9B4. Of interest for further study in *C. piscicola* LV17 is the fact that carnobacteriocins A and B are detected early and late in the growth cycle, respectively. It is not clear whether an "early" bacteriocin, such as carnobacteriocin A or leucocin A - UAL187 (Ahn and Stiles, 1992; Hastings *et al.*, 1991) is attributable to production of highly active compounds that are detected early in the growth cycle, differences in copy

number of the plasmids promoting higher production, or whether there is genetic control that determines time of production relative to stage of the growth cycle.

Production of lactococcin A by *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* WM4 is controlled by a cluster of four genes contained within a 5-kb fragment of plasmid DNA (Stoddard *et al.*, 1992). Upstream of the structural and immunity genes are three open reading frames (ORFs), two of which are implicated in secretion of the bacteriocin through their homology with proteins of signal sequence-independent secretion systems. A similar cluster of four genes in a 3.5-kb segment of plasmid DNA was associated with production and secretion of pediocin PA-1 from *Pediococcus acidilactici* in *Escherichia coli* (Marugg *et al.*, 1992). In this case all genes are located immediately downstream of the structural gene in a relatively compact configuration; however, it was previously shown for this strain of *P. acidilactici* that immunity to its own bacteriocin is chromosomally mediated (Gonzalez and Kunka, 1987). The structural gene for carnobacteriocin A is located on the 1.4-kb *Eco*RI fragment of DNA on which only one ORF was identified, indicating a considerable separation of the structural gene from its immunity and secretion genes. Evidence that the immunity gene is on the same plasmid was obtained by cloning different fragments of DNA downstream of the structural gene of carnobacteriocin A into a plasmidless strain of *C. piscicola* LV17 (data not shown). Analysis of the genetic control of carnobacteriocin A production is proceeding.

Although carnobacteriocin A differs in molecular size and amino acid composition from other bacteriocins in this group, it is similar to other bacteriocins by having an N-terminal extension that terminates in Gly-Gly- residues immediately prior to the cleavage site (Hastings *et al.*, 1991; Holo *et al.*, 1991; Marugg *et al.*, 1992; Muriana and Klaenhammer, 1991; Stoddard *et al.*, 1992; van Belkum *et al.*, 1991, 1992). The role of the N-terminal extension in synthesis or secretion of the active bacteriocin has yet to be demonstrated.

Nucleotide sequencing of the 2.4-kb *Eco*RI fragment containing the carnobacteriocin gene posed several problems. Nucleotide sequence could not be obtained with universal or reverse primers for the pUC118 MCS using double stranded templates. Several different plasmid extraction and purification techniques including cesium chloride ultracentrifugation, alkaline lysis and the ABI extraction protocol were used for sequencing, but with little success. Nucleotide sequencing was also attempted without success using the native plasmid and the degenerate probe created for hybridization studies. Sequence was finally obtained using single stranded DNA from the pUC118 clone. The same insert cloned in the opposite orientation was attempted but no useful sequence resulted. The 2.4-kb *Eco*RI fragment was then cloned into pK194 and sequencing of the complementary strand was accomplished.

In this fragment only one significant open reading frame was identified and it encodes the structural gene for carnobacteriocin A. Contained within the region just upstream of the start of the open reading frame is a putative ribosomal binding site spaced at the optimal number of base pairs upstream of the initiation codon for the carnobacteriocin A structural gene. The putative -10 promoter region contained a TG sequence upstream. This TG region is conserved in some Gram-positive promoters (Graves and Rabinowitz, 1986). Earlier work by Ahn (unpublished data) indicated that the structural and immunity genes on pCP49 are both on a 5.4-kb *Xba*I-*Pst*I fragment. The orientation of the structural gene in the *Eco*RI fragment indicates that the adjacent fragment probably contains the immunity gene(s).

Carnobacteriocin A, as described in this and previous studies (Ahn and Stiles, 1990a, b; 1992) is an interesting bacteriocin because its early production during the growth of *C. piscicola* LV17 gives the producer organism the potential for an ecological advantage in mixed fermentations. The antibacterial spectrum includes many lactic acid bacteria, and also *Enterococcus* and *Listeria* spp. (Ahn and Stiles, 1990b). Cloning and transformation

of the 25.5-kb *Bgl*II fragment of pCP49 into bacteriocinogenic *C. piscicola* UAL 26 resulted in the cloned organism acquiring the antibacterial spectrum of both bacteriocins (Ahn and Stiles, 1992). The ability to produce the cloned bacteriocin in a heterologous Bac$^+$ host, with expansion of the activity spectrum of the host strain, encourages the further study of producing "gene cassettes" of bacteriocins to broaden the antibacterial activity of a producer strain. The immunity and secretion genes have yet to be demonstrated. An understanding of the specific functions of the secretion proteins may allow secretion of several bacteriocins by a single secretion system.

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3. MOLECULAR ANALYSIS OF THE CARNOBACTERIOCIN A GENE CLUSTER DEDICATED TO THE PRODUCTION OF CARNOBACTERIOCIN A

3.1 INTRODUCTION

Over the past decade there has been major research interest in the bacteriocins produced by lactic acid bacteria. Bacteriocins are peptides (or proteins) that are antagonistic to closely related organisms. Lactic acid bacteria grow naturally on several foods, and this has prompted the study of the use of lactic acid bacteria and their bacteriocins for the preservation of appropriate foods, such as vacuum packaged meats. Initial studies involved characterization of the physiological properties of a broad range of bacteriocins. Preliminary genetic characterization of bacteriocins revealed the structural genes responsible for the antagonistic peptide itself. Klaenhammer (1993) divided the bacteriocins into four classes. Class I and II are the largest and best characterized groups. The class I bacteriocins are the lantibiotics that contain unusual amino acids and undergo extensive posttranslational modification to form lanthionine and β-methyllanthionine rings. Nisin is a class I bacteriocin that is in use as an important food preservative in numerous countries (Delves-Broughton, 1990). The genetic basis for nisin A production has been elucidated (Engelke *et al.*, 1992, 1994; Kuipers *et al.*, 1993; van der Meer *et al.*, 1993) and it has been shown that 15 kb of DNA is required for its production, immunity, processing and secretion. In contrast, most class II bacteriocins require less genetic information for their production. Typically, this class of bacteriocins has an 18 to 24 amino acid N-terminal extension that is cleaved on the carboxy-side of two glycine residues yielding the mature bacteriocin.

The production of class II bacteriocins in the absence of regulatory genes, is under the control of four or five genes, requiring 4 to 5 kb of DNA and including genes for the prebacteriocin, immunity, dedicated ATP-Binding Cassette (ABC) transporter and an

accessory protein, as reported for lactococcins A, B, and M (van Belkum *et al.*, 1991a, 1992; Holo *et al.*, 1991), leucocin A (van Belkum and Stiles, 1995), mesentericin Y105 (Fremaux *et al.*, 1995), and pediocin PA-1/AcH (Marugg *et al.*, 1992; Ray *et al.*, 1992). The ABC transporter protein is postulated to be involved in the secretion and cleavage of the N-terminal extension of the prebacteriocin (Håvarstein *et al.*, 1995). Also included in the class II bacteriocins, are some that have additional genes that encode a histidine kinase and a response regulator, homologous to signal transduction systems in bacteria, including Sakacins A and P (Holck *et al.*, 1992; Tichaczek, 1994), plantaricin A (Diep *et al.*, 1994), and carnobacteriocins B2 and BM1(Quadri *et al.*, 1996). For plantaricin A, an inducer peptide has also been reported (Diep *et al.*, 1995)

In our laboratory, we are studying the bacteriocins produced by *Carnobacterium piscicola* LV17, a lactic acid bacterium that was described by Collins *et al.* (1987), that has been isolated from vacuum packaged meats (Shaw and Harding, 1984; McMullen and Stiles, 1993). *C. piscicola* LV17 produces at least three bacteriocins, carnobacteriocins A, B2 and BM1 (Ahn and Stiles, 1992; Worobo *et al.*, 1994; Quadri *et al.*, 1994). The plasmids in *C. piscicola* LV17 were separated by Ahn and Stiles (1992) to give LV17A containing a 72-kb plasmid and producing carnobacteriocin A, and LV17B containing a 61-kb plasmid and producing at least two bacteriocins (Quadri *et al.*, 1994, 1996): carnobacteriocin B2 that is entirely associated with the plasmid; and carnobacteriocin BM1 that is under the control of genes on the plasmid, but the structural and putative immunity genes are on the chromosome (Quadri *et al.*, 1996). Carnobacteriocin A has particular interest for our studies because it is active against a wide range of lactic acid bacteria and strains of the foodborne pathogens, *Listeria monocytogenes*, *Enterococcus faecalis* and *Enterococcus faecium*. Production of carnobacteriocin A is detected early in the logarithmic stage of growth (Ahn and Stiles, 1990). This early production may provide a competitive advantage over other bacteria to prevent their growth in foods. Using reverse genetics, carnobacteriocin A was purified, sequenced and the structural

gene containing an 18 amino acid N-terminal extension was identified by nucleotide sequencing. No other genes were identified in the 1.4-kb fragment of plasmid DNA (Worobo *et al.*, 1994). Unlike the recently reported bacteriocins, divergicin A and acidocin B, that require approximately 0.5 kb of DNA and access the *sec*-pathway for their export from the cell (Worobo *et al.*, 1995; Leer *et al.*, 1995), production of carnobacteriocin A was only achieved when an additional 8.2-kb of contiguous plasmid DNA was included in the clone. In this chapter, the nucleotide sequence and genetic arrangement of the downstream region of the carnobacteriocin A structural gene is reported, as well as its relationship to the carnobacteriocin B2 genetic system.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains, cultures and plasmids

Bacterial strains and plasmids used in this study are detailed in Table 3.1. *Escherichia coli* strains were propagated at 37°C in LB broth or agar [1.5% (w/v)] (Sambrook *et al.*, 1989). Ampicillin, erythromycin, IPTG (isopropyl-β-D-thiogalactopyranoside), and X-gal (5-bromo-4-chlor-3-indolyl-β-D-galactopyranoside) were used at concentrations of 150, 200, 50 and 50 μg/ml, respectively. Erythromycin-resistant transformants of *E. coli* were selected on brain heart infusion agar plates (Difco Laboratories). Carnobacteria were propagated in APT broth (Difco Laboratories) or agar [1.5% (w/v)] at 25 °C. When appropriate, erythromycin or chloramphenicol was added to a concentration of 5 or 10 μg/ml, respectively.

3.2.2 Bacteriocin assay

To test for carnobacteriocin A production, cells of *C. piscicola* LV17A were spotted onto APT agar and incubated at 25 °C for 18 h. Soft APT agar [0.75 % (w/v)] was inoculated at 1 % with *Carnobacterium divergens* LV13 as the indicator strain. After 18 hours of incubation, the plates were examined for zones of inhibition. Immunity

Table 3.1. Bacterial strains and plasmids.

Bacterial Strain or Plasmid/phage Type	Description [a]	Source or Reference
Carnobacterium piscicola		
LV17A	Bac$^+$ Imm$^+$ containing pCP49	Ahn and Stiles, 1990b
LV17A.17	Bac$^+$ Imm$^+$ containing 17 MDa *Bgl*II religated fragment of pCP49	Ahn and Stiles, 1992
LV17C	Plasmidless strain	Ahn and Stiles, 1990b
Carnobacterium divergens		
LV13	Bac^s	B. G. Shaw, Bristol, U.K.
Escherichia coli		
MV 1193	Δ (*lac-pro*AB), *rps*L, *thr*, *end* A, *spc*B15, *hsd* R4, Δ (*srl-rec*A) 306:: Tn 10 (*Tet*^{r}) F' [*tra* D36, *pro* AB$^+$, *lac* I^q *lac* Z Δ M15]	Sambrook *et al.*, 1989
PLASMIDS		
pUC118	lacZ', Ap^r, 3.2 kb	Vieira and Messing, 1987
pK194	lacZ', Km^r, 2.4 kb	Jobling and Holmes, 1990
pCaT	Cm^r, 8.5 kb	Jewell and Collins-Thompson, 1989
pRW9.6	pCaT containing 9.6-kb *Pst*I fragment of pCP17; Cm^r, *CbnA*, *CbaI,X,R,K,T,C*, Bac$^+$ Imm$^+$, 18.1 kb	This study
pRW5.4C	pCaT containing 5.4-kb *Pst*I-*Xba*I fragment of pCP17;Cm^r, Bac$^-$ Imm$^+$, 13.9 kb	This study
pRW5.4	pUC118 containing 5.4-kb PstI-XbaI fragment of pCP17, Ap^r, 8.7 kb	This study
pRW5.2	pUC118 containing 5.2-kb *Eco*RI-*Xba*I fragment of pCP17, Ap^r, 8.5 kb	This study

[a] Bac$^+$ represents the production of carnobacteriocin A.
Imm$^+$ represents immunity to carnobacteriocin A

of the different strains was tested by deferred inhibition (as defined above) with the wild type *C. piscicola* LV17A as the producer and the strain in question as the indicator strain. In addition, a spot-on-lawn test of two-fold serial dilutions of cell-free supernatant was applied to APT agar and overlayered with 5 to 7 ml of soft APT inoculated at 1 % with the indicator strain.

3.2.3 DNA manipulation, cloning and transformation

Plasmids from *E. coli* and carnobacteria were extracted and purified as described previously (Sambrook *et al.*, 1989; and Chapter 2; respectively). Restriction endonucleases, Klenow fragment of *E. coli* DNA polymerase I, and T4 DNA ligase were obtained from Promega (Madison, WI), Bethesda Research Laboratories (Burlington, ON, Canada), Boehringer Mannheirm (Dorval, QU, Canada), or New England Biolabs (Mississauga, ON, Canada) and were used according to the recommendations of the supplier. Cloning and DNA manipulations were performed as described by Sambrook *et al.*, (1989). Competent *E. coli* cells were transformed by the method suggested in the Erase-a-base™ protocol. Transformation of carnobacteria was done by electroporation in 0.2 cm cuvettes using a Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, CA; settings 25 μF, 2.5 kV and 200 ohms) using the method described in Chapter 4 (see page 88).

3.2.4 DNA sequencing and computer analysis

Nucleotide sequencing analysis was performed by sequencing the DNA in both orientations by the dideoxy -chain method of Sanger *et al.*, (1977). DNA was sequenced by *Taq* DyeDeoxy Cycle sequencing on an Applied Biosystems 373A and Applied Biosystems Prism version 2.1.0 DNA sequencer (Applied Biosystems, Foster City, CA). For sequencing, stepwise deletion derivatives of cloned DNA fragments were made with the Erase-a-Base system from Promega. In addition, to fill in small gaps, a primer-

walking strategy was used for nucleotide sequencing. Synthetic oligonucleotides were made with an Applied Biosystems 391 PCR-Mate DNA synthesizer. Analysis of the nucleotide sequence was performed with DNASTAR (DNASTAR, Inc., Madison, WI) and DNA Strider 1.2 software programs. The search for homologies of the predicted amino acid sequences where compared with those contained in the SWISS-PROT protein sequence database release 31.0, March 1995; PIR release 45.0, June 1995; and GenBank release 91, October 1995. The homology comparisons and calculations were performed using the BLAST network service at the National Center for Biotechnology Information (NCBI).

3.3 RESULTS

3.3.1 Cloning of the genes involved in production of carnobacteriocin A

A 2.0-kb *Eco*RI fragment of a self-ligated 25.5-kb *Bgl*II fragment containing the *cbnA* structural gene was previously cloned into pUC118 and pK194, resulting in pRW2.4 and pRW2.4K (Figure 3.1). This fragment was cloned into the *Lactobacillus* cloning vector pCaT (Jewell and Collins-Thompson, 1989) but it did not produce carnobacteriocin A. A 5.4-kb *Pst*I-*Xba*I fragment cloned into pCaT also failed to produce carnobacteriocin, but it did confer immunity to carnobacteriocin A. With the inclusion of a 4.2-kb *Xba*I-*Pst*I fragment, yielding a 9.6-kb *Pst*I - *Pst*I fragment, full production of carnobacteriocin A and immunity was observed. This 9.6-kb *Pst*I - *Pst*I fragment cloned into pCaT showed full production and immunity to carnobacteriocin A. The 9.6-kb fragment was initially subcloned into 3 fragments and ligated into pUC118 (Figure 3.1). These three subclones were a 2.0-kb *Eco*RI - *Eco*RI fragment, a 3.2-kb *Eco*RI - *Xba*I fragment and a 4.2-kb *Xba*I - *Pst*I fragment. Further subcloning of the 3.2-kb *Eco*RI - *Xba*I fragment yielded a 1-kb *Eco*RI - *Sph*I and a 2.2-kb *Sph*I - *Xba*I fragment. Both were cloned into pUC118. The distal 4.2-kb *Xba*I - *Pst*I fragment was

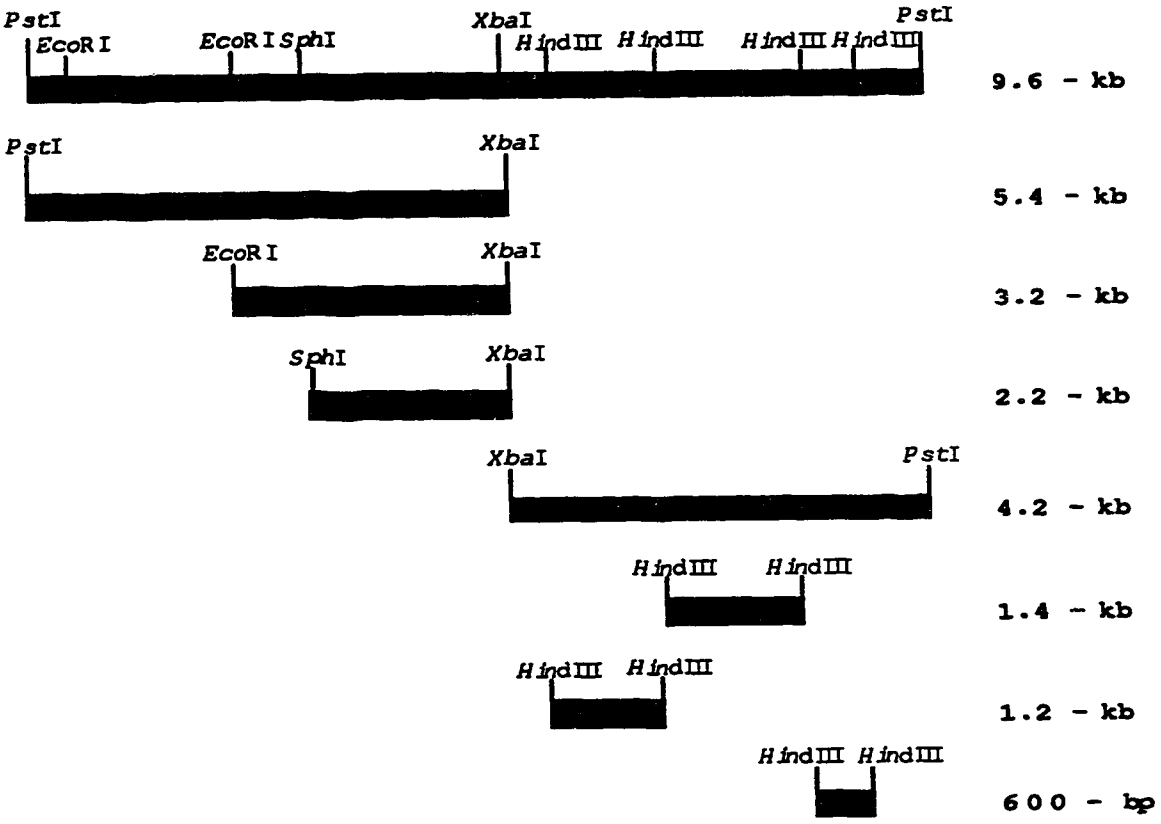

Figure 3.1. Diagrammatic representation of fragments used to clone the carnobacteriocin A genes.

subcloned as three *Hin*dIII fragments. All of the subclones were used to create step-wise deletion derivatives for sequencing (Figure 3.1).

Plasmids pRW5.4 and pRW2.4 were transformed into the plasmidless derivative *C. piscicola* LV17C. Immunity was restored with pRW5.4 but production of carnobacteriocin A was not observed. Preliminary results indicated that the immunity gene is located on this 3.2-kb fragment, but additional genetic information is required for expression of carnobacteriocin A. Transformation of pRW9.6 into *C. piscicola* LV17C restored full production of carnobacteriocin A and immunity, indicating that all of the information for bacteriocin production and immunity was present on this 9.6-kb *Pst*I fragment of pCP49.

3.3.2 Nucleotide sequence analysis

Restriction analysis confirmed the presence of the 2.1-kb *Eco*RI fragment containing the structural gene in the 9.6-kb *Pst*I fragment that was shown to be necessary for full bacteriocin production. The nucleotide sequence of 1.4-kb of this fragment is reported in Chapter 2. The additional 8.2-kb of information was sequenced in both directions by the dideoxy-chain termination method. The complete nucleotide sequence of the 9.6-kb *Pst*I fragment is shown in Figure 3.2. A total of seven ORFs, including the *cbnA* structural gene, was detected and they are shown diagrammatically in Figure 3.3. Downstream of the *cbnA* structural gene there is a small ORF of 153 nucleotides. It's translational product yields a peptide of 51 amino acids. It has a clearly distinguished ribosomal binding site 8 bases upstream of the start codon. Cloning of a 700-bp *Stu*I-*Hin*dIII fragment that contains this small ORF did not result in immunity to carnobacteriocin A, suggesting that it does not function as the immunity protein for carnobacteriocin A or that the regulation of this potential immunity gene is under the control of the response regulator (*cbaR*). For the carnobacteriocin B2 gene cluster, it was

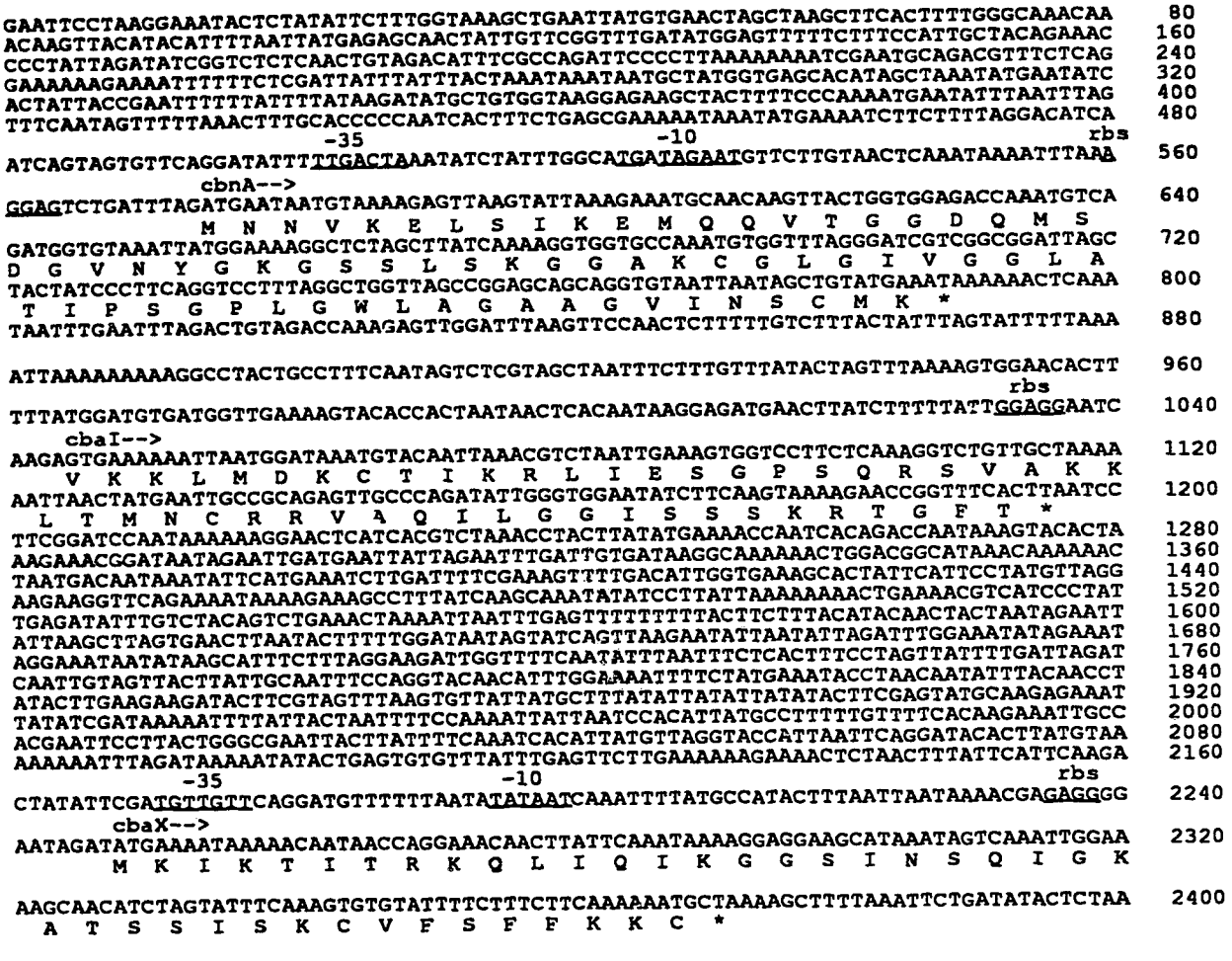

Figure 3.2. Nucleotide sequence of the 9.6-kb *Pst*I-*Pst*I fragment from the 25.5-kb self-ligated fragment of pCP17 from the pCP49 plasmid of *Carnobacterium piscicola* LV17, showing the structural, possible immunity and unidentified genes *cbnA*, *cbiA*, and *cbaX*, respectively. Putative ribosome binding sites (rbs) are underlinedm as are the -35 and -10 putative promotor regions.

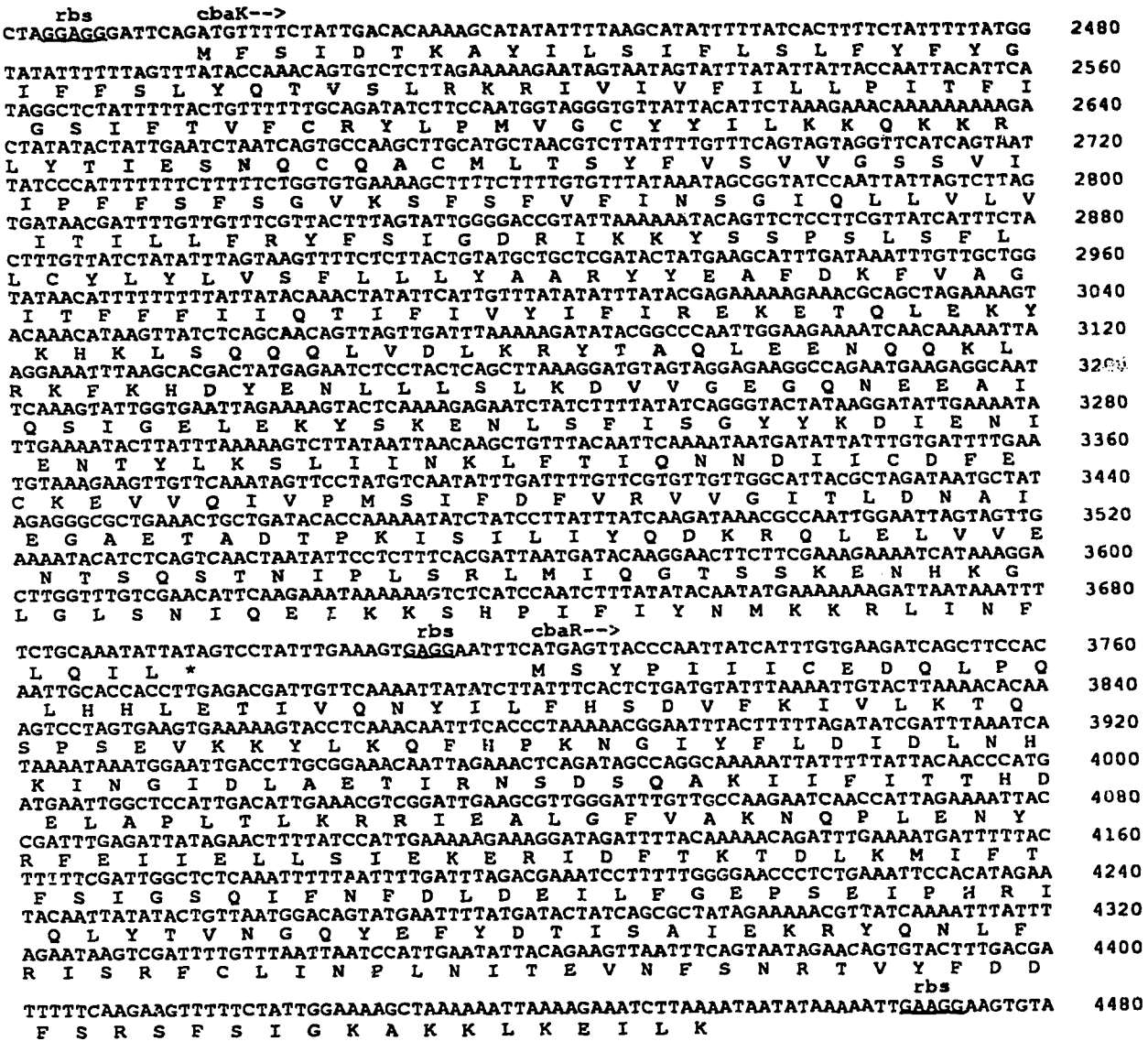

Figure 3.2. Cont. Showing the genes for the probable two-component regulatory system, *cbaK* and *cbaR*.

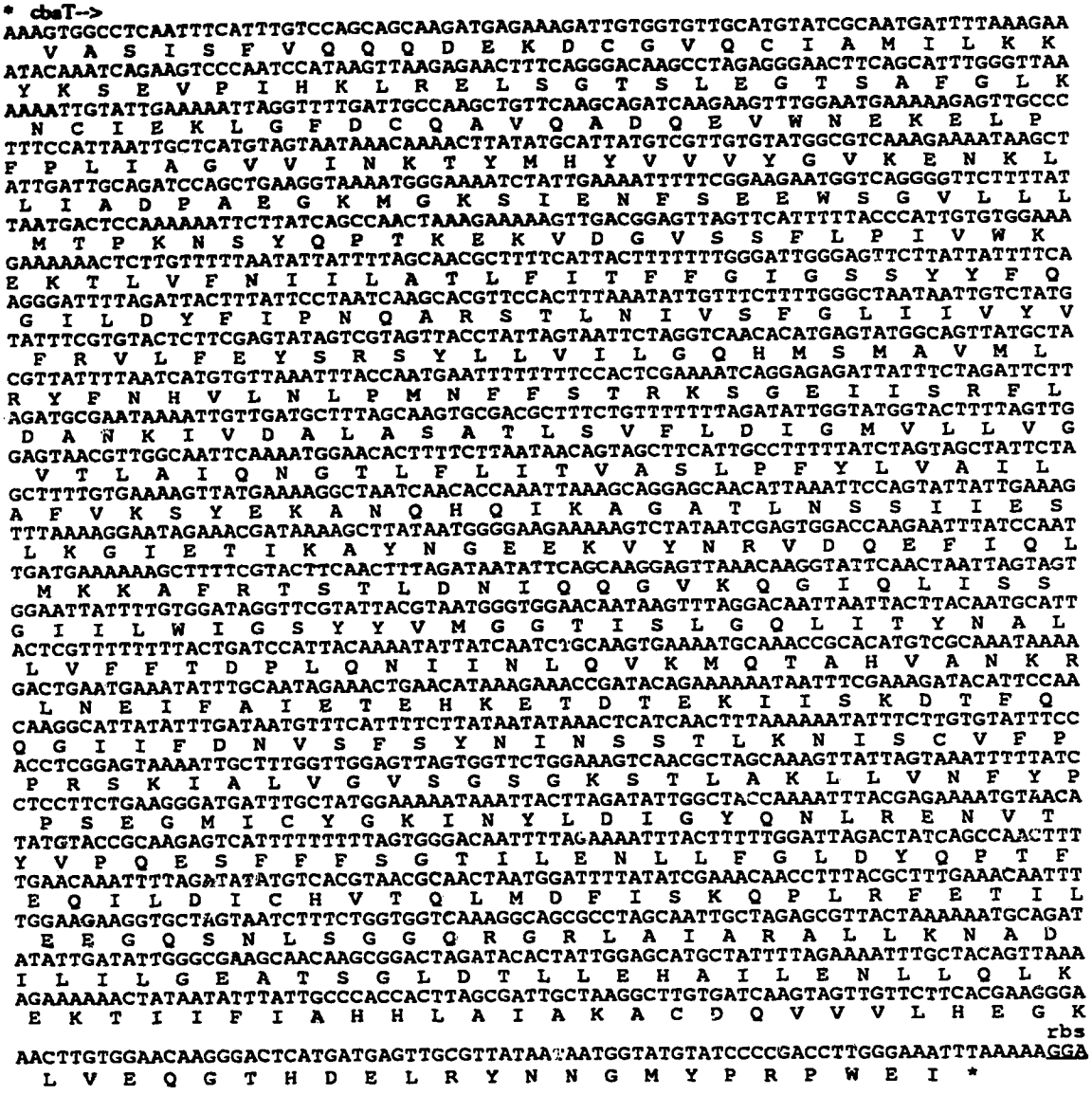

Figure 3.2. Cont. Showing the gene for the probable ABC translocator protein, *cbaT*.

```
    cbaC-->
GTTACCAATATGCAAACAAATAAGTGGCTTGATTCCTCCAGTGTATACAGCCAGCAACATAGTAAATTTTATCTTTGGGT   6720
         M  Q  T  N  K  W  L  D  S  S  S  V  Y  S  Q  Q  H  S  K  F  Y  L  W  V
GCTGTATCCCATCGGTGTTTTGTTTTTTTTATTAGGCCTATTTTTAGTTTTTGCCAGAAAAGAGGTAGTTAATCGTATTG   6800
 L  Y  P  I  G  V  L  F  F  L  L  G  L  F  L  V  F  A  R  K  E  V  V  N  R  I  A
CCCCCAAAAGTACAACCCAAACTATTAGTAAGTTACAAGCACCAATTGAAACCCAAATAACAGAAAATTACTTATACGAG   6880
  P  K  S  T  T  Q  T  I  S  K  L  Q  A  P  I  E  T  Q  I  T  E  N  Y  L  Y  E
AATAAAGTCGTAAAAAAAAGGTGAAATAGTGGTTGTTTTTGATACATTATCTTTAGAAAATGAACAAAAGCAATTTGAAGA   6960
N  K  V  V  K  K  G  E  I  V  V  V  F  D  T  L  S  L  E  N  E  Q  K  Q  F  E  D
TGAAGTTTTAGTTTTAGAGGAACAAAAGAAAGCAGCGCAGACCTTTATTATTAGTGTAGAGAATAATGAAAATCAATTTG   7040
 E  V  L  V  L  E  E  Q  K  K  A  A  Q  T  F  I  I  S  V  E  N  N  E  N  Q  F  V
TAGCAGATGATTCATTTGGGTATCCAAATCAATTAAATGCTCTATTTGCAGAACAGGAATCTCTTCAATATATTACACAA   7120
  Q  D  D  S  F  G  Y  P  N  Q  L  N  A  L  F  A  E  Q  E  S  L  Q  Y  I  T  Q
CAAGCAACAGATTTGAGTGAAATAAATCAAGAAGCTTACAAAAAAACAGAAGAGCAACTGGATTTTCAACTAACAAAACG   7200
Q  A  T  D  L  S  E  I  N  Q  E  A  Y  K  K  T  E  E  Q  L  D  F  Q  L  T  K  R
ATTAAATGCACAAAGTGAATGGGAACAAGTCAAAAAAGCTTGGGGAAATCAACAAGAAATACAAGAATTTTCTACGGAAA   7280
 L  N  A  Q  S  E  W  E  Q  V  K  K  A  W  G  N  Q  Q  E  I  Q  E  F  S  T  E  I
TTATTTCAAAATATAAAACTTGGCAATTACAAGTGAACGATGGTACTGAAGAACAAAAAAATCAAGTAATAGGAGCCATT   7360
  I  S  K  Y  K  T  W  Q  L  W  V  N  D  G  T  E  E  Q  K  N  Q  V  I  G  A  I
TTATCTACAATTGACGAAAATATTGGCGAATTAAAAAAAGAGATTGAACAAATTCAGGGTGAAAAAGCGAAACTTATTGG   7440
L  S  T  I  D  E  N  I  G  E  L  K  K  E  I  E  Q  I  Q  G  E  K  A  K  L  I  G
ACCCATTACATCAAAAAATGAAATAAATAGCGAAAATGCTAAGGTGAAACAAAACAAAGAACAACTATTAGCTAAGACTA   7520
 P  I  T  S  K  N  E  I  N  S  E  N  A  K  V  K  A  N  K  E  Q  L  L  A  K  T  K
AACAAGACATTATAGAATTTGATGACAAGCAAAAAAAAATTGAAGTGTCTATAAAACAATTAAAAGAAAAAATTCAACAG   7600
  Q  D  I  I  E  F  D  D  K  Q  K  K  I  E  V  S  I  K  Q  L  K  E  K  I  Q  Q
GGAATATTAAAGGCACCCATTGGCGGTACTATTTCACTTAATGAAGAATTTAAAACTATGATAGATATTCCTAAAGGGGC   7680
G  I  L  K  A  P  I  G  G  T  I  S  L  N  E  E  F  K  T  M  I  D  I  P  K  G  A
TCTTATTGCCGAAATTTATCCAACTACTGGAAGTAGGGAACAAACGTTTACCGCTCAACTACCCGCTAATGAAATGACAC   7760
 L  I  A  E  I  Y  P  T  T  G  S  R  E  Q  T  F  T  A  Q  L  P  A  N  E  M  T  R
GTATTAAAAAGGGAATGAATGTTCATTTTACATTGGATAAAAAAGGCGTCGCTGCAAAAATAGTTGATGGAAAATTAACA   7840
  I  K  K  G  M  N  V  H  F  T  L  D  K  K  G  V  A  A  K  I  V  D  G  K  L  T
GGAATTTCAGAAACAAGTGAAACAACAGAAAATGGAACTTTCTATACTGTTACAGGGAAAATTCAAATACCAAAAAACTT   7920
G  I  S  E  T  S  E  T  T  E  N  G  T  F  Y  T  V  T  G  K  I  Q  I  P  K  N  F
TAGTATTCGCTATGGCCTTACTGGGGAGATATCGTTGATTGTTGGAAAAAAAACATATTGGCAACAAATTAAAGATACAT   8000
 S  I  R  Y  G  L  T  G  E  I  S  L  I  V  G  K  K  T  Y  W  Q  Q  I  K  D  T  L
TATTAAATGTGGAATAATAAGTTAAAGACTTAGTTTGAAAAAATAAAAGAAAGAATAAATGATAAACTGGAGGGGGAGTA   8080
  L  N  V  E  *
CTTTGATAGGCATTATTGTAGCAATGAGCGGTTTGATAGGGATGATGTTACTTTATAAAAATGTAGAAATATACCCTATT   8160
TCTAATTTCGGATTTACTATTTATTGGACTAGCTTTACTATCTCTTTATTCTATGGTGTAGTTTCTAGTAAGATATAATG   8240
AGTATTAAATGGTCAACTATTGTAGATTTATTATTACTAGTAATGAGTTTAATAGGTATTATACTAGTTTTATCATCTTA   8320
TAAACCAAAGGAATTAACTAAACTAGCGCGCTACGGATATTTTTTAGTTATTTTTTATTTAATAGTTTTTAGCTGCATTA   8400
TTTTTTTAAGTATAGGTAAAATATGAAACTATTTTAAATTTAGAAATCTATGTTTTCTCTTACTATAAAGGAGGATGAGC   8480
GATGATAGAATTTATAATAGACGTCTTTTTAATAATGGCCGATACTATTCAATTAAAAAAGGTGAGAAAACAGTTGAAAA   8560
AAAAAAGAAAAAATCAATAAAATAAATGAATTACATCATATATTCTTCTACTAATTTAGAAGTGTGACAAAATGGTCTAA   8640
TTTTTTAGTTTATTATGTATAAAAAATAATTTGATGGCGATATCGGTGTAAGTAATATTCCAATGACAGATATTAATTTG   8720
GAATTTGGAGCAAAAAGTGTATCTGATATTGAAAAAGAAGAAAAAGTCAAAAACAAATAAAATGATTATAAAAATGACTT   8800
AAATAAACAAGTAAGGGCCTCACTAAAGTAGTATTTAGTGAGGCCCTTACTTGTTTATTCTTTCTCTTATTCTTCTACCC   8880
AATCTTCTAAACGTTTTTCTACAGTTTGATAGCATCCTATACAAGTAAATTTATCCGTATCGGGCTTCATAGAAGCTTTC   8960
AAAACTCTCAACTGCTTCTTCAAGCGTGAGATTAAATAGTTCTGAAACACATTGATAATAGTAAATATCTTTAGCTTCTC   9040
TACAACAATACCGACACTTCATAGGCATACCCCTTATCATATAGGACGTATGGATCACTTTACCTTATAAACAATTCTCA   9120
TCGCTTCTGGCATAGGTTGAACCGCTATGTTTTTTCTTACTCCAGTTTCAGTCCCAAACTCGTCATCTAACCAACGTTGC   9200
AGTTTAGAGCGTTCTGTAGACTCAAAATAAACCTTATGAGTCTCAATTCCAACTAATTTGAACAATTTTGTCAATTTTTA   9280
TGGATCTCCTTCAGAGATTATTTTTTTCTTTTGCATCAATTTTATCCAGGGTTAATTTTAAAATTTCCAAGTAAAGTCGA   9360
TCGTCTTTATTTTCTATCTTCACTTCTTTTTTAAGCTCAGAAAACAACAGTGCTGCTTTTTGGAAATTATCCATTTTATC   9440
CCTCTTTTTCTTGCTTACTTTACTTTAAAGTACCTTAATACGCCTTTAAAAGCAACTCATATATTAAATGACAATGAATT   9520
TTCCCAATCTGCATATGCCGAATTAGAATAATTAAACGTCTCTCCTTCAGACTGCAGGCATGCAAGCTTGGCACTGGCCG   9600
TCGTTTTACGAC                                                                        9612
```

*,stop codon

Figure 3.2. Completed. Showing the gene for the probable ABC translocator, accessory protein, *cbaC*.

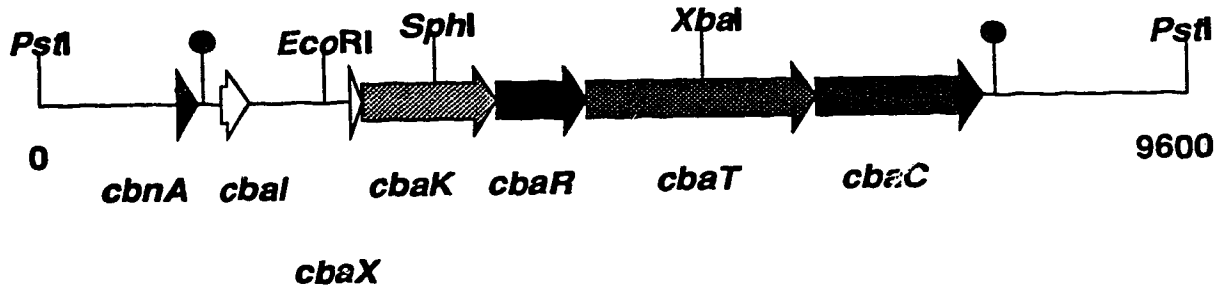

Figure 3.3. Diagramatic representation of the genes involved in carnobacteriocin A production showing unique restriction sites and possible *rho*-independent terminators shown as an ⊺.

shown that inactivation of the response regulator resulted in the loss of immunity (Quadri *et al.*, 1996). Further downstream of the possible immunity gene there is a small ORF designated *cbaX* that could encode a peptide of 41 amino acids. Immediately downstream of *cbaX* there is a fourth ORF (*cbaK*) that contains 426 codons. Following the TAG stop codon of *cbaK* is a fifth ORF designated *cbaR* that could encode a protein containing 245 amino acids A sixth ORF that has a GTG start codon and could encode a protein containing 716 amino acids was located immediately downstream of *cbaR* and was designated *cbaT*. Immediately downstream of the *cbaT* TAA stop codon was a seventh ORF designated *cbaC* that could encode a protein containing 456 amino acids. All six ORFs are preceded by probable ribosomal binding sites. Two possible promoters were identified upstream of the *cbaA* and *cbaI* genes. The putative promoter upstream of *cbaA* was described in Chapter 2. Another possible promoter sequence -35 (TGTTGTT) and -10 (TATAAT) was located upstream of *cbaX*. The spacing of 16 to 19 bases between the -10 and -35 regions agrees with the usual spacing found in LAB promoters. Possible rho-independent terminators were identified after the *cbnA*, *cbaI* and *cbaC* genes. No other ORFs were found in either strand in the 9.6-kb *PstI* fragment.

3.3.3 Similarity of *cbaX*, *cbaR* and *cbaK* gene cluster to two-component regulatory systems

Homology searches of the two putative proteins translated from *cbaR* and *cbaK* revealed significant homologies (32 to 58%) to established two-component bacterial regulatory systems, such as the *agr* -regulatory system that is found in *Staphylococcus aureus* (Vandenesch *et al.*, 1991). The ORF *cbaX*, showed highest homology (36%) to small ORFs upstream of two-component regulatory systems in bacteriocin-dependent secretion gene clusters such as ORF 4 in the sakacin A gene cluster (Axelsson and Holck, 1995) and 92% homology to ORF-6 of the carnobacteriocin B2 cluster (Quadri *et al.*,

1996). The role of this peptide is unknown at this time but it is likely that it is a signal peptide that induces production of the bacteriocin (Diep *et al.*, 1995). The homology comparisons for the three ORFs are shown in Table 3.2.

3.3.4 Similarity of *cbaT and cbaC* to ATP-dependent transport systems

Protein homology searches in the Swiss-Prot databases showed that the proteins deduced from *cbaT and cbaC* have marked homology with proteins of the HlyB-like family of ABC-transporters and accessory factors, respectively. This bacteriocin secretion system is independent of the signal peptide-dependent or general protein export system that is used in various prokaryotic organisms for the transport of proteins, such as proteases, fimbriae, β-lactamase, pili etc. (Von Heijne and Abrahmsén, 1989; Fath and Kolter, 1993). The ABC transporters have several highly conserved regions that include membrane spanning domains in the N-terminal region of the protein as well as ATP-binding domains (Håvarstein *et al.*, 1995; van Belkum and Stiles, 1995). The putative translational products of *cbaT and cbaC* show highest homologies to ABC-transporter proteins of *sapT* (51 %), *sppT* (50 %), *comA* (49 %), *mesI* and *lcnC* (48 %)and the accessory proteins of *lcaD* (26 %), *mesY* (25 %), *comB* (23 %) and *sapE* (21 %) (Axelsson and Holck, 1995, Huehne *et al.*, 1995, Hui and Morrison, 1991, Hui *et al.*, 1995, Fremaux *et al.*, 1995, Stoddard *et al.*, 1992, van Belkum and Stiles, 1995). Homology to other ABC-transporter and accessory proteins is shown in Table 3.3. Hydropathy profiles of *cbaT* gene product revealed the presence of several hydrophobic regions in the N-terminus of the protein. This correlates well with the presence of membrane spanning regions that are present in other ABC-translocators. Hydrophobicity analysis of the deduced *cbaC* product predicted mainly a hydrophilic protein with a strongly hydrophobic region in the N-terminus. The hydrophobic N-terminus and hydrophilic nature of the remainder of the protein is characteristic of other accessory proteins such as comB, a competence induction factor, as well as previously described

Table 3.2. Homology comparisons of three regulation proteins in the carnobacteriocin A gene cluster and other proteins reported in the data banks.

SOURCE	RESPONSE REGULATOR	HISTIDINE PROTEIN KINASE	ORF X
Carnobacteriocin A cluster from *C. piscicola*	CbaR	CbaK	X
Carnobacteriocin B2 cluster from *C. piscicola* (Quadri *et al.*, 1996)	CbnR 97%	CbnK 91%	orf-6 92%
Sakacin A cluster from *L. sake* (Axelsson and Holck, 1995)	SapR 58%	SapK 37%	orf-4 36%
agr-regulatory locus in *S. aureus* (Vandenesch *et al.*, 1991)	AgrA 32%	AgrB 26%	
Sakacin P cluster from *L. sake* (Huehne *et al.*, 1995)	SppR 30%	SppK 25%	
Plantaricin A regulatory genes in *L. plantarum* (Diep *et al.*, 1994)	Lpln3/4 33/32%	Lpln2 22%	

Note: Percentages were calculated using the BLAST network service at the National Center for Biotechnology Information (NCBI).

Table 3.3. Exporter and accessory protein homology comparisons to other proteins in the NCBI database using the BLAST program.

SOURCE	ABC EXPORTER	ACCESSORY PROTEIN
Carnobacteriocin A cluster from *C. piscicola*	CbaT	CbaC
Carnobacteriocin B2 cluster from *C. piscicola* (Quadri *et al.*, 1996)	CbnT 97%	CbnC 94%
Sakacin A cluster from *L. sake* (Axelsson and Holck, 1995)	SapT 51%	SapE 21%
Competence factor secretion system in *S. pneumoniae* (Hui and Morrision, 1991; Hui *et al.*, 1995)	ComA 49%	ComB 23%
Sakacin P cluster from *L. sake* (Huehne *et al.*, 1995)	SppT 50%	SppE 18%
Mesentericin Y105 secretion system in *L. mesenteroides* (Fremaux *et al.*, 1995)	MesI 48%	MesY 25%
Leucocin A secretion system in *L. gelidum* UAL187 (van Belkum and Stiles, 1995)	LcaC 48%	LcaD 26%
Lactococcin A secretion system in *L. lactic* subsp. *lactis* (Stoddard *et al.*, 1992)	LcnC 45%	LcnD 19%
Pediocin PA-1/AcH secretion system in *P. acidilactici* (Bukhtiyarova *et al.*, 1994; Marugg *et al.*, 1992)	PedD 39%	

Note: Percentages were calculated using the BLAST network service at the National Center for Biotechnology Information (NCBI).

bacteriocin secretion systems such as lcaD for leucocin A, mesY for mesentericin Y105 production, lcnD associated with lactococcin A, B and M secretion and well as sapE and sppE for sakacin A and P. As observed for the carnobacteriocin B2 and BM1 gene cluster, the cbaT of carnobacteriocin A contains two conserved amino acids, cysteine (residue 15) and histidine (residue 94) that have been shown to be proteolytic cleavage domains for lactococcin G (Håvarstein *et al.*, 1995) and pediocin PA-1(Venema *et al.*, 1995). The double glycine peptidase function is likely integrated with the ABC-translocator for cbaT in the same manner as previously described for bacteriocin translocator-peptidases.

3.3.5 Heterologous expression of carnobacteriocin A

All of the constructs containing various amounts of the original 9.6-kb fragment were transformmed into the heterologous host *C. divergens* LV13. They produced the same bacteriocin and immunity phenotypes as those observed for transformants in the plasmidless mutant LV17C. In some of the *C. piscicola* UAL 26 transformants there was an increased zone of inhibition. No increase in the size of the zone of inhibition was observed for *C. divergens* transformants.

3.4 DISCUSSION

Carnobacteriocin A is one of three bacteriocins produced by the wild type *C. piscicola* LV17 (Worobo *et al.*, 1994). The structural gene of carnobacteriocin A (*cbnA*) was originally identified and located on the 72-kb plasmid, pCP49. Initial characterization of carnobacteriocin A identified it as being a class II bacteriocin with all of the physical chkaracteristics and the Gly-Gly processing site of a class II bacteriocin. The size of carnobacteriocin A (5152 Da) was slightly larger than most class II bacteriocins that have been characterized to date. Holck *et al.* (1994) identified and

reported the same bacteriocin produced by *C. piscicola* LV61, designated piscicolin 61. Comparison of amino acid sequence and molecular mass revealed that carnobacteriocin A and piscicolin 61 are identical bacteriocins produced by different strains of *C. piscicola.* Both of these strains originated from the same culture collection and were originally isolated from vacuum packaged meat by Shaw and Harding (1984). These reports did not contain additional information about the genetic features of these bacteriocins, and it is not known if strain LV61 produces a bacteriocin equivalent to carnobacteriocin B2, or failing that if carnobacteriocin BM1 is encoded on the chromosome (Quadri *et al.*, 1994, 1996).

Class II bacteriocins from LAB generally have a second ORF immediately following the structural gene. This has been shown to be the immunity gene for carnobacteriocin B2 (Quadri *et al.*, 1995), leucocin A (van Belkum and Stiles, 1995), and pediocin PA-1 (Venema *et al.*, 1995). It is postulated that the immunity gene protects the producer cell from its own bacteriocin. Most immunity genes encode a protein of 110 to 115 amino acids. Analysis of the nucleotide sequence downstream of the *cbnA* structural gene did not show an ORF in the equivalent, contiguous position or of a size that could encode a protein of this size. Immediately following the structural gene there is a possible rho-independent terminator and there are numerous stop codons in all three phases of translation. Immunity to carnobacteriocin A was achieved with transformants that included the 3.2-kb *Eco*RI-*Xba*I DNA fragment located 1-kb downstream of the structural gene. Sequence analysis of this fragment revealed the presence of ORFs with translation products that are large proteins that show homology to histidine protein kinases and response regulators. Further examination of the downstream region of *cbnA* revealed a small ORF encoding a peptide containing 51 amino acids with a ribosomal binding site and a -10 and -35 promoter consensus sequence upstream of the ORF. Subcloning of this small region in front of a P32 promoter did not show the immunity phenotype to carnobacteriocin A.

Little or no homology has been reported between immunity genes. The only similarity that they share has been their size. The only bacteriocin immunity gene that shows any similarity to the putative immunity gene *cbaI* for carnobacteriocin A is *dviA* of divergicin A (Chapter 4) that also has an immunity peptide containing 51 amino acids. There was no apparent amino acid or nucleotide sequence homology between these two immunity proteins when they were compared through the BLAST search program. The immunity phenotype is only detected when the downstream region containing the regulatory genes is present. This indicates that the immunity gene for carnobacteriocin A is probably under the regulation and control of the response regulator and protein histidine kinase genes for this bacteriocin.

To achieve full production of carnobacteriocin A it was necessary to include the 7.4 kb of DNA downstream of the structural gene. It was originally postulated that this region contained the dedicated secretion machinery for carnobacteriocin A. The results of this study showed that, in addition to genes encoding the two transport proteins, there are genes for two proteins that show homology to regulatory proteins comprised of a response regulator and protein histidine kinase. It is proposed that these four genes together synchronize the initiation (regulation), production, transport and maturation of carnobacteriocin A.

When the four ORFs were analyzed using homology searches it was observed that they were highly homologous to other proteins involved in regulation and secretion of proteins and peptides. The highest homology of 92 to 94% identity existed with the carnobacteriocin B2 secretion and regulation proteins. These proteins were also found to be highly homologous with proteins associated with regulation and secretion of other bacteriocins, including: leucocin A, mesentericin Y105, lactococcin A, pediocin PA-1, sakacin A, plantaricin A and colicin V. Dedicated transport systems for nonbacteriocin proteins, such as hemolysin B and competence induction factors, also showed homology

to the proteins associated with carnobacteriocin A transportation (Felmlee *et al.*, 1985, Vandenesch *et al.*, 1991). The similarity of the four putative proteins associated with carnobacteriocin A to the regulatory and secretion proteins of the other bacteriocin systems suggests that these proteins are involved in the production of carnobacteriocin A.

Near identical amino acid and nucleotide sequences to those of the characterized carnobacteriocin B cluster suggests that a similar loss of production of carnobacteriocin A would occur if any of the secretion and regulation genes are inactivated. All four of these genes may be integral to the production and regulation of carnobacteriocin A which supports earlier observations of the lack of production with less than 9.6 kb of the DNA.

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4. A SIGNAL PEPTIDE SECRETION-DEPENDENT BACTERIOCIN FROM *CARNOBACTERIUM DIVERGENS*[1]

4.1 INTRODUCTION

Bacteriocins are antagonistic peptides or proteins that typically inhibit the growth of closely related bacteria. Klaenhammer (1993) divided the bacteriocins of lactic acid bacteria (LAB) into four classes based on their chemical, structural and functional properties. The class I and II bacteriocins are the best characterized. They are small, heat stable peptides that are inactivated by proteolytic enzymes of the intestinal tract. This makes them interesting as potential preservatives for foods. Class III and IV bacteriocins are not as well characterized and they are generally heat labile, large proteins or protein conjugates, respectively. The class I bacteriocins are known as lantibiotics because they contain unusual amino acids, lanthionine and β-methyllanthionine. The lantibiotics are not exclusive to LAB but they include nisin which is an important food preservative produced by *Lactococcus lactis* ssp. *lactis* (Delves-Broughton, 1990). Their production is characterized by extensive posttranslational modification of the gene product to produce the active peptide. Fusion of the N-terminal extension of subtilin in front of the structural element of nisin resulted in the production of nisin suggesting the involvement of the N-terminal extension in the secretion of lantibiotics (Kuipers *et al.*, 1993a). Nisin has a polycistronic gene cluster that requires close to 10 kb of DNA to control its production, secretion and posttranslational modification (Kuipers *et al.*, 1993b). Other LAB produce lantibiotics, such as carnocin UI49 produced by

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Carnobacterium piscicola (Stoffels *et al.*, 1992), but these lantibiotics have not been extensively characterized.

Class II bacteriocins produced by LAB are the largest group that have been characterized. They are produced as prebacteriocins that comprise the precursor of the mature peptide with an 18 to 24 amino acid N-terminal extension that is cleaved during secretion or maturation of the bacteriocin. They are minimally modified, apart from cleavage of the N-terminal extension at the Gly-Gly- (-2, -1) site. The role of the N-terminal amino acid extension of the class II bacteriocins has not been established, but it may be important in recognition of the prebacteriocin by the transport and maturation machinery of bacteriocin dependent secretion systems. The N-terminal extensions of class II bacteriocins have marked homology of their hydrophobicity profiles (Fremaux *et al.*, 1993; Håvarstein *et al.*, 1994; Quadri *et al.*, 1994). The class II bacteriocins, such as the lactococcins (Stoddard *et al.*, 1992) and pediocin PA-1/AcH (Marugg *et al.*, 1992; Motlagh *et al.*, 1992) require dedicated secretion and maturation systems to produce the extracellular, mature bacteriocin. They require less genetic information than nisin for their production, but they generally have structural and immunity genes as an operon, with the secretion genes in close proximity in a relatively condensed gene package contained in approximately 3.5 to 4.5 kb of DNA (Marugg *et al.*, 1992; Van Belkum, 1994).

Initial cloning vectors for Gram-positive bacteria were constructed as chimeric plasmids carrying replicons for both Gram-positive and Gram-negative organisms, but the need to construct these vectors was alleviated with the discovery of small cryptic lactococcal plasmids that were functional in lactococci and *Escherichia coli*: for example, 2.3 kb plasmid pWV01 from *Lactococcus lactis* subsp. *cremoris* Wg2 (Kok *et al.*, 1984); 2.1 kb plasmid pSH71 from *L. lactis* subsp. *lactis* (de Vos, 1987); and 5.5 kb plasmid pDI25 from *L. lactis* subsp. *lactis* 5136 (Xu *et al.*, 1990, 1991). A family of lactococcal

cloning vectors was constructed from pDI25 using a 1 kb fragment encoding chloramphenicol resistance from the staphylococcal plasmid pC194 (Xu *et al.*, 1991). In our laboratory, we used the native, chloramphenicol resistance plasmid pCaT from *Lactobacillus plantarum* as a cloning vector, but it is too large for cloning purposes and it relies on antibiotic resistance as a selection marker (Ahn *et al.*, 1992). Several small cryptic plasmids from nonlactococcal lactic acid bacteria have been reported: for example, 2.14 kb plasmid pC30i1 from *Lb. plantarum* (Skaugen, 1989); 2.49 kb plasmid pLC2 from *Lb. plantarum* NCDO 1088 (Bates and Gilbert, 1989); 3.55 kb plasmid pLB4 from *Lactobacillus curvatus* LTH683 isolated from raw sausage (Klein *et al.*, 1993); 2.4 kb plasmid p353-2 from *Lactobacillus pentosus* MD353 and a 1.9 kb plasmid p8014-2 from *Lb. plantarum* ATCC 8014 (Leer *et al.*, 1992). A catalase gene from *Lactobacillus sake* LTH677 has been cloned that has the potential to serve as a food-grade marker gene (Knauf *et al.*, 1992). In our studies, we are interested in developing bacteriocin production or immunity as a food-grade selection marker.

The focus of our research on bacteriocins of meat-related LAB, *Carnobacterium piscicola* [currently proposed as *Carnobacterium maltaromicus* (Collins *et al.*, 1991)] and *Leuconostoc gelidum* (Hastings *et al.*, 1991; Quadri *et al.*, 1995; Worobo *et al.*, 1994), has been to characterize these bacteriocins with a view to enhancing their antibacterial spectrum through site-directed mutagenesis or production of two or more bacteriocins within "gene cassettes". *C. piscicola* LV17 produces three bacteriocins (Quadri *et al.*, 1994; Worobo *et al.*, 1994), and contains at least two independent secretion systems for the three bacteriocins, although regulation of production of these bacteriocins is interrelated (Saucier *et al.*, 1995). In contrast, lactococcins A, B, and M share a common secretion system for all three bacteriocins (Van Belkum, 1994). This indicates that for multiple bacteriocins to be expressed by one organism may require more than one secretion system and multiple immunities. There are no reports of immunity proteins capable of expressing immunity to more than one bacteriocin or of

secretion proteins involved in the production of multiple, heterologous bacteriocins. As a result of these bacteriocin dependent immunity and secretion genes, gene cassettes would be difficult to produce with class II bacteriocins because of the large amount of DNA required for immunity to, and production of, each bacteriocin. In this study we report the purification and sequence of a novel bacteriocin, divergicin A, that functions as a signal peptide secretion-dependent bacteriocin, that does not require bacteriocin dedicated secretion machinery. We also report the complete nucleotide sequence of pCD3.4 with a view to its use for a food grade cloning vector for use in lactic acid bacteria.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 4.1. Carnobacteria were grown in APT broth (Difco Laboratories Inc., Detroit, MI) at 25 °C, and *Escherichia coli* was grown in Luria-Bertani (LB) broth (Sambrook *et al.*, 1989) on a rotary shaker at 250 rpm at 37 °C. Solid media were prepared by adding 1.5% agar to the broth media; overlay media were prepared with 0.75% agar. Stock cultures of carnobacteria were maintained in Cooked Meat Medium (Difco) at 4 °C and at -70 °C in APT broth (Difco) containing 40% (v/v) glycerol. *E. coli* strains were stored in LB broth with 40% (v/v) glycerol at -70 °C. When appropriate, media were supplemented with antibiotics in the following concentrations: ampicillin and erythromycin at 200 µg/ml for *E. coli*; and erythromycin at 10 or 5 µg/ml for broth and solid media, respectively, for carnobacteria.

Table 4.1. Bacteria and plasmids used to study bacteriocin production by *Carnobacterium divergens* LV13.

Bacterial Strain	Description[a]	Source or Reference
C. divergens		
LV13	*Dvn*$^+$ *Dvi*$^+$ (containing pCD3.4)	NCFB[c]
AJ	Dvn^S (Indicator strain)	laboratory isolate
C. piscicola		
LV17C	Dvn^S (indicator strain), plasmidless	(Ahn and Stiles, 1990)
LV17A	*Cbn*A (containing pCP49), Bac$^+$ Dvn^S	(Ahn and Stiles, 1990)
LV17B	*Cbn*B2 & BM1 (containing pCP40)	(Ahn and Stiles, 1990)
UAL 26	Bac$^+$ Dvn^S	(Ahn and Stiles, 1990)
L. lactis subsp. *lactis*		
MG1363	Dvn^r, plasmidless	(Gasson, 1983)
IL1403	Dvn^r, plasmidless	(Chopin *et al.*, 1984)
Escherichia coli		
DH5α	F$^-$, *end* A1, *hsd* R17(r_k^-, m_k^+), *sup*E44, *thi*-1, λ^-, *rec*A1, *gyr*A96, *rel*A1, Δ (*arg*F-*laczya*)UI69, Ø80*dlac* Z Δ M15]	BRL Life Technologies Inc.
MH1	MC1061 derivative; *ara*D139, *lac*X74, *Gal*U, *gal*K, *hsr*, *hsr*$^+$*str*A	(Casadaban and Cohen, 1980)

Table 4.1. Cont. Bacteria and plasmids used to study bacteriocin production by *C. divergens* LV13.

Plasmids		
pCD3.4	*Dvn*$^+$, *Dvi*$^+$, 3.4 kb	this study
pCD4.4	pCD3.4 containing 1.0 kb *Eco*RI Cm^r gene of pGS30; Cm^r, *Dvn*$^+$, *Dvi*$^+$, 4.4 kb	this study
pUC118	lacZ', Amp^r, 3.2 kb	(Vieira and Messing, 1987)
pGS30	pUC7 containing 1.0 kb *Pst*I Cm^r gene of pC194; Cm^r, 3.7 kb	G. Venema[b]
pKM1	pUC7 containing 1.3 kb *Pst*I Km^r gene of pUB110; Km^r, 3.7 kb	G. Venema[b]
pGKV259	Em^r, Cm^r, 5.0 kb	(van der Vossen *et al.*, 1987)
pRW5.6	pGKV259 containing a 583 bp *Eco*RV-*Acc*I fragment; Em^r, *Dvn*$^+$, *Dvi*$^+$, 5.6 kb	this study
pRW6.0	pGKV259 containing divergicin signal peptide fused to alkaline phosphatase	this study

[a] *Dvn*$^+$ divergicin production gene; *Dvi*$^+$ divergicin immunity gene; Dvn^r divergicin resistant strain; Dvn^s divergicin sensitive strain; Bac$^+$ bacteriocinogenic strain; Em^r Erythromycin resistance; Cm^r Chloramphenicol resistance; Amp^r Ampicillin resistance; Km^r Kanamycin resistance.

[b] G. Venema laboratory strain, Department of Genetics, University of Groningen, Haren, The Netherlands.

[c] NCFB: National Collection of Food Bacteria, Reading, U.K.

4.2.2 Production and purification of divergicin

Partial purification of divergicin was done with a 1% inoculum of an overnight culture of *C. divergens* LV13 in 2 liters of APT broth, maintained at pH 7.5 with 2 N NaOH using a pH stat (Chem-Cadet, Cole Palmer) while stirring gently at 25 °C. The culture was adjusted to pH 5.6 with 5 N HCl and heated to 70 °C for 35 min before centrifugation (9000 x *g*, 5 min, 4 °C). The bacteriocin in the supernatant was precipitated with ammonium sulfate (700g/L) by stirring for 24 h at 4 °C, and harvested by centrifugation (9000 x *g*, 20 min, 4 °C). The precipitate was dissolved in 60 ml of 20 mM 2-(N-Morpholino) ethanesulfonic acid (MES; Sigma Chemical Co., St. Louis, MO) pH 5.5 (fraction A). Ammonium sulfate (200g/L) was added to fraction A and precipitated proteins were removed by centrifugation (9000 x *g*, 20 min, 4 °C). The clarified fraction A was loaded onto a 45 ml octyl sepharose CL4B (Pharmacia, Uppsala, Sweden) chromatography column equilibrated with 20% ammonium sulfate in 20 mM MES, pH 5.5. The column was washed with 1.5 volumes of 20%, 15%, 10%, 5% and 0% ammonium sulfate in 20 mM MES pH 5.5 followed by washes with 1.5 volumes of water, 10% and 70% ethanol. The active fraction was concentrated by rotary evaporation and resuspended in 6 M urea and loaded onto Sephadex G-75 column (2.5 x 120 cm; Pharmacia) equilibrated with 6 M urea. Elution was monitored by absorbance at 228 nm. All fractions were assayed for bacteriocin activity by spot-on-lawn test (Ahn and Stiles, 1990). The urea was removed by dialysis in 3500 molecular weight cut-off dialysis tubing (Spectra Por, Spectrum Medical Industries Inc., Los Angeles, CA) and lyophilized.

Complete purification was achieved using the method previously described by Hastings *et al.* (1991), except that the culture was grown in 5 liters of APT broth

supplemented with 1% glucose. After incubation for 20 h, the cells were removed by centrifugation and the culture supernatant was applied directly to an Amberlite XAD-8 column (4 x 40 cm; BDH Chemicals Ltd., Poole, England) washed with 3 liters of 0.05% trifluoroacetic acid (TFA), 2 liters of 20% and 35% ethanol in 0.05% TFA. The active fraction eluted with 50% ethanol in 0.05% TFA (1.5 L) was concentrated by rotary evaporation and 10% (5 ml) was loaded onto a Sephadex G-50 column (2.5 x 120 cm; Pharmacia) that had been equilibrated with 0.05% TFA. The active fractions were concentrated to 1 ml by rotary evaporation and applied in 100 µL portions to a C_4 column (Waters Delta-Pak, 10 x 200 mm, 15 µm, 300 Å, flow rate 1.5 ml/min, mobile phase: A, 0.05% TFA in water; B, 95% ethanol in 0.05% TFA) and eluted using a gradient method: first 50% to 63% B solvent in 7 min and then 63% to 64% in 6 min. Fractions were monitored for absorbance at 218 nm and for activity against the indicator strain. Purity of the fractions was checked by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). Bacteriocin production and immunity were tested using deferred inhibition assay (Ahn and Stiles, 1990).

4.2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Purified divergicin preparations were examined for the presence of contaminating proteins using 15% polyacrylamide gels and 3 M Tris-HCl, pH 8.8. Electrophoresis was done at constant current of 20 mA as described by Laemmli (1970). Gels were fixed in 50% (v/v) methanol, 10% (v/v) acetic acid for 30 min and stained with Coomassie Brilliant Blue (Bio-Rad Laboratories Ltd., Mississauga, ON). To test for activity, the polyacrylamide gel was washed with water (2 x 1 L) for 2.5 h. The gel was placed onto an APT plate and overlayered with soft APT agar inoculated with 1% of a sensitive indicator strain.

4.2.4 N-terminal amino acid sequence and amino acid analysis

Partially purified divergicin obtained using octyl sepharose CL4B and Sephadex G-75 was subjected to SDS-PAGE, the proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 50 V for 3 h in blotting buffer 10 mM $NaHCO_3$, 3 mM Na_2CO_3, pH 9.9 in 20% ethanol (Dunn, 1986). The blotted membrane was stained with 0.25% Coomassie Brilliant Blue in 40% methanol and destained with 50% methanol. The protein band corresponding to activity on the overlayered gel was excised and used for N-terminal amino acid sequencing by Edman degradation analysis on an automated gas phase sequencer (Applied Biosystems model 470A, Foster City, CA) with on-line phenylthiohydantoin derivative identification by reversed-phase HPLC (Applied Biosystems model 120A). Amino acid analysis was done on a similarly excised band containing the peptide. The amino acids were derivatized with phenylisothiocyanate on an Applied Biosystems (model 420A) derivatizer and separated by HPLC (Applied Biosystems model 130A) using a C-18 column. Data were recorded and analyzed on an Applied Biosystems (model 920A) data analyzer system.

4.2.5 Mass spectrometry

The mass spectra of divergicin were determined by Mr. Paul Semchuk of Protein Engineering Centres of Excellence (PENCE), University of Victoria, B.C., Canada. Electrospray mass spectra were acquired on a Fisons VG Quatro instrument. Divergicin, which had been purified by HPLC, was introduced by direct infusion (1 µg/ml) in 50% aqueous acetonitrile containing 0.5% TFA through a sample loop. Fisons software was employed to determine the molecular mass from the envelopes of multiply charged peaks in the *m/z* spectra.

4.2.6 DNA isolation, manipulation and sequence analysis

Large scale plasmid preparation from *C. divergens* LV13 was done as previously described for *C. piscicola* LV17A (Worobo *et al.*, 1994). Other DNA manipulations

were based on those described by Sambrook *et al.* (1989). *Pfu* DNA polymerase (Stratagene, LaJolla, CA), restriction endonucleases and T4 DNA ligase were obtained from Promega (Madison, WI), Bethesda Research Laboratories (Burlington, ON), Boehringer Mannheim (Dorval, PQ), New England Biolabs (Mississauga, ON) and used according to the suppliers' recommended procedures. Step-wise deletion derivatives for sequencing were prepared using the Erase-a-Base® system (Promega) and DNA fragment recovery was done using Geneclean II® (Bio 101 Inc., LaJolla, CA). Carnobacteria were transformed by resuspending a 3-hour culture from a 2% inoculum in one tenth of the volume of cold Milli-Q water and collecting the cells by centrifuging at 6000 x *g*. The cells were washed twice with electroporation buffer (ETB; 0.5 M sucrose, 2.5 mM $CaCl_2$) and resuspended in 1/40th of the original volume of ETB. The cells were electroporated with a Gene Pulser™ (Bio-Rad) using the method recommended by the supplier. Immediately following electroporation, 1 ml of APT containing 0.5 M sucrose was added, the cells were incubated at room temperature for 3 hours, and plated onto appropriate selective media. Oligonucleotides prepared as sequencing and PCR primers were synthesized on an Applied Biosystems (model 391) PCR Mate synthesizer. Double stranded DNA was sequenced by *Taq* DyeDeoxy Cycle sequencing on an Applied Biosystems (model 373A) sequencer. For amplification of the DNA encoding the mature part of alkaline phosphatase, primers

KLR 179 (5' GCGCAAGCTTCTGCTCGGACACCAGAAATGCCTGTT 3') and

KLR 180 (5' GGCCAAGCTTGCCATTAAGTCTGGTTGCTA 3') were used with the *E. coli* C_4F_1 (Torriani, 1968) alkaline phosphatase gene as a template.

4.2.7 Assay for alkaline phosphatase

Cells from 1.5 ml of an overnight culture grown in LB broth were centrifuged (9000 x *g*, 5 min, 25 °C) and washed in an equal volume of STE (50mM NaCl, 10 mM

Tris pH 8.0, 1 mM EDTA pH 8.0). The culture media and periplasmic fractions were assayed for alkaline phosphatase. Periplasmic fractions were prepared by resuspending the washed cells in 0.5 ml of 20% sucrose with 50 µl of 0.5 M EDTA and 25 µl of lysozyme (10 mg/ml) and incubating at room temperature for 15 min. The samples were centrifuged (9000 x *g*, 5 min, 25 °C) and the supernatant was assayed for alkaline phosphatase activity (Torriani, 1968) by absorbance at 405 nm.

4.2.8 Nucleotide sequence accession number

The DNA sequence of 541 bp of DNA containing the structural and immunity genes for divergicin A was submitted to GenBank (Los Alamos, NM) and was given the accession number L37791.

4.3 RESULTS

4.3.1 Production and purification of divergicin

Carnobacterium divergens LV13 produces a bacteriocin, divergicin A that is active against the indicator strains *C. piscicola* LV17C and *C. divergens* AJ. The amount of divergicin produced by *C. divergens* LV13 in APT broth at pH 7.5 at 25 °C for 18 h was 2 mg/L. Different media and pH conditions were tested for increased bacteriocin production. Divergicin was not produced in the semi-defined medium CAA used by Hastings *et al.* (1991). Of the media tested, only APT supported bacteriocin production. Best production was achieved at pH 6.5 with 1% glucose added to the APT broth. For purification of divergicin using ammonium sulfate precipitation followed by octyl sepharose chromatography, all of the activity was eluted in the 70% ethanol wash. Using size exclusion chromatography (Sephadex G-75) most of the activity was detected in the second peak. This fraction was concentrated and subjected to SDS-PAGE and transferred onto a PVDF membrane. The band corresponding to activity on the SDS-PAGE gel that was overlayered with the sensitive *C. piscicola* LV17C indicator strain,

was excised from the membrane and used for Edman degradation and amino acid analysis. Divergicin is rapidly inactivated in acetonitrile that was used in our initial HPLC purification protocol but it was stable in ethanol. Using ethanol as the carrier solvent enabled further purification of divergicin A and the determination of its molecular mass. HPLC purified bacteriocin for mass spectrometry was obtained using Hastings *et al.* (1991) protocol with gradients of ethanol and water containing TFA to elute the pure divergicin.

4.3.2 N-terminal sequence and amino acid analysis of divergicin

The excised bacteriocin band from the electroblotted PVDF membrane enabled the following 21 amino acid N-terminal sequence to be determined: Ala-Ala-Pro-Lys-Ile-Thr-Gln-Lys-Gln-Lys-Asn-X-Val-Asn-Gly-Gln-Leu-Gly-Gly-Met-Leu-Ala-. It matches the amino acid content derived from the nucleotide sequence and amino acid analysis except for the apparent absence of methionine in the amino acid analysis. Methionine was not detected in the amino acid analysis probably due to the small amount of bacteriocin contained on the blotted membrane. The unidentified amino acid at position 12 was subsequently shown to be cysteine by interpretation of the nucleotide sequence (Figure 4.1a).

4.3.3 Mass spectrometry of divergicin

Mass spectral analysis using positive ion fast atom bombardment with an electrospray interface gave multiply charged molecular ions (Figure 4.2) which allowed calculation of the average molecular mass of 4223.9 ± 0.1 Da.

4.3.4 Identification and expression of the divergicin structural and immunity genes

The native plasmid pCD3.4 was completely sequenced (Figure 4.1a and 4.1b) and four possible open reading frames were identified. The restriction map of pCD3.4 is shown in Figure 4.3. The probable replication protein (repA) is shown on the restriction

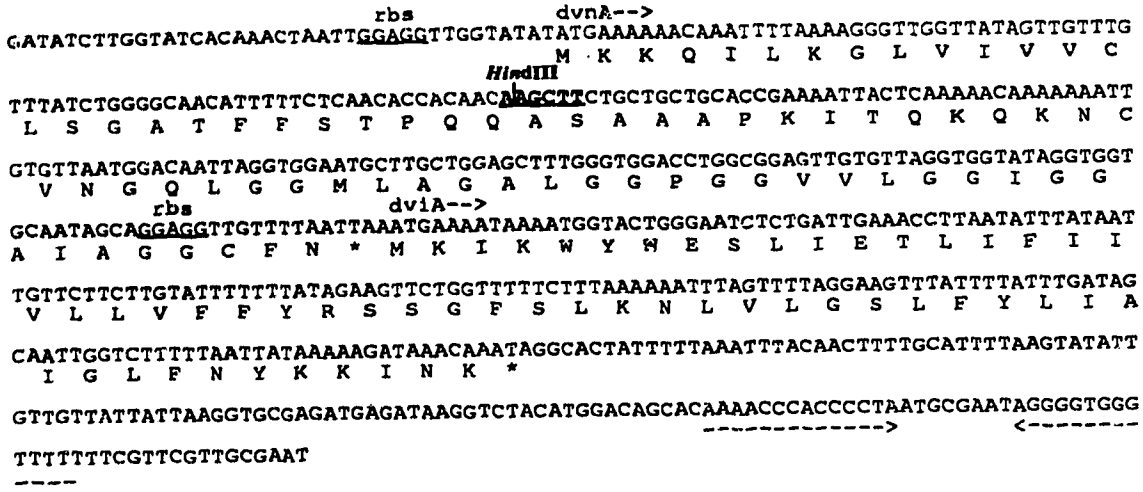

Figure 4.1a. Nucleotide sequence of a 581-bp fragment from pCD3.4, a native plasmid of *Carnobacterium divergens* LV13, showing the deduced amino acid sequence for the precursor of divergicin A and its putative immunity protein below the nucleotide sequence. A potential *rho*-independent termination site is indicated by reversed arrows on the nucleotide sequence and the putative ribosome-binding site (RBS) is underlined.

```
                                                ACGAACGTGTGGGTTAGAGACAACTTGCGAGATTATCGTCTACACAGCCTAACCAATGA   640
TCCACTAGTATTAATACTAGTCCCCCAC AAAGTGGAGCAATAACCAATGAGATA GGGTTTCCATAAACAGCACCCCCTT   720
TCAGGGGCAAGTTGCCACTTACTAATATAGCACAGCTCCTTTATTGTTCTTAGTCTAAATCTGATAAATCTTTTCTTGTT   800
CACAAATATAGACCACTTAAAAGCTTATAACGGTACTAGATTTTTCAGATACCCCAATTACCTACTTAAAACGTCTCTCT   880
TTTTCGTTTTAAGATGTTTAAAATTATTTTCTATGAATTATACACAAATGTGCTTAAATCGTCTTAAATCGTCTTAAAAT   960
GTGGTCTGTGTTGAGAATACAACGACTTTGTTTGGTCGTACCTCTAAATCTGTTTGCTGTGGGCGAGGGTAGCGAAGTGA  1040
ACTTTTTGTGCTAACGCTCTTGGTTTTGTCTTTTGATTTTATAAAATGTGGATGTAATCCACATACTCAAGCTTGCATGC  1120
CTTTAGAAAATAAAGGAGCTTGCGAATGCAAGGTGCCCTTTTTTCTTTGTCTGACTACTAGGGACAAATTATCTGAGTAT  1200
GAACAAGATTTTGTCTGTTCTTGCGCGTATTTATTAATATATATTTTAAGAGATATTTTAAGAGATATTTTAAAACCTTT  1280
TTAGGGGTGAGCTCAGCCTTAGAGAGAGTAATACTTGTAGCATAGTGCTAGGGACACATTATCTGACTACTAGGGATAAA  1360
TTATCTGACTACTAGGGACAAATTATCTGACTACTAGGGAGAAATTATCTGTCTCCTAGGGACGCACTTTACTTTGTGTA  1440
TCGTATCGTTTATAATCTTTATATGTGAGGGGAGGTCGAAAGGATTGGAAAAGAAAACGAATTTAAAAATTGCATATCCA  1520
AAATGAATTGAATCTGGTTCCACTTAAAAATTTCAATGCTAAACAAATGGATTTATTCTTTGCTTTGTGTGCCCGAATGA  1600
AAAATAAAGGGCTTAGAAAGGTATCTTTTACGTTTGAAGAACTAAGAGAACTAAGTGATTACAAGATAACTGCTATCGAA  1680
CCGTTCACGAATGATTTAGAACAATTATACAAAAAAATGTTAAACCTAACATACAGAACGGAGACAGAAACAAAAATCAG  1760
TTATTTCGTTTTATTTACTGGGTTGTGATTGATAAATCAGACCAAATTGTTGAAGTTAGTGTAAACCCAGACTTAGAACA  1840
TATCATTAACGGTATCTCTAGTGAGTTCAATAAATTTGAGTTTCTAGCATTCACAAGTATCCAGTCGAAATATACGAAAA  1920
                    repA-->
CACTCTTTAGATTGCTTATGCAGTTTCAATCAACTGGGTTTTATGTGGTTAAAATTGAAGATTTCAGAGCACTTTTAGAC  2000
                      M  Q  F  Q  S  T  G  F  Y  V  V  K  I  E  D  F  R  A  L  L  D

ATTCCAAAATCTTATCAAATGACTGACATAACCCAACGGATATTGAAACCTAGTTTAATTGAGTTAAGTCAGTACTTTAA  2080
I  P  K  S  Y  Q  M  T  D  I  T  Q  R  I  L  K  P  S  L  I  E  L  S  Q  Y  F  N

TGATTTAAAAGTTAATAAAATTAAAGCTCGAAAGGGTAATAAAATAGACCGTTTAGAATTCACTTTCTCCGGTCTAAAGA  2160
 D  L  K  V  N  K  I  K  A  R  K  G  N  K  I  D  R  L  E  F  T  F  S  G  L  K  T

CTGATTTACCTAAAGTTCCATTGCACGACTGGACGAAATAAAAAAAGGACCTCCCCCTCACATTTAAGCAAGTAGGAACG  2240
  D  L  P  K  V  P  L  H  D  W  T  K  *
                                                                    rbs
TCCCTCGCAATCCACGAAGACTGCTGATTCATTTTAGCATATATTGTGCGGGACTTCTAAATAAATTATATTTGGAGGTC  2320
      orfX-->
ATTTTTATGTCGAATAAGTACTTGAAAAAAAGAAAGCGTCAAGCTAAGCAGGTAGCTGATTTGTACGATTTAATTATTGG  2400
      M  S  N  K  Y  L  K  K  R  K  R  Q  A  K  Q  V  A  D  L  Y  D  L  I  I  G

GGTTGAACATGCTGGCAGCTCGTTAATTGCGTTGTATGAGGGAATTAAACCCTCTCAATATCGAATTTTTATTCTTTTGT  2480
 V  E  H  A  G  S  S  I  I  A  L  Y  E  G  I  K  P  S  Q  Y  R  I  F  I  L  L  S

CTTATTCTAGTTTTGAAAATAAATTAAACTTATACAACAAAGCGATTTTAAGAACTGAAGTTTATTCTTTAGAAAAAAAA  2520
  Y  S  S  F  E  N  K  L  N  L  Y  N  K  A  I  L  R  T  E  V  Y  S  L  E  K  K

ATTAAACGAAAAAATAAATGCTCAAATCACAATTGCGCAAAAAAAATAAAAAAGAAATTGCGGTAATTGATTTCACAAAAC  2600
I  K  R  K  N  K  C  S  N  H  N  C  A  K  K  *
```

Figure 4.1b

```
AAAAAGAAAAACTCAAAAGAGAATTACTTAGTTTTGAAAATGATAAAGAAATGAAACTTATGGATTCGCAATTAAAACAA   2680
TTTCATGAAAATAAAACGTTAGCTGATATTAATGATCAGTTTTTTATGACGGTACAAAATAGTTTAATTTTGCTGCATAA   2760
AAAAGCACCTTTAACATTAAAATTAATTTGTTTGAAAAATTATATTCGCCTTTGCAAAAATTATTTTCTAAAGAATATAT   2840
TTTAATGTTTTTTGAAAAAAATAGTAACATGGGAACATGTTGCTCTGCTCGCAAAAGGAAAAATATTTAAACTAATAAAA   2920
AACCGTCGGAGACCACCCAACCAATAGGTTGGCTTTAAGTTTAAGCCTACGTTGACAACTGTCAATGTATAAGTGCGCCC   3000
TTTGGCTGTTTTATTTTTTGTTTAACTATTATTTTCTGCATAGGTTTTTTATTTTTATTAATTTGATTTTCAAGAAAGGG   3080
ATGAACCTAAAATGATTTATAAACAAAAAAAGAAAGAAGATGTTTTTGGATTTCCTAAAGTTTTAACAATTGCTGATTTG   3160
AGTACGAGATGGAAAATGTCACGTCAAGCTATCCATAAAAAAATTCGAGAAGATTTATTATTTCCTATGCCTGTTCAAAT   3240
TGTCTCAAATGGAAAAATTAAATTGTTTTTATTTGTTGATAGTGAAAAATACGAAAAAAATCGTCCGTGGTTATTAGACA   3320
TTAATTATCGAAATGAGCGACAACTTTGGATTTACACAAATGGTTTTTTTAAATAGCAAGTTAGTCAATTACCTTATACC   3400
TTGTTGGATATCTTTGGATAAAAAAATAGTTGTAT                                                3435
```

Figure 4.1b. Nucleotide sequence of the remaining 2854 bp of pCD3.4 showing the deduced amino acid sequence for two ORFs repA and orfX. A potential ribosome-binding site (RBS) preceeding orfX is underlined.

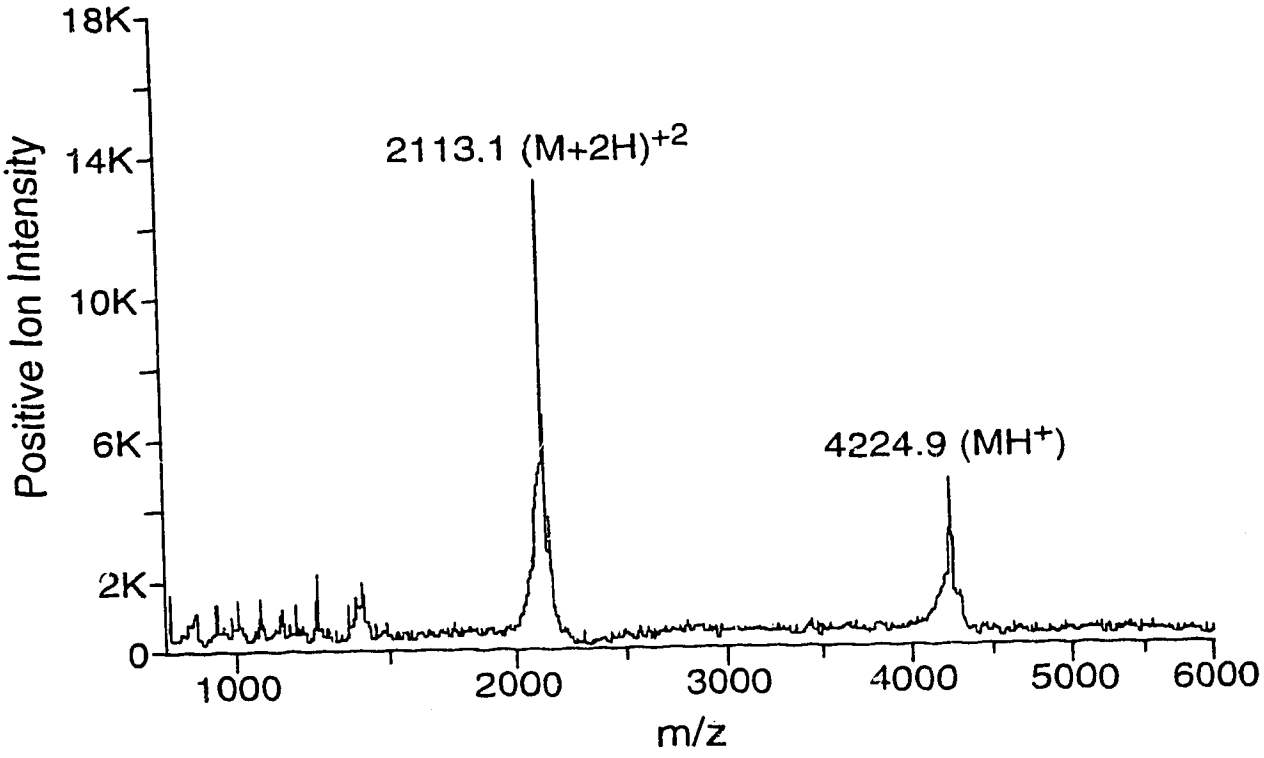

Figure 4.2. Electrospray FAB mass spectrum of divergicin A showing multiply charged molecular ions from which atomic mass was calculated.

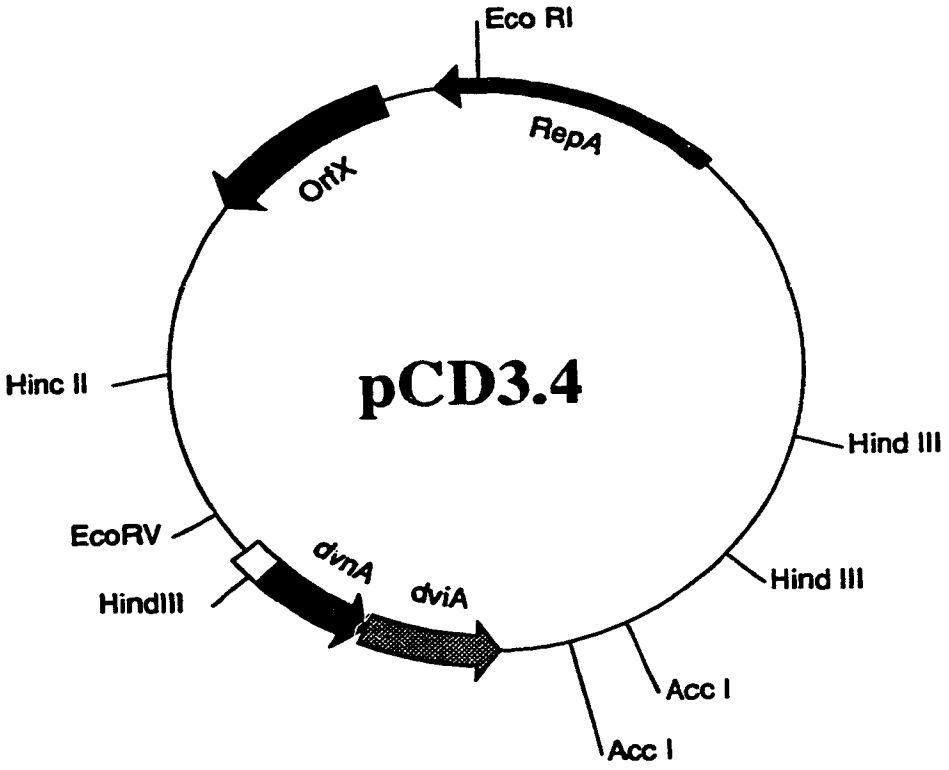

Figure 4.3. Restriction map of pCD3.4 and assignment of the genetic determinants of divergicin A.

map but further confirmation of its role in the replication process is required. The N-terminal amino acid sequence of divergicin was observed in the nucleotide translation product in the open reading frame (ORF) containing 225 nucleotides. This ORF is contained in the 514-bp *Eco*RV - *Acc*I fragment of pCD3.4 (Figure 4.1), starting with the alanine residue at position 30 of the structural gene for divergicin A (*dvnA*). This gene encodes a 75 amino acid prepeptide consisting of a 29 amino acid N-terminal extension and a 46 amino acid bacteriocin. Immediately following the divergicin structural gene is a second ORF that encodes 56 amino acids that could be the protein for divergicin immunity. Two probable ribosome binding sites (GGAGG) for the divergicin structural gene and possible divergicin immunity gene are located 9 and 13 base pairs, respectively, upstream of the initiator codons. Downstream of the second ORF (nucleotides 530 to 564) there is a 14 base inverted repeat with a 7 base loop that is a potential *rho*-independent terminator. An unique *Ssp*I site contained within the divergicin immunity gene (*dviA*) was used to insert a kanamycin resistance marker from pKM1 and to inactivate the immunity gene. Nucleotide sequencing of the immunity gene was done to confirm insertion into the *dviA* gene and the loss of immunity was accompanied by the strain still producing divergicin A but growth of these organisms was poor.

The native plasmid pCD3.4 with a chloramphenicol resistance marker from pC194 inserted into the *Eco*RI site (not shown) to produce pCD4.4 was used to transfer the plasmid to heterologous carnobacteria hosts including *C. piscicola* LV17A, B and C and UAL 26 (Table 4.1). Transformation of pCD4.4 into heterologous *C. piscicola* and *C. divergens* hosts resulted in chloramphenicol resistance as well as production of, and immunity to, divergicin. To utilize the *Pst*I site immediately following the P59 promoter of pGKV259, the 514-bp *Eco*RV - *Acc*I fragment and the *Pst*I site of pGKV259 were blunt-ended and the fragment was cloned into the *Pst*I site, resulting in the construct

pRW5.6. The presence of the correct fragment and orientation for the expression of divergicin A was confirmed by restriction enzyme analysis. The presence of the structural and possible immunity genes for divergicin was also confirmed by nucleotide sequencing. Electroporation of the pRW5.6 construct into other *Carnobacterium* hosts resulted in full divergicin production and immunity determined by deferred inhibition assays (see Figure 4.5, page 105). The "half moon" halo effect in the zones of inhibition shown in Figure 4.5, panel B was created by spotting pronase E in close proximity to the producer organism, confirming that the inhibition was due to bacteriocin production. The pRW5.6 construct was also electroporated into two *Lactococcus* strains MG1363 and IL1403. Expression was achieved in both of these strains with more divergicin produced by strain MG1363 than by strain IL1403. Divergicin immunity in the *Lactococcus* strains could not be tested because the host strains are normally resistant to divergicin.

4.3.5 Alkaline phosphatase fusion

PCR-generated DNA encoding the mature part of alkaline phosphatase with *Hin*dIII linking ends was inserted into the *Hin*dIII site (Fig 4.1a) of the divergicin signal peptide. The *dvnA* Ala-Ser-Ala- cleavage site was regenerated by incorporation of these three residues into the PCR primer. The presence of phosphatase activity was screened by direct plating of the ligation mixture onto LB agar containing X-Phosphate (X = 5-bromo-4-chloro-3-indolyl) and appropriate antibiotics. Blue colonies were selected and the tranformants were cultured in liquid medium. The periplasmic and culture media fractions were assayed for phosphatase activity using a spectrophotometric method (Torriani, 1968) that uses the hydrolysis of *p*-nitrophenylphosphate and the production of *p*-nitrophenol (*p*NP) for quantitative assay (Table 4.2). The data in Table 4.2 show that the alkaline phosphatase activity for the phosphatase fusion is located in the culture

N-terminal extension of class I & II bacteriocins

Class I	
Nisin A	M S T K D F N L D L V S V S K K D S G A S P R -
Class II	
Pediocin PA-1	M K K I E K L T E K E M A N I I G G -
Leucocin A	M M N M K P T E S Y E Q L D N S A L E Q V V G G -
Carnobacteriocin A	M N N V K E L S I K E M Q Q V T G G -

Signal peptide of divergicin A

M K K Q I L K G L V I V V C L S G A T F F S T P Q A S A -

Figure 4.4. Amino acid sequence of the signal peptide of divergicin A compared with the sequence of the N-terminal amino acid extensions of well characterized class I and class II bacteriocins of lactic acid bacteria.

Table 4.2. Alkaline phosphatase assay.

Sample	Enzyme units[a]
MH1 supernatant	4.37
MH1 periplasmic	0.18
MH1 + pRW6.0 supernatant	167.00
MH1 + pRW6.0 periplasmic	89.35

[a] One enzyme unit = 1 μmol *p*-nitrophenol min^{-1}. Activity units calculated per ml of original culture.

media and the periplasm. The amount of phosphatase activity in the fusion construct compared to the control shows active production and secretion of phosphatase.

4.4 DISCUSSION

We are interested in the development of a food grade vector suitable for use in our studies of bacteriocin production by lactic acid bacteria that were isolated from meats. The plasmid pCD3.4 in *C. divergens* LV13 was of interest because of its high copy number and the possibility that it was responsible for the production of divergicin A. Initially it was thought that the plasmid was too small to contain the genetic information for bacteriocin production, or that bacteriocin production was controlled by genes on the plasmid and on the chromosome, as in the case of carnobacteriocin BM1 (Quadri *et al.*, 1994). The purification of divergicin A and the determination of its N-terminal sequence enabled the position of the structural gene for divergicin to be located on pCD3.4. From the precise agreement of the calculated and determined molecular mass of divergicin A of 4223.89 Da. and 4223.9 Da, respectively, it was evident that no posttranslational modifications occurred, such as a disulfide bridge between the cysteine residues at positions 12 and 44. Formation of disulfide bridges in mature bacteriocins has been observed for carnobacteriocins A and B2 (Quadri *et al.*, 1994; Worobo *et al.*, 1994), leucocin A (Hastings *et al.*, 1991) and pediocin PA-1 (Marugg *et al.*, 1992).

Divergicin A has a novel amino acid sequence, N-terminal extension and processing site. Its small size, hydrophobic nature and thermostability are indicative of a Class II bacteriocin as defined by Klaenhammer (1993). This class of bacteriocins includes pediocin PA-1, sakacins A and P, curvacin A, lactacin F, lactococcins A, B, G and M, carnobacteriocins A, BM1 and B2, mesentericin Y105 and leucocin A-UAL 187. All of the Class II bacteriocins for which the nucleotide sequence has been characterized are produced as precursors that contain an N-terminal extension with Gly-Gly- processing

site at positions -1 and -2 of the cleavage site. These N-terminal extensions have marked homology in their amino acid sequences (Fremaux *et al.*, 1993; Håvarstein *et al.*, 1994; Klaenhammer, 1993; Quadri *et al.*, 1994). However, the N-terminal extension of divergicin has no homology with that of the Class II bacteriocins and, compared with other LAB bacteriocins, it has an unique processing site of Ala-Ser-Ala-. No homology exists with a recently identified peptide antibiotic AS-48 produced by *Enterococcus faecalis* that is postulated to contain a signal peptide in the N-terminal region of the immature antibiotic (Martínez-Bueno *et al.*, 1994). The N-terminal extension of the divergicin structural gene resembles a signal peptide (see Figure 4.4, page 101), comparable to signal peptides of α- and β- amylase, alkaline phosphatase, outer cell wall proteins, β-lactamase, fimbriae and proteases that access the *sec* -dependent pathway in bacteria (Von Heijne and Abrahmsén, 1989). Signal peptides have three distinct domains referred to as the N, C and H regions (Perlman and Halvorson, 1983; Von Heijne and Abrahmsén, 1989). The N terminus region has at least one lysine residue in the first 6 to 8 amino acids; the H region contains 8 to 15 amino acids and has strong hydrophobicity; and the C region contains the cleavage site that consists of neutral amino acids with small side chains at positions -3 and -1, respectively (Von Heijne, 1983, 1986a). The 29 amino acid N-terminal region of divergicin complies with all of the Von Heijne rules for a signal peptide. The signal peptide for divergicin also contains a proline located two residues upstream of the -3 Ala. This is thought to bend the signal peptide so that it can expose the cleavage site to the peptidase (Von Heijne, 1986b).

Class I and II bacteriocins that have been characterized from LAB require a dedicated secretion apparatus that in most cases has been shown to be genetically linked to, or associated with, the bacteriocin structural and immunity genes. The possibility that all of the genetic information required for divergicin expression was contained on a small 3.4-kb plasmid seemed likely because transformation of the pCD3.4 into heterologous, bacteriocinogenic and nonbacteriocinogenic hosts resulted in divergicin production and

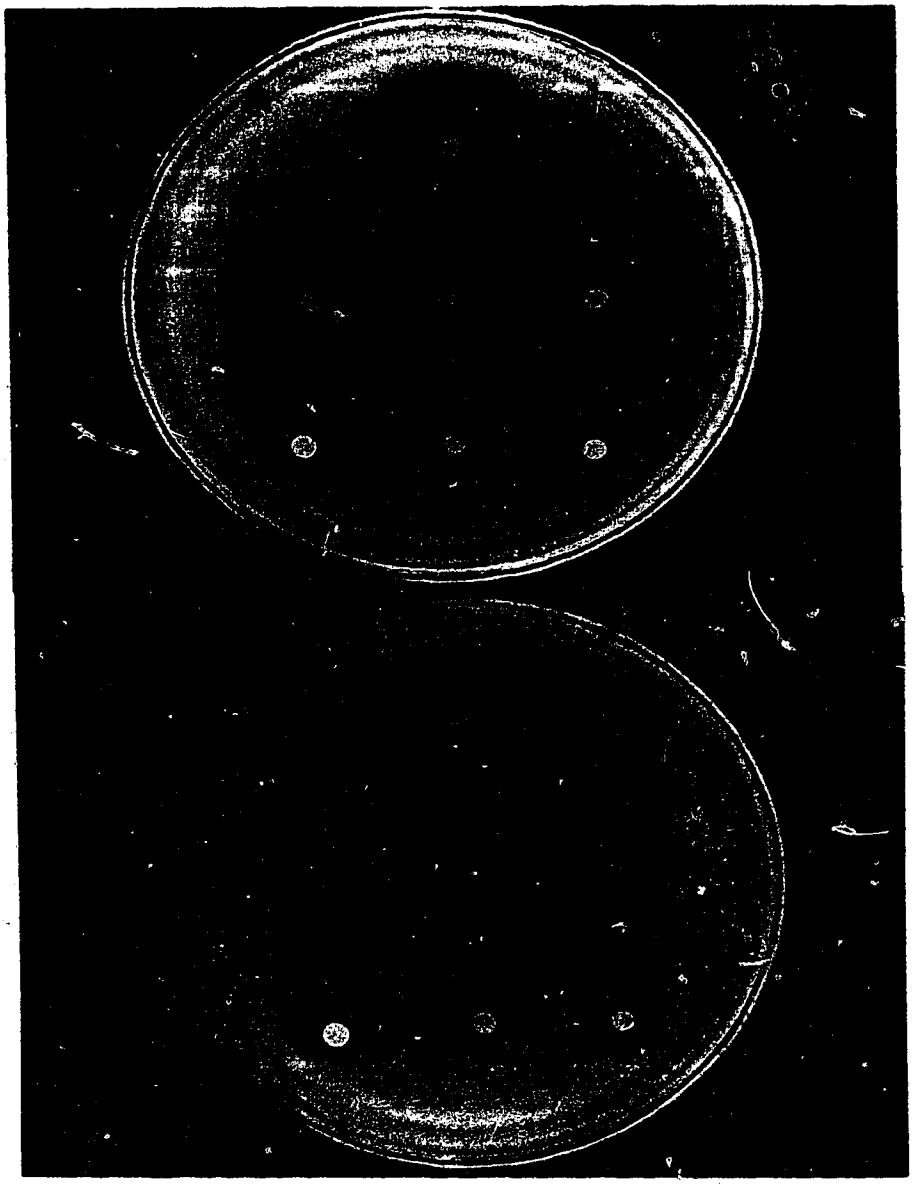

Figure 4.5. **Deferred inhibition of *Carnobacterium divergens* AJ by homologous and heterologous hosts transformed with the divergicin A gene.**

Panel A: a) *Carnobacterium divergens* LV13; (b) *Carnobacterium piscicola* LV17C containing pRW5.6; (c) *Lactococcus lactis* MG1363 containing pRW5.6; (d) *Lactococcus lactis* IL1403 containing pRW5.6; (e) plasmidless strain of *Carnobacterium piscicola* LV17C; (f) plasmidless strain of *Lactococcus lactis* MG1363; (g) plasmidless strain of *Lactococcus lactis* IL1403.

Panel B: Producer strains as described above. This illustrates the inactivation of divergicin A by pronase E.

immunity. The remaining 2.9-kb portion of pCD3.4 is too small to encode the different proteins necessary for secretion of the bacteriocin equivalent to those for pediocin PA-1, lactococcins A, B and M, or nisin A. All of these bacteriocins rely on two or more large proteins for their activity, including an ATP binding cassette (ABC) translocator protein (Klaenhammer, 1993).

Comparison of the N-terminal sequence with established signal peptides and the absence of additional secretory and maturation genes indicated that divergicin may be utilizing existing signal sequence dependent general export pathways in the host. This was confirmed by cloning the dedicated genes for divergicin production and immunity present on the 514-bp *Eco*RV-*Acc*I fragment of pCD3.4 behind the P59 promoter of pGKV259. Production of divergicin A was also observed in heterologous hosts such as *C. piscicola* LV17C, *L. lactis* strains IL1403 and MG1363. *L. lactis* IL1403 contains a set of genes on the chromosome involved in production and secretion of lactococcins (Stoddard *et al.*, 1992). However, production of divergicin in IL1403 was not greater than that observed in MG1363, indicating that a dedicated bacteriocin secretion machinery is probably not involved in divergicin production. Conclusive evidence that the N-terminal extension of divergicin acts as a signal peptide was achieved with the production of alkaline phosphatase in *E. coli* when it was fused behind the signal sequence for divergicin. Divergicin requires as little as 0.5-kb for its production; whereas most bacteriocins require 3.5 to 10 kb for independent bacteriocin production. Although the spectrum of activity for divergicin A is narrow, the signal peptide creates interesting possibilities for secreting other bacteriocins via the *sec*-dependent pathway. With gene fusions between the signal peptide of divergicin and structural genes of other bacteriocins it may be possible for bacteriocins of interest to be secreted without the specific secretion and maturation proteins.

Most LAB bacteriocins have a regular pattern of hydrophobic and hydrophilic domains. Divergicin A is a very hydrophobic molecule with a calculated pI of 9.2 and only one small hydrophilic region. Several class II bacteriocins have been shown to contain a positively charged residue at the N-terminus of the mature molecule. This would most likely inhibit secretion through the general export pathway (Izard and Kendall, 1994). The class II bacteriocins have a common mode of action (Abee *et al.*, 1994; Bhunia *et al.*, 1991; Maftah *et al.*, 1993; Van Belkum *et al.*, 1991). They are membrane active compounds that cause cell permeabilization with loss of proton motive force and efflux of intracellular components. The immunity protein of divergicin has the unusual feature of being a small, very hydrophobic molecule and an unusual topology because the second of the two transmembrane segments is flanked by positive charges. Divergicin A may have a different mode of action or immunity. Divergicin A contains seven Gly-Gly- paired residues in the mature peptide. The Gly-Gly- motif is a feature of microcin B17 that is an inhibitor of DNA replication (Davagnino *et al.*, 1986). The unique nature of the Ala-Ser-Ala- processing site and the signal peptide indicate that divergicin A does not fit established classes of bacteriocins. Further study will indicate whether divergicin A represents a fifth class of bacteriocins (Klaenhammer, 1993). Detection of a similar system in *Enterococcus faecalis* (Martínez-Bueno *et al.*, 1994) represents another bacteriocin that may access the *sec* pathway of bacteria.

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5. GENERAL DISCUSSION AND CONCLUSIONS

The genus *Carnobacterium* was originally identified as "atypical lactobacilli" among the lactic acid bacteria on vacuum packaged meats (Shaw and Harding, 1984; McMullen and Stiles, 1993). Subsequent studies revealed that carnobacteria are more widespread than in the microflora of vacuum packaged meats (Franzmann *et al.*, 1991), but on meats they do not seem to cause overt spoilage despite the fact that they are heterofermentative. We obtained several strains of LAB from B. G. Shaw (Meat Research Institute, Bristol, UK) in 1986. These included atypical lactobacilli that were subsequently shown to be *C. divergens* (strain LV13) and *C. piscicola* (strain LV17). *C. piscicola* LV17 was shown to be bacteriocinogenic and *C. divergens* LV13 was a good indicator organism for the bacteriocin(s) produced by strain LV17 as well as many other bacteriocinogenic LAB isolated from meats (Ahn and Stiles, 1990a). Initial studies on *C. piscicola* LV17 revealed that it produced at least two plasmid-mediated bacteriocins. The plasmids mediating production of these carnobacteriocins were separated into different strains LV17A, LV17B and a nonbacteriocinogenic, plasmidless strain LV17C (Ahn and Stiles, 1990b, 1992). This set the ground work for further study of these bacteriocins. The bacteriocin produced by strain LV17A was of particular interest because it is detected early in the growth cycle of the producer strain, compared with the bacteriocin(s) produced by LV17B which are only detected at the end of the growth cycle. Subsequent studies revealed that these bacteriocins require their own bacteriocins to induce production (Saucier *et al.*, 1995) but whether early and late detection of the bacteriocins is a concentration or a genetic effect is currently under investigation. The early production of a bacteriocin is considered a potential advantage for a producer strain intended for use in biopreservation of meats. The characterization of carnobacteriocin A was undertaken (by Worobo) and is reported in Chapter 2 of this thesis (Worobo *et al.*, 1994); and carnobacteriocin B by Quadri *et al.* (1994).

5.1 CARNOBACTERIOCINS

The genetic information for the two bacteriocins is encoded on separate plasmids, which facilitated their study. It became apparent that the B bacteriocins were more complex than the A bacteriocin. Two B bacteriocins were identified and characterized, carnobacteriocins BM1 and B2 (Quadri *et al.*, 1994). The genetic information for carnobacteriocin B2 is located entirely on the plasmid, whereas the structural and putative immunity genes for carnobacteriocin BM1 are located on the chromosome. Production of carnobacteriocin BM1 depends on proteins encoded on the B2 plasmid. The immunity gene for carnobacteriocin B2 was confirmed (Quadri *et al.*, 1995) and an additional four genes involved in the production of carnobacteriocin B2 (and probably BM1) have been characterized (Quadri *et al.*, 1996). Involvement of the genes *cbnK, cbnR, cbnT* and *cbnD* in bacteriocin production and immunity has been studied. Frame-shift mutations in these genes eliminated or, in the case of *cbnK*, reduced the production of carnobacteriocins and reduced the levels of immunity to carnobacteriocins BM1 and B2. Translational products of *cbnK* and *cbnR* have homology with elements of bacterial two-component signal transduction systems, while those of *cbnT* and *cbnD* have homology with ATP-dependent translocators and accessory proteins of the bacterial signal-sequence independent secretion machinery. Results indicated that the carnobacteriocins are exported by secretion machinery formed by CbnT and CbnD, and that production of, and immunity to, the carnobacteriocins are regulated by CbnK and CbnR. The B bacteriocins are complex, require at least 10 kb of DNA to encode the genes necessary for their production, and may include at least two other bacteriocins or possible inducers (Quadri *et al.*, 1996). From this information it is apparent that *C. piscicola* LV17 produces multiple bacteriocins and that the genetic information for some of them is interrelated.

The objective of the study of carnobacteriocin A production was to determine the chemical composition of the bacteriocin and to characterize its genetic control.

Subsequent research was directed toward the development of a food grade plasmid vector for production of multiple bacteriocins that would have a broad spectrum of antibacterial activity. The genetic analysis of carnobacteriocin A was more difficult than that for carnobacteriocins B and other bacteriocins, because the genes for carnobacteriocin A production are less compact that those for carnobacteriocins BM1 and B2. The discovery of a novel bacteriocin from *C. divergens* LV13 dramatically influenced the direction of this study and greatly improved the possibility of genetic modification of LAB to produce a battery of bacteriocins. The production of multiple bacteriocins by one "starter organism" was not accomplished in this study, leaving this exciting possibility for further study and for application to food systems.

The first task in identifying the gene responsible for carnobacteriocin A was the purification of the peptide to homogeneity to enable amino acid sequencing. Using hydrophobic interaction chromatography, size exclusion and reverse phase HPLC, carnobacteriocin A was purified and the amino acid sequence of the N-terminus of the mature peptide was elucidated using Edman degradation. A span of seven amino acids was used to design a degenerate oligonucleotide probe to locate the structural gene that encodes carnobacteriocin A. Although it appeared that there may be three different bacteriocins produced by strain LV17A, N-terminal amino acid sequencing and mass spectrometry data revealed that, unlike strain LV17B, only one active compound was produced and that the other peaks represented different chemical modifications of the same compound. A 2-kb *Eco*RI fragment was cloned and sequenced but only revealed the structural gene for carnobacteriocin A. This bacteriocin is active against *Listeria monocytogenes* (Ahn and Stiles, 1990), but compared with other "*Listeria*-active" bacteriocins (Klaenhammer, 1993), it lacks the YGNGV consensus sequence near the N-terminus of the mature bacteriocin. The lack of the YGNGV region suggests the possibility of an alternative active site for the targeting of *Listeria* cells. Presently, no research has been done to establish the correlation between the YGNGV consensus

region and the sensitivity of *Listeria*. Similarities to other bacteriocins can be observed in specific conserved amino acids in the mature molecule and in the N-terminal extension of the bacteriocin.

Initial studies revealed that the structural gene alone was insufficient for production of the bacteriocin phenotype. With the addition of 7.4-kb of DNA downstream of the structural gene, production of carnobacteriocin A and immunity was restored. Subcloning and sequencing of this downstream region elucidated the genes involved in producing the mature bacteriocin and immunity. Within the 7.4-kb a total of six genes was identified. They were designated *cbaI*, *cbaX*, *cbaR*, *cbaK*, *cbaT* and *cbaC*. Four of these genes showed high homology to other bacteriocin dependent secretion systems (Håvarstein *et al.*, 1995; Héchard *et al.*, 1995; van Belkum and Stiles, 1995), in particular to the carnobacteriocin B2 system. These are comprised of a response regulator, histidine protein kinase, ATP-binding cassette protein and an accessory protein. The fifth gene, *cbaI*, is postulated to encode the immunity protein of carnobacteriocin A, but like other immunity proteins it shows no homology to other immunity proteins. Production of a carnobacteriocin A immune phenotype was not achieved with subcloning of this gene into homologous and heterologous hosts. It is postulated that this may be due to the strict regulation of the immunity and structural genes by the regulation system for the carnobacteriocin A gene cluster located immediately downstream of the putative immunity gene. As indicated above, the regulation and secretion genes of carnobacteriocin A are almost identical in amino acid and nucleotide sequence to carnobacteriocin B2; however, there is no complementation of the A system with production of the carnobacteriocin BM1 located on the chromosome. A major difference in carnobacteriocins A and B2 is that carnobacteriocin A is detected early in the growth cycle and has higher activity against most sensitive organisms. Studies done on the mode of action of carnobacteriocin (van Belkum, personal communication) suggest that carnobacteriocin A is bactericidal, whereas carnobacteriocin B2 is bacteriostatic. The

main difference between these bacteriocins on a genetic basis is the organization of their operons. The carnobacteriocin A cluster is in a tandem array with the structural gene followed by the 5 genes. In carnobacteriocin B2, the secretion and regulation genes are upstream of the structural and immunity genes (Quadri *et al.*, 1996). It is possible that the organization or different promoters induce greater or earlier production of carnobacteriocin A. The total amount of DNA required for carnobacteriocin A production was 9.6 kb, with very little nonencoding regions of DNA.

5.2 PLASMID pCD3.4 AND DIVERGICIN A

By definition, bacteriocins are usually only active against closely related bacteria. This limits their value for application as biopreservatives in food systems. Initial research was directed towards the incorporation of several bacteriocins into one strain to provide one organism with a broad range of activity. For such a multiple bacteriocin system to be accepted, a "food grade" vector for the incorporation of the bacteriocins was required. "Food grade status" has been a controlling aspect of these studies, and was interpreted for this study as meaning that the bacteria came from a food (meat) source, and only bacteriocins or genes associated with these bacteriocins would be considered for use in a modified, bacteriocinogenic organism.

The meat isolate, *C. divergens* LV13, was observed to contain a small, high copy number plasmid. The plasmid, designated pCD3.4, was shown to be capable of replicating in other carnobacteria and in *E. coli*. pCD3.4 also has the benefit of high electrotransformation efficiency in Gram-positive hosts and it contains several unique restriction sites which enhance its suitability for use as a vector. Incorporation of a chloramphenicol gene provided a selective marker for the study of the plasmid. Sequencing of the 3485 bp plasmid revealed the presence of a repA-like gene and two other genes, a bacteriocin and its corresponding immunity gene. The immunity gene

contained on the plasmid may provide a food grade marker that is needed for a food grade vector. Further work on this vector and the identification of essential regions of the plasmid require further study.

The discovery that pCD3.4 encodes a bacteriocin was surprising because the smallest amount of DNA required to encode production of pediocin PA-1 (Marugg *et al.*, 1992) or lactococcin A (van Belkum *et al.*, 1991) was 4 to 5 kb. Other bacteriocins, especially those with regulatory genes, require up to 10 kb of DNA (Holck *et al.*, 1992; Tichaczek, 1994; Diep *et al.*, 1994; Quadri *et al.*, 1996), and nisin requires 15 kb of DNA (Siegers and Entian, 1995). Even with 4 to 5 kb of DNA for each bacteriocin, it would make the production of multiple bacteriocins from a single vector very difficult to achieve. Some researchers have investigated the complementation of secretion systems, but with limited success (Fremaux *et al.*, 1993). Successful complementation occurs with similar bacteriocins but full restoration is not achieved. Divergicin A was the first LAB bacteriocin discovered that lacked a dedicated secretion system for its bacteriocin. Divergicin A is secreted in amounts equivalent to the wild type strain with the cloning of only the structural (*dvnA*) and immunity (*dviA*) genes. This unique and valuable characteristic of divergicin allows bacteriocin production with only 500 bp of DNA. This is due to the presence of a signal peptide that is common to the *sec*-dependent secretion pathways. The divergicin A structural gene contains the typical features of a signal peptide, i.e., a positively charged N-terminus, a hydrophobic core and an Ala-X-Ala cleavage site. Confirmation that the *sec*-pathway is utilized for secretion was done by fusing the divergicin signal peptide in front of the mature portion of the alkaline phosphatase gene that utilizes the *sec*-pathway to secrete the protein. As final evidence that divergicin is produced by the *sec*-pathway of the cell, constructs producing divergicin were put into *sec*-pathway mutants and antibodies were raised to divergicin and used to immunoprecipitate the divergicin in the *sec* mutants. This work is in progress

in our laboratory, and the results of these experiments are in the final stages of completion.

The discovery of divergicin A facilitates the development of bacteriocin gene cassettes for the production of multiple bacteriocins. The successful secretion of alkaline phosphatase with the divergicin signal peptide and the production of divergicin in *E. coli*, suggests the potential for biotechnology applications for small and large protein secretion using this system. The addition of purified, multiple bacteriocins to foods as preservatives on an industrial level is not realistic. However, the potential to produce multiple bacteriocins with a broad range of antibacterial activity in nonaciduric LAB as "starter organisms" is more realistic. The production of bacteriocin gene cassettes is on-going in our laboratory. Once the construction is completed, the next step will be to assess the feasibility of such a "starter" in selected food systems. It must be determined whether adequate control of target organisms is achieved and it must be established that the "starter" organism do not cause premature spoilage of the food. Much of the bacteriocin research has overlooked or ignored the presence of resistance in normally sensitive cells. The point is rapidly approaching in bacteriocin research where the focus of the research effort must be directed towards the application of these bacteriocins in the biopreservation of foods.

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