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BIOCHEMICAL AND GENETIC CHARACTERIZATION OF ANTIMICROBIAL PEPTIDES PRODUCED BY *CARNOBACTERIUM PISCICOLA* LV17B

by

Luis E. N. Quadri

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN

PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

IN

Food Microbiology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Spring 1996

ISBN 0-612-10628-4

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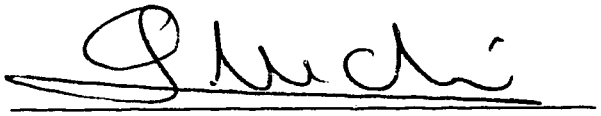
DEGREE: Doctor of Philosophy

YEAR THIS DEGREE GRANTED: 1996



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January 19, 1996

UNIVERSITY OF ALBERTA

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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 **Biochemical and Genetic Characterization of Antimicrobial Peptides Produced by *Carnobacterium piscicola* LV17B** submitted by **Luis Edmundo Nereo Quadri** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Food Microbiology**.

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DEDICATION

To my wife Ana and to my parents and sister Nelly, Luis and Andrea

ABSTRACT

Carnobacteriocins BM1 and B2 are class II bacteriocins produced by *Carnobacterium piscicola* LV17B. The production of, and immunity to, the carnobacteriocins are dependent on the presence of a 61-kb plasmid (pCP40) in the producer strain. These bacteriocins were purified and characterized and their genetic determinants were cloned and sequenced. The genetic determinants of carnobacteriocins B2 and BM1 (*cbnB2* and *cbnBM1*) are located on pCP40 and on the chromosome of *C. piscicola* LV17B, respectively.

Downstream of *cbnB2* is the gene *cbiB2* encoding the protein that confers immunity to carnobacteriocin B2. Production of CbiB2 in different hosts indicated that this protein confers immunity to carnobacteriocin B2, but not to carnobacteriocin BM1. The protein conferring immunity to carnobacteriocin BM1 is probably encoded by *cbiBM1*, a gene located of downstream *cbnBM1*. The immunity protein was purified and characterized. Immunolocalization studies indicated that the majority of the intracellular pool of the immunity protein is in the cytoplasm.

Cloning of a 10-kb fragment of DNA from pCP40 into the plasmidless strain *C. piscicola* LV17C restored production of the plasmid-encoded carnobacteriocin B2, the chromosomally-encoded carnobacteriocin BM1, and their immune phenotypes. This fragment also allowed expression of carnobacteriocin B2 and its immunity in a heterologous host. In addition to *cbnB2* and *cbiB2*, the fragment contains several open reading frames including *cbnK*, *cbnR*, *cbnT* and *cbnD*. CbnK and CbnR form a two-component signal transduction system that regulates production of, and immunity to, the carnobacteriocins. CbnT and CbnD are ATP-dependent transporter and accessory proteins of the bacterial signal-sequence independent secretion machinery involved in carnobacteriocin secretion.

ACKNOWLEDGMENTS

I would like to express my appreciation and gratitude to my supervisor, Dr. M. E. Stiles, for his guidance and encouragement during my graduate studies. I am especially thankful to him for his help and support beyond his professional obligations.

I would like to thank Dr. John C. Vederas and Dr. Miloslav Sailer from the Department of Chemistry for their excellent team work, without which this thesis would have not been possible. I am especially grateful to Dr. John C. Vederas for his help and advice regarding the next step in my career.

I would like to thank Dr. Kenneth Roy for sharing his knowledge and experience and allowing me to work in his laboratory. Without his support and advice this thesis would have not been possible.

I am very grateful to Dr. Lynn Elmes and Patricia Murray for their valuable technical assistance and to Dr. Peter Sporns, Dr. Lynn M. McMullen, Gordon Alton, Dr. Joseph Boothe and Mauricio R. Terebiznik for their help and stimulating discussion.

I would like to extend my sincere gratitude to my friends who helped me in many ways.

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1. INTRODUCTION AND REVIEW OF LITERATURE

1.1. General introduction

1.1.1. Taxonomy and general description lactic acid bacteria (LAB)

The microorganisms regarded as LAB comprise a diverse group of Gram-positive, catalase-negative, nonsporulating bacteria that are coccus-, coccobacillus- or rod-shaped. LAB have a guanine plus cytosine (G+C) content that is less than 50 mol% and, based on the 16S and 23S ribosomal ribonucleic acid sequence information, they belong to the so-called *Clostridium* branch of Gram-positive bacteria (Schleifer and Ludwig, 1995). LAB are currently divided into eleven genera: *Aerococcus, Alloiococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus,* and *Vagococcus* (Pot et al., 1994). They are facultative or strict anaerobes, but all of them require a fermentable carbohydrate as energy source. During the fermentation process, the carbohydrates are degraded to lactic acid by homofermentative LAB, and to lactic acid, acetic acid and (or) ethanol and carbon dioxide by the heterofermentative LAB. LAB are widely distributed in a variety of habitats. The ecological niches of LAB include not only foods such as dairy products, sourdough bread, fermented vegetables and meats, and beverages, but also they can be found in silage, sewage, on plants, and in the genital, intestinal and respiratory tracts of humans and animals (Hammes et al., 1991).

1.1.1.1. Genus *Carnobacterium*

Because this thesis deals with strains of *Carnobacterium* and antimicrobial peptides that they produce, a brief description of the genus is presented in this section. The first report of microorganisms that subsequently became the genus *Carnobacterium*

was published in 1957. The organisms were isolated from chilled chicken meat and characterized as *Lactobacillus*-type organisms that were unable to grow in acetate agar at pH 5.6 (Thornley, 1957). After several reports of *Lactobacillus*-type organisms isolated from chill-stored meats, and because of their inability to grow on acidified agar, the group was generally referred to as nonaciduric *Lactobacillus*-type organisms and, later, they were included in the genus *Lactobacillus*. Two new species of lactobacilli were proposed: *Lactobacillus divergens* (Holzapfel and Gerber, 1983) and *Lactobacillus carnis* (Shaw and Harding, 1985). The similarity of *Lactobacillus carnis* to a newly identified fish pathogen, *Lactobacillus piscicola*, triggered the proposal for the new genus *Carnobacterium* (Collins et al., 1987). Today, this genus includes *Carnobacterium piscicola* and *Carnobacterium divergens*, the former *Lactobacillus carnis* and *Lactobacillus divergens*, respectively, and *Carnobacterium gallinarum* and *Carnobacterium mobile*. Recently, a new name, *Carnobacterium maltaromicus*, has been proposed for *Carnobacterium piscicola* (Collins et al., 1991). The type species of the genus is *Carnobacterium divergens* and the genus has been described as follows: "Nonsporeforming, straight, slender rods usually occurring singly or as pairs but sometimes as short chains. Cells are Gram-positive. May or may not be motile. Heterofermentative, producing predominantly L-(+)-lactic acid from glucose. Gas production is variable (depending on the substrate) and frequently negative. Growth occurs at 10 °C; most strains grow at 0 °C but not at 45 °C. No growth in 8 % NaCl. Does not grow on acetate agar or broth. Catalase and benzidine negative. Nitrate is not reduced to nitrite" (Collins et al., 1987).

1.1.2. Role of LAB in foods

In recent years LAB have become the focus of an increasing amount of fundamental and applied research. This trend is mainly due to the importance of many of these organisms in food and feed technology. LAB are utilized for the manufacture of a

wide variety of fermented food and beverage products. The development of a specific LAB population in the food determines product attributes such as taste, flavor, texture, and it contributes to the improvement of shelf-life and safety of the product (McKay and Baldwin, 1990; Vandenbergh, 1993; De Vuyst and Vandamme, 1994a). The significance of the overall impact of LAB on the organoleptic properties of the final product is product-specific and it is mainly associated with the release into the environment of several metabolic products generated at the expense of food components. Among these metabolic products, organic acids such as lactic and acetic acids, ethanol, amino acids and small peptides, volatile compounds as diacetyl, and exopolysaccharides such as dextrans are among the most important contributors to the sensory attributes of the products (De Vuyst and Vandamme, 1994a). Similarly, the improvement of shelf-life and microbiological safety is mainly due to the production of compounds with antimicrobial activity that are released into the environment and contribute to the inhibition or retardation of growth of undesirable microorganisms, such as pathogens and organisms with high spoilage potential. Among these compounds are the short chain organic acids, hydrogen peroxide, diacetyl, enzymes, and bacteriocins (antimicrobial peptides or proteins; Lindgren and Dobrogosz, 1990; Vandenbergh, 1993; De Vuyst and Vandamme, 1994b).

1.1.2.1. Genus *Carnobacterium* in foods

Carnobacterium strains can be isolated from many meats and meat products, poultry and fish products, where they grow as part of the adventitious microflora. Strains from the genus *Carnobacterium* represent an important fraction of the LAB microflora that prevails in chill-stored meat and poultry products packaged under vacuum or in modified atmosphere with elevated carbon dioxide content (Schillinger and Holzapfel, 1995). Under these packaging and storage conditions, a Gram-positive psychrotrophic microflora of nonputrefactive LAB that includes *Carnobacterium* spp. develops,

replacing the Gram-negative psychrotrophic microflora that is predominant under aerobic storage and produces putrefactive spoilage. Although the adventitious LAB microflora eventually causes nonputrefactive spoilage of the product due to flavor and odor defects, the conditions that favor the growth of LAB often produce a significant extension of the storage life and an enhancement of microbiological safety of the products (Egan, 1983; Stiles and Hastings, 1991).

1.1.3. Antimicrobial peptides produced by LAB: definitions and classification

Lactic acid bacteria, as is the case with several other groups of prokaryotic organisms, are capable of producing proteins and peptides with antimicrobial activity, called bacteriocins. The early definition of bacteriocins was based on, and referred to, proteins like colicins produced by several Gram-negative microorganisms.(Jacob et al., 1953). The identification of antimicrobial proteins and peptides among Gram-positive bacteria that shared characteristics with colicins, but showed clear differences from them, called for a redefinition of bacteriocins. This prompted Tagg et al. (1976) to establish six criteria to define an antimicrobial compound as a bacteriocin: "(i) a narrow spectrum of inhibitory activity centered about the homologous species; (ii) the presence of an essential, biologically active protein moiety; (iii) a bactericidal mode of action; (iv) attachment to specific cell receptor; (v) plasmid-borne genetic determinants of bacteriocin and of host cell bacteriocin immunity; (vi) production by lethal biosynthesis (i.e., commitment of the bacterium to produce bacteriocin will ultimately lead to cell death)." Understanding the diversity already existing among these antimicrobial compounds, Tagg suggested that an antimicrobial compound should meet at least criteria (ii) and (iii) to be recognized as a bacteriocin. Later, Klaenhammer (1988) defined bacteriocins as "proteins or protein complexes with bactericidal activity against species that are usually closely related to the producer bacterium" and in 1993 he proposed a specific

classification for the bacteriocins produced by LAB that grouped the known bacteriocins into the following four classes (Klaenhammer, 1993):

I. The lantibiotics, small membrane-active peptides (< 5 kDa) containing the unusual amino acids lanthionine, β-methyllanthionine, and dehydrated residues.

II. Small-heat stable, nonlanthionine-containing membrane-active peptides (<10 kDa) characterized by Gly-Gly^{-1}-Xaa^{+1} processing site of the bacteriocin precursor. The mature bacteriocins are predicted to form amphiphilic helices with varying amounts of hydrophobicity, β-sheet structure. They have moderate (100 °C) to high (121 °C) heat stability. Three subgroups were defined within the class II bacteriocins:

IIa. *Listeria*-active peptides with a consensus sequence in the N-terminal of: Thr-Gly-Asn-Gly-Val-Xaa-Cys.

IIb. Poration complexes consisting of two proteinaceous peptides for activity.

IIc. Thiol activated peptides requiring reduced cysteine residues for activity.

III. Large heat-labile proteins (> 30 kDa).

IV. Complex bacteriocins, composed of proteins plus one or more chemical moieties (lipids, carbohydrates) required for activity.

Although not all of the bacteriocins produced from LAB can be defined using the criteria of Tagg et al. (1976) or the definition of Klaenhammer (1988), most of them can be included in one of the four classes mentioned above. The use of expressions such as "peptide antibiotic" and "antagonistic or antimicrobial peptide or protein" to refer to

bacteriocins can be found throughout the literature. Throughout this thesis, the expressions "antimicrobial peptide" or "bacteriocin" will be used.

1.1.3.1. Uses of bacteriocins and bacteriocin-producing LAB in food systems

Bacteriocins could play a role in the microbial ecology of food by adversely affecting sensitive microorganisms and conferring a competitive advantage to the producer strains (Klaenhammer, 1988). Bacteriocin-producing LAB have been isolated from many foods and beverages, and active bacteriocins have been extracted from several food systems (Nielsen et al., 1990; Sobrino et al., 1991; Luchansky et al., 1992; Garver and Muriana, 1993; Jiménez-Díaz et al., 1993; McMullen and Stiles, 1993; Garriga et al., 1994; Leisner et al., 1995). The use of bacteriocin-producing LAB or bacteriocins as additives to extend shelf-life and enhance the safety of food represents a promising strategy (Daeschel, 1993; Vandenbergh, 1993). The antimicrobial activity of bacteriocins against foodborne pathogens and organisms with high spoilage capability makes these compounds candidates for use as biopreservatives in conjunction with, or in place of, traditional chemical preservatives. Addition of bacteriocins or the *in situ* production of bacteriocins by specific LAB strains, adds a "hurdle" to the growth of undesired microorganisms (Daeschel, 1993; Vandenbergh, 1993).

The best example of the application of a bacteriocin as a biopreservative is the use of the class I lantibiotic nisin A, the most characterized bacteriocin produced by LAB. Nisin A is produced by strains of *Lactococcus lactis* subsp. *lactis*, and it is active against a wide range of Gram-positive bacteria (Hurst, 1981). Inhibition of the outgrowth of *Clostridium* spores is the most relevant feature of the spectrum of antimicrobial activity of nisin. This property, in addition to the ability to inhibit several other foodborne pathogens, has promoted its use as a food preservative (Vandenbergh, 1993). The use of nisin A as a food preservative is approved in over 40 countries and applications have

been developed for processed cheese, dairy desserts, milk, fish, bacon, frankfurters, fermented beverages and several canned foods (Delves-Broughton, 1990; Vandenbergh, 1993).

1.2. Literature review

1.2.1. Bacteriocins produced by LAB: genetic organization of bacteriocin loci

In recent years, there has been a growing interest in the preservation of foods by lactic acid bacteria. The possibility of using bacteriocins, or bacteriocin producing strains of LAB, to inhibit the growth of spoilage and pathogenic bacteria in foods has stimulated the study of these antimicrobial peptides produced by this group of organisms. Although the study of antimicrobial properties of LAB has lead to many reports of bacteriocins or bacteriocin-like compounds produced by different species of LAB (see De Vuyst and Vandamme, 1994c and Ralph et al., 1995 for reviews) only a few of these bacteriocins have been extensively characterized and they will be considered in detail in the following sections.

Extensive studies of some bacteriocins produced by LAB have allowed the identification of genes encoding proteins involved in: (i) bacteriocin secretion; (ii) cleavage of the leader peptide, (iii) formation of dehydrated amino acid residues and lanthionine and β-methyllanthionine residues in lantibiotics, (iv) immunity (resistance of the producer organism to its own bacteriocin) and (v) regulation of bacteriocin expression. In most cases, the genes required for bacteriocin production and immunity are clustered and located on the chromosome or on plasmids present in the producer organism. The best characterized gene clusters and operons, containing genetic information required for Bac^+ Imm^+ phenotype, are shown in Figure 1.1.

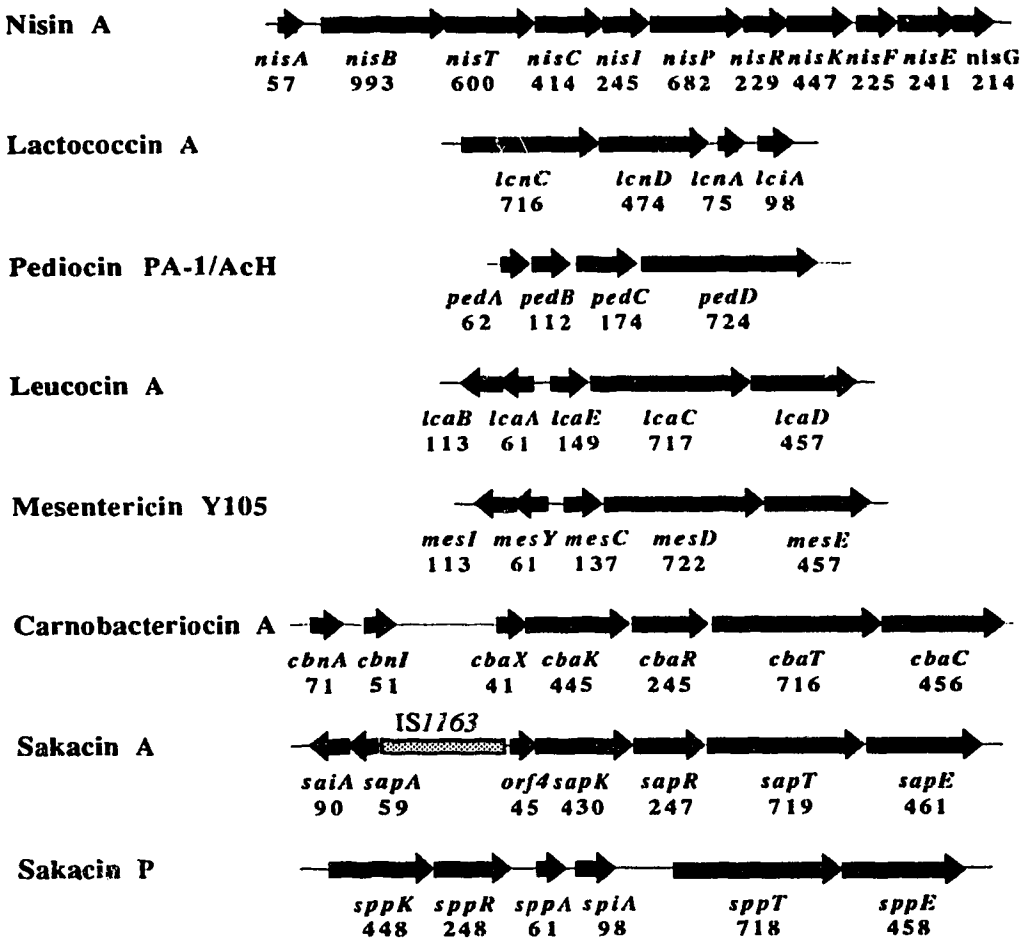

Figure 1.1. Organization of the genes involved in the production of and immunity to: nisin A (Kuipers et al., 1993; Engelke et al., 1994; Siegers and Entian, 1995); lactococcin A (Stoddard et al., 1992); pediocin PA-1/AcH (Marugg et al., 1992; Bukhtiyarova et al., 1994); mesentericin Y105 (Fremaux et al., 1995); leucocin A (Van Belkum and Stiles, 1995); carnobacteriocin A (Worobo, 1995); sakacin A (Axelsson and Holck, 1995); sakacin P (Huehne et al., 1995). The number of amino acids of each gene product is indicated.

1.2.2. Posttranslational modification and secretion of bacteriocins

Bacteriocins are ribosomally synthesized as precursor proteins containing unusual leader peptides. These leader peptides do not generally fit the consensus of signal sequences involved in the secretion of proteins by the bacterial *sec*-dependent secretory pathway (Gierasch, 1989). Leader peptides of bacteriocin precursor molecules are cleaved during the bacteriocin maturation process to release the fully processed and active bacteriocin (Håvarstein et al., 1995; de Vos et al., 1995). Mature bacteriocins are found extracellularly and, in most cases, their transport outside of the cell requires a dedicated translocation machinery. This translocation machinery is formed by proteins belonging to the family of bacterial transporters containing an ATP binding cassette (ABC) and referred to as ABC exporters (Fath and Kolter, 1993). In the case of class II bacteriocins, the translocation machinery is comprised of an ABC exporter and an accessory factor protein. In at least two cases, the ABC exporters have been shown to possess leader peptidase activity responsible for the cleavage of leader peptides of the prebacteriocins (Håvarstein et al., 1995; Venema et al., 1995).

Class I bacteriocins, or lantibiotics, undergo several posttranslational modifications in addition to the cleavage of the leader peptide. Lantibiotics require specific enzymes for the maturation process and a dedicated translocation machinery is required for the secretion of the bacteriocin. In class I bacteriocins, ABC exporters are involved in bacteriocin secretion; however, unlike class II bacteriocins, no accessory factor proteins have been identified, suggesting that no other specific protein is required for secretion. The peptidase activity responsible for the cleavage of the leader peptide of lantibiotics does not reside in the translocation machinery. In at least one case, the specific peptidase activity has been found in a membrane bound peptidase (Van der Meer et al., 1993).

Secretion and maturation of nisin A, lactococcins A and G, and pediocin PA-1 has been studied in some detail and information on this is presented in the following sections.

1.2.2.1. Posttranslational modification, secretion and leader peptide cleavage of class I bacteriocins: Nisin A, a model system

Lantibiotics are ribosomally synthesized as prepeptides composed of a N-terminal extension or leader peptide that possesses distinct characteristics and differ from those found in the typical signal sequences involved in secretion via the *sec*-dependent pathway (Gierasch, 1989; de Vos et al., 1995). The leader peptides are followed by the probacteriocin, in which specific enzymes introduce modifications to produce the characteristic structural features of this class of bacteriocins (Schnell et al., 1988; Jung 1991).

The chain of events leading to the production of a fully posttranslationally modified nisin A, the best characterized class I bacteriocin produced by lactic acid bacteria, is beginning to be elucidated, and this information was recently reviewed by de Vos et al., (1995). The posttranslational modifications, secretion and cleavage of the leader peptide in prenisin require the gene products of *nisB*, *nisC*, *nisT* and *nisP*, four genes that are located downstream of *nisA* in the nisin gene cluster (Fig. 1.1; Steen et al., 1991; Engelke et al., 1992, 1994; Van der Meer et al., 1993; Kuipers, 1995). The same gene organization has been found in the gene cluster of nisin Z, a structural variant of nisin A (Mulders et al., 1991; Immonen et al., 1995) and homologous genes have been found in the gene cluster of other lantibiotics (de Vos et al., 1995). NisB and NisC are probably membrane proteins that are involved in formation of dehydrated amino acid residues and lanthionine and β-methyllanthionine residues that form several intramolecular thioether bridges. When these genes are inactivated, the producer strain fails to produce fully modified nisin A and only produces prenisin (Kuipers, 1995). These experiments suggest a role for NisB and NisC in the posttranslational modification

leading to the formation of a prenisin, containing all of the modifications typical of lantibiotics. NisT is an ABC exporter responsible for the secretion of prenisin into the medium. The inactivation of this gene leads to the intracellular accumulation of prenisin (Engelke et al., 1992; Kuipers et al., 1993; Kuipers, 1995).

NisP is the subtilisin-like serine protease (leader peptidase) responsible for cleavage of the leader peptide in the prenisin molecule (Van der Meer et al., 1993). Inactivation of *nisP* leads to the accumulation of prenisin in the medium. Furthermore, incubation of prenisin with the membrane fraction from NisP-producing strains leads to the cleavage of the leader peptide and the release of active nisin A. Based on the primary structure of nisin P and using computer programs, a signal sequence located at the N-terminus and a hydrophobic membrane anchor located at the C-terminus of the protein can be predicted. This prediction is in agreement with the experimental data that indicated that the protein is attached to the membrane (Van der Meer et al., 1993).

1.2.2.2. Secretion and leader peptide cleavage of Class II bacteriocins: lactococcins A and G, pediocin PA-1 as model systems.

Class II bacteriocins are synthesized as prebacteriocins containing a N-terminal extension or leader peptide that is cleaved to yield the mature antimicrobial peptide (Klaenhammer, 1993). The majority of the leader peptides of class II bacteriocins have distinct characteristics, and they differ from those found in the typical signal sequences involved in secretion via the *sec*-dependent pathway (Gierasch, 1989). The best conserved feature of these leader peptides is the presence of two glycine residues at positions -2 and -1 of the leader peptide (Håvarstein et al., 1995).

The secretion of most of the class II bacteriocins is believed to require a dedicated secretion machinery composed of an ABC transporter and an accessory factor (Fath and Kolter, 1993). Bacteriocin secretion and leader peptide cleavage has been most extensively studied for pediocin PA-1 and lactococcin G. In these two cases, the ABC

transporter involved in the secretion of the bacteriocins was shown to have a peptidase domain responsible for the cleavage of the leader peptides of the prebacteriocins (see below). The translocation machinery involved in the secretion of lactococcin A has also been studied in some detail. These three bacteriocins contain the double glycine motif at the cleavage site of the leader peptides and the genes encoding their secretion machinery have been cloned and sequenced (Marugg et al., 1992; Stoddard et al., 1992; Håvarstein et al., 1995).

In the lactococcin G gene cluster, the genes *lagD* and *lagE*, specify the proteins LagD and LagE with homology to ABC transporters and accessory factors from the *sec*-independent secretion machinery, respectively (Fath and Kolter, 1993). These proteins are believed to be involved in the secretion of lactococcin G. The ABC transporter, LagD, is also responsible for the cleavage of the leader peptide of the prebacteriocin. The peptidase activity of LagD is associated with a 150 amino acid N-terminal cytoplasmic domain of the translocator (Håvarstein et al., 1995).

In the case of pediocin PA-1, *pedC* and *pedD*, located in the pediocin gene cluster (Fig. 1.1), encode the accessory factor PedC and an ABC transporter PedD required for pediocin PA-1 production (Marugg et al., 1992). The lack of the functional ABC transporter PedD, or accessory factor PedC, leads to intracellular accumulation of precursor molecules, or precursor and mature pediocin molecules, respectively. Furthermore, a peptidase domain of 170 amino acids, located at the N-terminus of the ABC transporter, is responsible for cleavage of the leader peptide of the pediocin precursor (Venema et al., 1995). The topology of the accessory factor PedC has also been studied (Emond et al., 1995). Computer algorithms predicted the presence of one or two membrane spanning segments in PedC. Random in-frame translational fusions of PedC to β-galactosidase or alkaline phosphatase were constructed. The analysis of the activity of the reporter proteins and the membrane localization of several fusions allowed

the formulation of a model for PedC topology. According to the model, PedC is an integral membrane protein with two membrane spanning segments; the first, including amino acid residues 9 to 29 and the second, including amino acid residues 126 to 146. The first 8 amino acid residues at the N-terminus and the last 28 amino acid residues at the C-terminus, are exposed to the cytoplasm. The large segment of the protein between the two membrane spanning segments, forms an extracellular loop (Emond et al., 1995).

Studies of the gene cluster involved in the production of lactococcin A have shown that *lcnC* and *lcnD* are two genes essential for the production of lactococcin A (Fig. 1.1; Stoddard et al., 1992). The genes specify the proteins LcnC and LcnD, an ABC transporter and an accessory factor, respectively, that are involved in the secretion of lactococcin A. The topology of the accessory factor LcnD has been studied in some detail by analyzing in-frame translational fusions of LcnD to the reporter proteins β-galactosidase or alkaline phosphatase (Venema et al., 1995). The results support a model in which the 21 N-terminal amino acids of LcnD are located intracellularly and a transmembrane helix formed by residues 22 to 43 spans the cytoplasmic membrane, thereby leaving the C-terminal region of the protein exposed to the outside of the cell. The results were in agreement with the topology predicted from the deduced amino acid sequence, and they indicated that LcnD is an integral membrane protein (Stoddard et al., 1992).

1.2.3. Regulation of bacteriocin expression

Bacteriocin production by LAB is affected by the composition and pH of the medium, and by the presence of specific inducing factors (Barefoot and Klaenhammer, 1984; Joerger and Klaenhammer, 1986; Parente and Hill, 1992; Barefoot et al., 1994; Saucier et al., 1995). Although this might suggest the existence of regulatory pathways, in most cases the molecular basis for these phenomena has not been studied.

Recently, studies on the production of bacteriocins by *Carnobacterium piscicola* LV17, LV17A and LV17B (see below) have shown that bacteriocin production in these strains is an inducible process. Production of bacteriocins by strains of *C. piscicola* is lost when the culture is inoculated below $1x10^4$ cfu ml^{-1}. In a nonproducing culture, bacteriocin production can be restored by adding sterile supernatant from a bacteriocin-producing culture or purified bacteriocins, or by growing the nonproducing strains in solid media (Saucier et al., 1995). From these results, Saucier concluded that production of bacteriocins by *Carnobacterium piscicola* LV17, LV17A and LV17B is an autoregulated process; however, the molecular basis for regulation has not been studied.

Two examples of regulation of bacteriocin production by signal transduction are well documented. The first example is the regulation of bacteriocin production in *Lactobacillus plantarum* C11 by plantaricin A, a bacteriocin-like peptide (Diep et al., 1995). The second example is the autoregulation of nisin A production in *L. lactis*. In this system, nisin A is the signal that regulates its own synthesis. Elements of the bacterial two-component signal transduction system have shown to be required for production of the class II bacteriocin sakacin A; however, this system has not been characterized as well as the ones mentioned above.(Axelsson and Holck, 1995). Elements of the bacterial two-component signal transduction system have also been identified in the gene clusters of the bacteriocins sakacin P and carnobacteriocin A. Although these genes are probably required for production of the bacteriocins and immunity, they have not been individually inactivated and their involvement has not been jet demonstrated (Huehne et al., 1995; Worobo, 1995).

1.2.3.1. Regulation of bacteriocin production in *L. plantarum* C11

It has been demonstrated that the production of bacteriocin in *L. plantarum* C11 is a regulated process that requires the presence of an inducing factor to trigger bacteriocin production. The inducing factor has been identified as the mature, extracellular

plantaricin A peptide (Diep et al., 1995). Plantaricin A is a small extracellular, cationic peptide synthesized as a precursor containing 48 amino acids (Diep et al., 1994). The precursor contains a leader peptide of 22 amino acids with homology to those present in many class II bacteriocins and it contains the Gly-Gly motif at the cleavage site. The genetic determinant of plantaricin A (*plnA*) is located in the same operon as an *agr*-like, two-component signal transduction system. Three genes, encoding a possible histidine kinase (*plnB*) and two possible response regulators (*plnC* and *plnD*), are located downstream of *plnA*. The genes *plnABCD* form part of the same transcriptional unit (Diep et al., 1994). Plantaricin A not only induces bacteriocin production in *L. plantarum* C11, but it also regulates its own synthesis by triggering transcription of the plantaricin operon *plnABCD*. It has been postulated that PlnB is the sensor protein for the presence of extracellular plantaricin A and that PlnC and PlnD regulate the transcription of the plantaricin operon and the genes required for bacteriocin production (Diep et al., 1995).

1.2.3.2. Autoregulation of nisin A production in *L. lactis*

Production of the lantibiotic nisin A is a regulated process. The autoregulation phenomenon of this lantibiotic has been studied in considerable detail, and it is beginning to be understood at the molecular level (Kuipers et al., 1995). Autoregulation of nisin A takes place by a two-component signal transduction system comprised of NisR and NisK. These two proteins, encoded by *nisR* and *nisK*, are located in the nisin gene cluster and they are required for nisin A production and immunity (Fig. 1.1; Kuipers et al., 1993, 1995; Engelke et al., 1994). NisK is the histidine protein kinase that senses extracellular nisin A molecules and NisR is the response regulator that activates transcription from two promoters within the nisin A gene cluster. The promoter located upstream of *nisA* and the promoter located upstream of *nisFEG* were identified as nisin-inducible promoters, and in all likelihood they are the targets of phosphorylated NisR (de Ruyter et al., 1995).

1.2.4. Immunity to bacteriocins produced by LAB

Because the bacteriocins synthesized by a strain could be active against the producer organism, specific mechanisms that confer "immunity" to their own bacteriocins are required in the producer strains. It is clear that the immunity mechanism(s) should counteract the mechanism of antimicrobial activity of the bacteriocins. The mode of action of several bacteriocins from LAB has been studied in some detail (Ruhr and Sahl, 1985; Gao et al., 1991; van Belkum et al., 1991b; Chikindas et al., 1993; Venema et al., 1993; Abee et al., 1994). Bacteriocins target the cytoplasmic membrane of sensitive cells, and they cause dissipation of the proton motive force and leakage of intracellular components through the formation of multimeric pores. The molecular basis of the mechanism(s) whereby the producer organisms protect themselves against the antimicrobial effect of their bacteriocins, is poorly understood. Proteins of the producer organism that confer immunity to the specific bacteriocins have been identified (Van Belkum et al., 1991, 1992; Axelsson and Holck, 1995; Van Belkum and Stiles, 1995; Venema et al., 1994; Worobo et al., 1995). Immunity to the bacteriocins nisin A and lactococcin A are best characterized.

1.2.4.1. Immunity to Nisin A

Several genes of the nisin A gene cluster have been implicated in immunity to this lantibiotic (Kuipers et al., 1993; Engelke et al., 1994; Siegers and Entian, 1995). The first gene from the nisin A gene cluster that was shown to be involved in immunity was *nisI*. When NisI, the translational product of *nisI*, was expressed in nisin A sensitive strains of *L. lactis* or *E. coli*, it conferred significant levels of immunity to externally added nisin A (Kuipers et al., 1993). The deduced amino acid sequence of NisI showed the presence of a consensus lipoprotein signal sequence, suggesting that NisI is an extracellular membrane-anchored lipoprotein. It has been suggested that the protein protects the

producer organism by specifically binding nisin A molecules and preventing their insertion into the membrane (Kuipers et al., 1993).

Three other genes from the nisin A cluster, *nisF nisE* and *nisG*, have also been implicated in immunity (Fig. 1.1; Siegers and Entian, 1995). The gene products NisF and NisE, showed amino acid sequence similarity to proteins in the family of ABC exporters involved in immunity to the lantibiotic subtilin and the microcin B17. NisG, is a predominantly hydrophobic protein that resembles proteins involved in immunity to several colicins. Analysis by gene disruption indicated that *nisF, NisE* and *nisG*, are involved in immunity (Siegers and Entian, 1995). All of the mutants were more sensitive to externally added nisin than the wild type strain. It has been suggested that NisF could form a membrane complex with NisE, or alternatively with NisG, that could protect the cells by transporting active nisin molecules into the cytoplasm where they would be degraded. An alternative function postulated for the hydrophobic protein NisG is based in the mode of action of several immunity proteins of colicins. NisG might interact with pore-forming nisin molecules in the membrane, preventing the antimicrobial effect of nisin A (Siegers and Entian, 1995).

1.2.4.2. Immunity to Lactococcin A

Genetic studies have shown that *lciA* , located downstream of the lactococcin A genetic determinant, encodes the lactococcin A immunity protein (Fig. 1.1; Holo et al., 1991; Van Belkum et al., 1991a). Immunolocalization experiments, using antibodies against LciA, showed that part of the cellular pool of the immunity protein was associated with the membranes from immune strains (Nissen-Meyer et al., 1993). More detailed studies using right side out and inside out membrane vesicles and a monoclonal antibody that recognized an epitope in LciA located between amino acid residues 60 and 80 were used to reveal the topology of LciA. The protein has a putative amphiphilic α-helix encompassing amino acid residues 29 to 47, that spans the cytoplasmic membrane. The

N-terminal and the C-terminal region of LciA are located in the cytoplasmic and the extracellular part of the membrane, respectively (Venema et al., 1994).

Based on the topology of LciA, the existence of a putative membrane receptor for lactococcin A, and several experiments using fusion of right side out vesicles of immune and sensitive cells, a molecular model for the mode of action of the immunity protein was proposed (Venema et al., 1994). According to this model, the immunity protein is associated with the receptor molecule through the hydrophilic side of the transmembrane α-helix. The presence of the immunity protein associated with the receptor, prevents the successful insertion of lactococcin A into the cytoplasmic membrane to form pores (Venema et al., 1994).

1.2.5. Bacteriocins produced by *Carnobacterium* species

Production of bacteriocins by strains of carnobacteria has been reported (Ahn and Stiles, 1990b; Schillinger and Holzapfel, 1990; Lewus et al., 1991; Buchanan and Klaenhammer, 1992). The *Carnobacterium* strains identified as bacteriocin producers were, in most cases, isolated from fish or meat products (Ahn and Stiles, 1990a,b; Schillinger and Holzapfel, 1990; Stoffels et al., 1992); however, in one instance, a bacteriocin-producing strain was isolated from soft cheese (Herbin et al., 1995). The best characterized antimicrobial peptides produced by this group of organisms are the class II bacteriocins: carnobacteriocins A (Worobo et al., 1994) and carnobacteriocins B2 and BM1 (described in this thesis). These three bacteriocins are produced by *Carnobacterium piscicola* LV17. Another well characterized bacteriocin is divergicin A, produced by *Carnobacterium divergens* (Worobo et al., 1995). The lantibiotic carnocin UI49 produced by *Carnobacterium* sp and the class II bacteriocin piscicolin 61, with the same amino acid sequence of carnobacteriocin A and produced by *Carnobacterium piscicola* LV61, have been characterized to a lesser extent (Stoffels et al., 1992; Holck et al., 1994).

This thesis deals with the antimicrobial peptides produced by *Carnobacterium piscicola* LV17B, a derivative of *Carnobacterium piscicola* LV17. A brief description of the previous studies on the bacteriocinogenic activity of this strain is presented.

1.2.5.1. Bacteriocins produced by *Carnobacterium piscicola* LV17

Production of bacteriocins by *Carnobacterium piscicola* LV17 was first reported by Ahn and Stiles (1990a). *Carnobacterium piscicola* LV17 was isolated from vacuum-packaged meat by B. G. Shaw (Langford, Bristol, UK). A preparation of partially purified bacteriocin from *Carnobacterium piscicola* LV17 showed antimicrobial activity against strains of carnobacteria, lactobacilli, pediococci, enterococci and *Listeria* (Ahn and Stiles, 1990b). Heat stability studies (100 °C, 30 min.) and sensitivity to different proteases indicated that the antimicrobial compound(s) was heat stable and proteinaceous in nature. Plasmid isolation from *Carnobacterium piscicola* LV17 revealed the presence of three plasmids: pCP9, pCP40 and pCP49, of 9, 40 and 49 mDa, respectively (Ahn and Stiles, 1990b). Plasmid curing and conjugation experiments demonstrated that two different bac^+ imm^+ phenotypes were associated with the plasmids pCP40 and pCP49 and indicated the possibility of two bacteriocins being produced by this strain. Curing experiments also showed that a plasmidless strain (LV17C) did not produce bacteriocin and that it was sensitive to the bacteriocins produced by the derivatives containing only pCP40 (strain LV17B) or pCP49 (strain LV17A) (Ahn and Stiles, 1992).

Further work was done to study and characterize the bacteriocin produced by *Carnobacterium piscicola* LV17A. Cloning experiments revealed that the genetic information required for bacteriocin production and immunity was located in a 25.5-kb *Bgl*II fragment of the plasmid pCP49. The introduction of this fragment into the plasmidless strain derived from LV17, or into the heterologous host, *Carnobacterium piscicola* UAL26, resulted in transformants that produced the bacteriocin from the 25.5-kb fragment (Ahn and Stiles 1990b, 1992). Subsequent deletion analysis, indicated that a

9.6-kb *Pst*I fragment from pCP49 was required for full bacteriocin production and immunity (Ahn, PhD thesis, 1991). The bacteriocin produced by *Carnobacterium piscicola* LV17A, carnobacteriocin A, has been purified and characterized and its genetic determinants have been sequenced. Carnobacteriocin A is a class II bacteriocin synthesized as a precursor of 71 amino acids containing an 18 amino acids leader peptide of the Gly-Gly type (Worobo et al., 1994). Recently, the sequence of the 9.6-kb *Pst*I fragment from pCP49 was determined and revealed the presence of several open reading frames (Fig. 1.1). The gene products of these genes are probably required for carnobacteriocin A production (Worobo, 1995).

The bacteriocin activity associated with the strain *Carnobacterium piscicola* LV17B that contains only pCP40, was not previously investigated, and it is the subject of this thesis.

1.3. Objective

The main objective of this research was to study and characterize the bacteriocins produced by *C. piscicola* LV17B. The research was conducted in three stages:

1. Biochemical characterization of the substances responsible for the antimicrobial activity of *C. piscicola* LV17B and the identification of the bacteriocin genes.

2. Homologous and heterologous host expression of the bacteriocins and characterization of a protein involved in immunity to one of the bacteriocins produced by *C. piscicola* LV17B.

3. Identification and characterization of four genes required for bacteriocin production and immunity.

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2. CHEMICAL AND GENETIC CHARACTERIZATION OF BACTERIOCINS PRODUCED BY *CARNOBACTERIUM PISCICOLA* LV17B [1]

2.1. Introduction

The potential to use bacteriocins or bacteriocinogenic lactic acid bacteria to suppress the growth of spoilage and pathogenic bacteria in foods has stimulated extensive study of these inhibitory peptides (Klaenhammer, 1993). The best known is the lantibiotic, nisin A, produced by *Lactococcus lactis* subsp. *lactis*. This is an extensively posttranslationally modified peptide of 34 amino acids containing lanthionine ring structures (Jung and Sahl, 1991). Nisin inhibits a broad spectrum of Gram-positive bacteria and the outgrowth of *Bacillus* and *Clostridium* spores (Delves-Broughton, 1990). Its poor solubility and instability at the normal pH (5.5 to 5.8) of unprocessed meat and the mesophilic nature of *L. lactis* preclude the use of nisin or its producer strains in meat. Lactic acid bacteria isolated from chilled fresh and unfermented processed meats, or commercially fermented meats have been shown to produce bacteriocins. These compounds are generally small hydrophobic peptides that do not undergo substantial posttranslational modification. Examples include curvacin A from *Lactobacillus curvatus* (Tichaczek et al., 1992), sakacins A and P from *Lactobacillus sake* (Holck et al., 1992; Tichaczek et al., 1992), leucocin A from *Leuconostoc gelidum* (Hastings et al., 1991), and pedio[illegible]A-1 from *Pediococcus acidilactici* (Marugg et al., 1992).

Our studies have led to examination of *Carnobacterium* spp. which prevail on chilled meats stored under vacuum or in modified atmospheres containing elevated levels of carbon dioxide (McMullen, and Stiles, 1993). Although carnobacteria are

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[1] A version of this chapter was previously published: Quadri, L. E. N., M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles. 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. J. Biol. Chem. 269:12204-12211.

heterofermentative, they do not produce large amounts of gas, and therefore have potential for commercial application. The production of bacteriocins by *C. piscicola* LV17 has been reported by our laboratory (Ahn and Stiles, 1990), and it was demonstrated that the presence of two plasmids of 74- and 61-kb molecular size are associated with bacteriocin production and immunity. By curing and plasmid mobilization experiments the plasmids were separated and introduced into the plasmidless host strain (LV17C) as LV17A and LV17B containing the 74- and 61-kb plasmids, respectively (Ahn and Stiles, 1992). The chemical characteristics and the structural gene for carnobacteriocin A from LV17A have been described (Worobo et al., 1994). In this study we report the purification, chemical characterization, and the genetic determinants of two different bacteriocins produced by *C. piscicola* LV17B, which contains only the 61-kb plasmid.

2.2. Materials and methods

2.2.1. Bacterial strains, culture conditions and bacteriocin assay

The bacterial strains and plasmids used in this study are described in Table 2.1. *Carnobacterium* cultures were grown in APT broth (Difco Laboratories, Detroit, MI) as previously described (Ahn and Stiles, 1990). For purification of bacteriocins, *C. piscicola* LV17B was grown in CAA[2] medium (Hastings et al., 1991). *Escherichia coli* cultures were grown in Luria-Bertani (LB) broth or on LB agar (Sambrook et al., 1989). Ampicillin (100 µg/ml) was added to LB media for growth and selection of *E. coli* transformants. Bacteriocin activity was monitored against *Carnobacterium divergens* LV13 by the spot-on-lawn inhibition tests and expressed in arbitrary activity units (AU)

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[2] The abbreviations used are: CAA, semidefined casamino acids medium; TFA, trifluoroacetic acid; Et_2O, diethyl ether; SDS, Sodium dodecylsulfate; MeCN, acetonitrile; Me_2SO, dimethyl sulfoxide; HPLC, High performance liquid chromatography; AcOH, acetic acid; AU, arbitrary units of inhibitory activity; ORF, open reading frame(s); RBS, ribosome binding site(s).

Table 2.1. Bacterial strains and plasmids

Bacterial strains and plasmids	Relevant phenotype and properties	Reference
Bacteria		
C. piscicola		
LV17B	Bac$^+$, containing pCP40	Ahn and Stiles, 1990
LV17C	Bac$^-$, plasmidless mutant derived from *C. piscicola* LV17B	Ahn and Stiles, 1990
C. divergens		
LV13	Cbn^S indicator strain	Shaw[a]
E. coli		
MV1193	cloning host	Sambrook et al., 1989
LQ5.21	*E. coli* MV1193 containing pLQ5.21	This study
LQ7.2	*E. coli* MV1193 containing pLQ7.2	This study
Plasmids		
pUC118	3.2-kb cloning vector, Amp^R, lacZ'	Vieira and Messing, 1987
pCP40	61-kb plasmid conferring Bac$^+$ Imm$^+$ phenotype	Ahn and Stiles, 1990
pLQ5.21	pUC118 containing a 1.9-kb *Hin*dIII fragment of pCP40	This study
pLQ7.2	pUC118 containing a 4.0-kb *Eco*RI-*Pst*I genomic fragment from *C. piscicola* LV17C	This study

[a] Supplied by Dr B G. Shaw, Institute of Food Research, Langford, Bristol U. K.
Cbn = carnobacteriocin(s), ^{S} = sensitive

based on the reciprocal of the greatest dilution that is inhibitory to the indicator strain (Ahn and Stiles, 1990).

2.2.2. Production and isolation of bacteriocins

Production of bacteriocins was determined under controlled pH conditions (5.5, 6.0, 6.2 and 6.5) in CAA medium. For purification of the bacteriocins, an 18-h culture of *C. piscicola* LV17B was inoculated (1%) into 5 liters of CAA medium maintained at pH 6.2 with a pH controller (Chem-Cadet®, Cole-Parmer, Chicago, IL) by addition of 1 M NaOH, and gently stirred under N_2 atmosphere (40 ml/min) at 25 °C for 24-28 h. Isolation procedures were based on those previously described (Sailer et al., 1993). The supernatant was loaded directly onto an Amberlite XAD-8 (BDH Chemicals, Toronto, ON) column (4.5 x 50 cm) preequilibrated with 0.1% TFA, and sequentially eluted with increasing concentrations of ethanol (2 liters of each of 0, 20 and 35%; 1.5 liters of 50%; and 1 liter of 80%). The most active fraction (50% ethanol) was concentrated *in vacuo* at 30 °C to *ca.* 20 ml, mixed with an equivalent volume of MeCN and applied onto a Sephadex LH-60 (Sigma, St. Louis, MO) column (5 x 25 cm) equilibrated with 50% MeCN/0.1% TFA. After elution, the active fractions were pooled and concentrated to 10 ml. The separation of active compounds was accomplished by reversed-phase HPLC with a C_8 VYDEC column (10 x 250 mm, 10 µm particle size, 300 Å pore size, flow rate 2.5 ml/min, monitored at 218 nm) using a gradient from 20 to 31% MeCN in 0.1% TFA. Active fractions were identified and the bacteriocins were finally purified to homogeneity by isocratic elution (MeCN/0.1% TFA) on the same column. The pure bacteriocins were lyophilized and stored at -70 °C.

2.2.3. Stability of B carnobacteriocins

Lyophilized bacteriocin fractions were redissolved in aqueous solution (50 µg/ml) and tested for stability at different temperatures, in selected organic solvents and in

distilled water, and at the following pH levels: pH 3.0 (25 mM glycine-HCl); pH 5.0 (50 mM citric acid-HCl); pH 7.0 (50 mM Tris-HCl); pH 9.0 (50 mM Tris-HCl). Organic solvents included: 90% ethanol; 95% MeCN; 95% tetrahydrofuran; and 95% Me_2SO. Residual bacteriocin activity was determined after storage at 25 °C for 24 h. All aqueous bacteriocin solutions were tested for heat resistance by boiling for 30 min. All tests were repeated at least twice.

2.2.4. Enzymatic cleavage

Two sequence grade endoproteases were used: a specific Glu-C protease from *Staphylococcus aureus* V8 (Sigma) in 0.1 M ammonium acetate buffer (pH 4.0, at 37 °C); and chymotrypsin from bovine pancreas (Boehringer Mannheim Canada, Laval, PQ) in 0.1 M ammonium formate buffer (pH 8.0, 24 °C). Both were used with a 20:1 molar ratio of peptide to enzyme. The digestions were stopped by addition of HCOOH (10% final concentration), and the peptide fragments were immediately separated by HPLC on a reverse phase C_{18} column (VYDEC, 4 x 250 mm, 5 µm, 300 Å, flow rate 1 ml/min) using a 30 min linear gradient from 0 to 35% MeCN in 0.1% TFA.

2.2.5. Synthesis of nonapeptides

Chemical synthesis of the authentic nonapeptide, Ala-Ser-Ser-Leu-Ala-Gly-Met-Gly-His, was accomplished using standard solid phase FMOC (fluorenyl-methoxycarbonyl) methodology on a PS3 peptide synthesizer (Rainin Instruments, Emeryville, CA). The resulting resin-bound peptide (1 g) was cleaved from the resin and fully deprotected by stirring at 20 °C for 2 h in TFA/water/anisole/thioanisole (20/1/1/1). After removal of TFA *in vacuo* at 30 °C, the residual peptide was dissolved in 10% AcOH, precipitated with Et_2O, and washed twice with ethyl acetate, once with dichloromethane, and twice with Et_2O. The crude nonapeptide (*ca.* 20 mg) was purified by HPLC on a reverse phase C_{18} column (VYDEC, 4 x 250 mm, 5 µm, 300 Å, flow rate

1 ml/min) using a 30 min linear gradient from 0 to 50% MeCN in 0.1% TFA; retention time was 16.42 min. This yielded 14 mg of peptide (≥97% purity) whose mass spectra and HPLC retention time matched those of the fragment derived from degradation of carnobacteriocin BM1 (see below). A reference sample of the corresponding nonapeptide having the methionine residue oxidized to a sulfoxide was obtained by allowing the synthetic sample (*ca.* 2 mg) to stand exposed to the atmosphere in 10% aqueous TFA for two weeks at 20 °C.

2.2.6. Amino acid and N-terminal sequence analyses

Purified bacteriocins and their proteolytic degradation fragments were analyzed for amino acid composition (cysteine was determined as cysteic acid) by the Alberta Peptide Institute (University of Alberta). The N-terminal amino acid sequencing employed Edman degradation on an automated gas phase sequencer (Applied Biosystems model 470A) with on-line phenylthiohydantoin derivative identification by reversed-phase HPLC (Applied Biosystems model 120A).

2.2.7. Mass spectrometry

The mass spectra of carnobacteriocins B1 and B2 were obtained by Drs. Thomas Oglesby and Eric Johnson of Finnigan MAT (San Jose, CA). The samples were analyzed by direct infusion of their solution (1 μg/μl in 50% methanol/0.1% AcOH) using a Finnigan MAT TSQ® 700 triple quadrupole instrument with an electrospray ionization source (Analytica, Branford, CT). Finnigan MAT BioMass software was used to determine the molecular mass from the envelope of multiple charged peaks in the *m/z* spectra. The mass spectra of carnobacteriocin BM1 were obtained by Dr. Guenter Eigendorf (Chemistry Department, University of British Columbia, Vancouver, BC) on a Kratos Concept II spectrometer using Cs^+ liquid secondary ion mass spectrometry (LSIMS) by direct infusion of a solution of the bacteriocin in 50% MeCN/0.1% HCOOH.

The fragments derived from proteolytic digestions of carnobacteriocin B1 and carnobacteriocin BM1 were measured in the same laboratory using a 3-nitrobenzyl alcohol matrix.

2.2.8. Isolation of plasmid and chromosomal DNA

Small and large scale isolations of *E. coli* and *C. piscicola* LV17B plasmid DNA were done according to methods previously described (Sambrook et al., 1989; Ahn and Stiles, 1990). Chromosomal DNA was isolated from an overnight culture of *C. piscicola* LV17C (40 ml) grown to an optical density of 0.6 at 600 nm and harvested by centrifugation. The pellet was washed twice with 0.5% NaCl (20 ml) and resuspended in 3 ml of 25% sucrose - 50 mM Tris-HCl - 5 mM EDTA, pH 8.0. Lysozyme (Sigma) was added to a final concentration of 20 mg/ml and, after incubation at 37°C for 1 h, 3 ml of 1% SDS-100 mM EDTA was added. The lysate was mixed and incubated at 65 °C for 15 min. Proteinase K (Boehringer Mannheim, Dorval, PQ) was added to a final concentration of 100 µg/ml and the mixture was incubated at 56 °C for 6 to 8 h. After incubation, 0.6 ml of 1 M NaCl was added and the preparation was extracted twice with phenol-chloroform and once with chloroform. The DNA was precipitated with two volumes of 95% ethanol, washed three times with 70% ethanol, and resuspended in 2.5 ml of TE buffer (10 mM Tris-HCl - 1 mM EDTA) pH 8 with 100 µg/ml of RNase A (Sigma).

2.2.9. DNA manipulation and molecular cloning of carnobacteriocin BM1 and B2 structural genes

Restriction digestions, 5'-labeling of probes with γ-^{32}P-ATP, Southern and colony blot hybridization analyses on nylon membrane, electroelutions and DNA ligation were done by standard procedures (Sambrook et al., 1989). Restriction enzymes, T4 polynucleotide kinase and T4 DNA ligase were purchased from Boehringer Mannheim

(Dorval, PQ). pUC118 plasmid was used as the cloning vector and *E. coli* MV1193 was used as the host (Table 2.1). Transformation of *E coli* competent cells, selection and screening of transformants were done by established methods (Sambrook et al., 1989).

2.2.10. Synthesis of oligonucleotide probes and hybridization conditions-

A 24-mer oligonucleotide probe for carnobacteriocin B2 (B2 probe): 5'-GTAAACTGGGGACAAGCCTTTCAA-3', and a pool of 8 guessmer probes of 53 deoxynucleotides each for carnobacteriocin BM1 (BM1 probe): 5' AATAAAGCTGAAAATAAACAAGCTATTAC(AT)GG(AT)ATTGTTATTGG(AT)G G(AT)TGGGC-3' were synthesized on an Applied Biosystems 391 PCR MATE DNA synthesizer. Hybridizations were done in 6 x SSPE (0.18 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA) hybridization solution at 47 °C, with or without 20% formamide, using probes BM1 and B2, respectively.

2.2.11. DNA sequencing analysis

DNA was sequenced bidirectionally and analyzed in an Applied Biosystems 373A DNA Sequencer using fluorescent dyedeoxy-chain terminators. The recombinant plasmids pLQ5.21 and pLQ7.2 were used as templates (Table 2.1). The reactions were primed with forward and reverse universal primers for the pUC plasmid series, probes BM1 and B2, or with specific oligonucleotides newly synthesized using information from the sequences. Overlapping sequences were assembled, scanned for the presence of ORF and interpreted using the DNA Star program (Madison, WI).

2.2.12. Protein sequence analysis

Secondary structure prediction, membrane spanning segment identification and hydropathic profiles were determined using the program PC/GENE (Stratagene Cloning Systems). Search for homology with other proteins was done in the following data bases:

Brookhaven Protein Data Bank, June 1993 release; SWISS-PROT 26.0, August 1993; and PIR 37.0 (complete), June 30, 1993. The computation was performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service. Nucleotide sequences were submitted to GenBank (Los Alamos, NM) and were given the accession numbers L29058 and L29059 for the 1.9-kb and the 0.67-kb, respectively.

2.3. Results

2.3.1. Production, purification, and characterization of B carnobacteriocins

During static cultivation of a 1% inoculum of the producer strain in APT broth without pH regulation, the first traces of inhibitory activity in the supernatant were detected after 6 to 9 h of incubation. Cell growth and bacteriocin production reached a maximum at approximately 24 h (400 AU/ml), accompanied by a decrease in pH from 6.4 to 4.5. A critical dependence of bacteriocin production on pH was observed under controlled pH conditions. Low activity was detected at pH 5.5 and 6.5, with a maximum of 400 AU/ml and 1,200 AU/ml, respectively, after 24 h of incubation. A significant increase was observed at pH 6.0 and 6.2 with maxima of 5,800 and 6,500 AU/ml, respectively, after approximately 22 h of incubation. No differences in kinetics of growth were observed at different pH levels, and in all cases bacteriocin production was associated with the growth phase of the producer strain. A major reduction of the activity in the supernatant was observed at pH 6.0 and 6.2 after 24 h.

A yield of 3.0 to 3.5 x 10^7 AU was obtained from 5 liters of culture broth grown at pH 6.2. Adsorption on an Amberlite column gave recovery of 70% of the activity. Separation on a Sephadex LH-60 column partially increased the specific activity of the sample five-fold with 80% recovery of active material. HPLC separation, followed by biological assay of the inhibitory activity, showed the presence of more than one bacteriocin. Three inhibitory compounds termed carnobacteriocins B1, BM1 and B2,

with HPLC retention times of 12.43, 20.05, and 23.45 min, respectively, were isolated and purified to homogeneity. The specific activities (AU/μg) against *C. divergens* LV13 were *ca.* 18, 93 and 56 with total yields of 1.0, 1.4 and 2.6 mg for carnobacteriocins B1, BM1 and B2, respectively. The individual purified bacteriocins displayed differences in specific activity against selected indicator strains (Table 2.2).

All B carnobacteriocins were stable after freeze-drying. No significant decrease of activity was found in distilled water, or in any of the buffers tested (pH 3.0, 5.0, 7.0, and 9.0), or in 0.1% TFA, 95% ethanol, 95% MeCN or 95% Me_2SO after 24 h at room temperature. However, 75% of the initial activity was lost in 95% tetrahydrofuran-water after 24 h at room temperature. The bacteriocins were also stable after boiling for 30 min in aqueous solutions at pH 3 to 5.

N-terminal amino acid sequencing of the purified bacteriocins established 35 amino acid residues for carnobacteriocin B2. Similarly, the first 38 residues of both carnobacteriocins BM1 and B1 were determined, and shown to have identical sequences. The enzymatic cleavage of carnobacteriocin B2 at residue 24 with Glu-C endoprotease followed by Edman degradation of two fragments separated by HPLC elucidated the complete sequence of 48 amino acids. Mass spectrometric data and the calculated molecular weights based on amino acid sequence for the pure parent bacteriocins are shown in Fig. 2.1 and Table 2.3.

The Glu-C specific endoprotease treatment of carnobacteriocins BM1 and B1 resulted in two fragments from each bacteriocin (residues 1 to 21 and 22 to 43). No cleavage was obtained at residue Glu 13. This may be due to a particular conformation of the region as a result of the disulfide bridge between the cysteine residues 10 and 15 (see below) or the chemical environment created by the lysine residues flanking the glutamic acid residue. The N-terminal fragments (residues 1 to 21) of carnobacteriocins B1 and

Table 2.3. Antimicrobial spectrum of the purified B carnobacteriocins.

Indicator		Specific activity AU/µg		
		B1	BM1	B2
Carnobacterium divergens	LV13	18	93	56
Carnobacterium piscicola	UAL26	16	128	64
Lactobacillus plantarum	ATCC 4008	-	8	2
Pediococcus parvulus	ATCC 19371	-	8	8
Listeria monocytogenes	ATCC 15313	8	64	32
Listeria innocua	ATCC 33090	4	32	16
Enterococcus faecium	ATCC 11576	-	16	16
Enterococcus faecalis	ATCC 19433	2	32	16
Enterococcus faecium	ATCC 19434	2	8	4

The bacteriocins were tested against the indicator strains at a concentration of 50 µg/ml using the spot-on-lawn technique. - : no activity detected

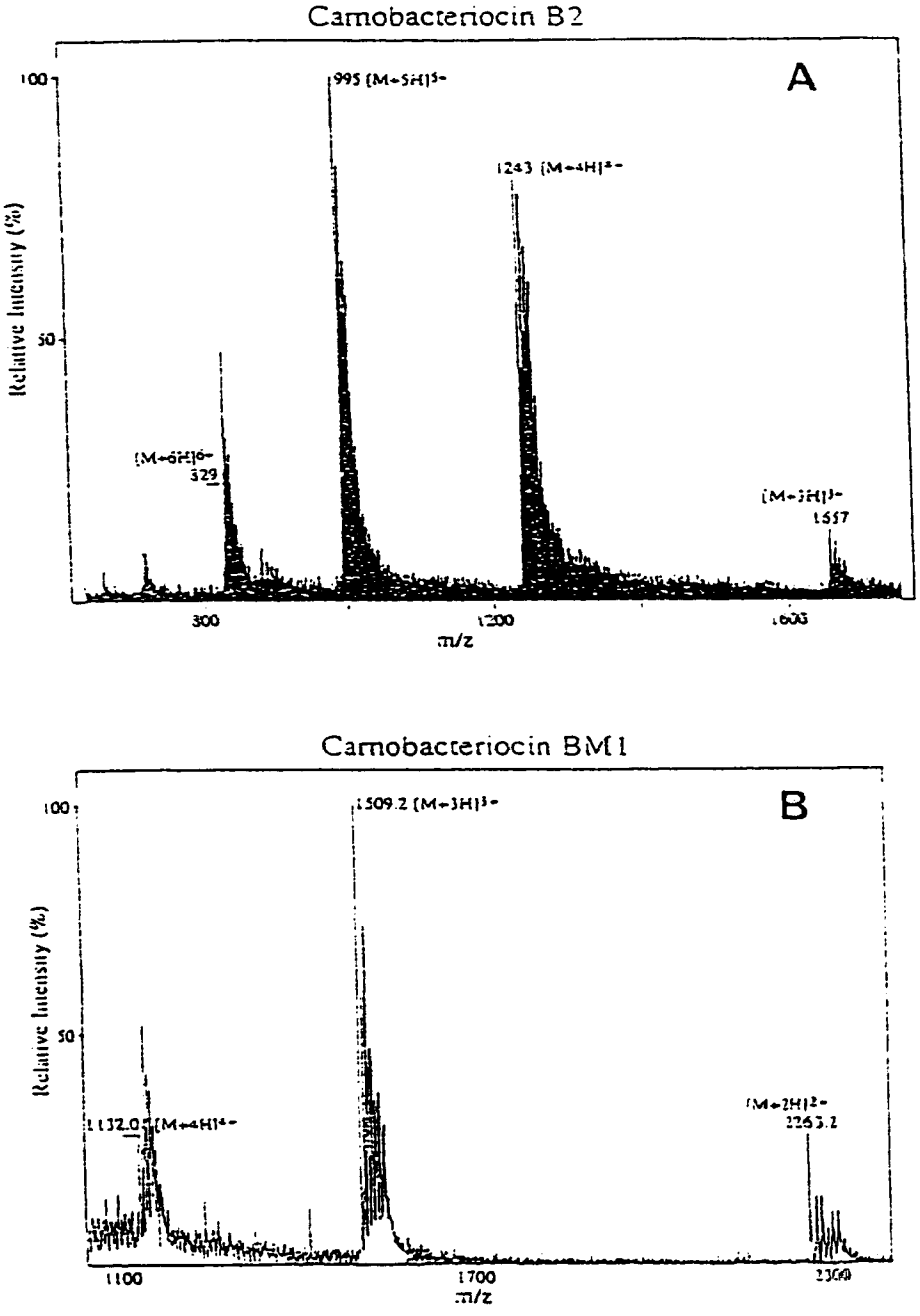

Figure 2.1. Mass spectra of carnobacteriocins B2 and BM1. Electrospray Fast Atom Bombardment mass spectra of carnobacteriocins B2 (A) and BM1 (B) with multiple charged molecular ions.

Table 2.3. Molecular mass and calculated molecular weight of the purified bacteriocins and fragments derived by enzymatic proteolysis

Peptide	Molecular mass from mass spectroscopy	Calculated molecular weight	Difference
B1	4541.9 ± 0.6	4527.12	+14.8 ± 0.6
B1(residues 1-21)	2374.3 ± 0.4	2376.66	-2.3 ± 0.4
B1(residues 22-43)	2185.2 ± 0.4	2168.46	+16.4 ± 0.4
BM1	4524.6 ± 0.6	4527.12	-2.5 ± 0.6
BM1(residues 1-21)	2374.3 ± 0.4	2376.66	-2.3 ± 0.4
BM1(residues 22-43)	2168.5 ± 0.3	2168.46	-
B2	4969.9 ± 0.7	4969.49	-

BM1 had the same HPLC retention time as well as average molecular mass (Table 2.3). The -2 Da difference from the calculated molecular weight indicates the presence of adisulfide bridge between the two cysteine residues at positions 10 and 15. The C-terminal pair of fragments (residues 22 to 43) had different HPLC retention times and masses (Table 2.3). They were individually cleaved by chymotrypsin at the Trp 34 residue. Edman degradation of both fragments derived from carnobacteriocin BM1 (residues 22 to 34 and 35 to 43) elucidated the complete amino acid sequence of carnobacteriocin BM1. The amino acid composition was confirmed by amino acid analysis (data not shown) and was in agreement with the calculated molecular weight.

The C-terminal nonapeptides (residues 35 to 43) derived from carnobacteriocins BM1 and B1 showed different HPLC retention times. A nonapeptide corresponding to the last nine C-terminal residues of carnobacteriocin BM1 was synthesized and displayed identical HPLC retention time and mass spectra to that of the nonapeptide obtained by degradation of this bacteriocin. It was found that the synthetic nonapeptide slowly oxidized upon standing in aqueous trifluoroacetic acid at room temperature at the Met 41 residue (+16.4 Da increase, Table 2.3) to a peptide whose HPLC retention time corresponded with that of the C-terminal nonapeptide of carnobacteriocin B1. The slow transformation (*ca.* 5% per week) of the parent carnobacteriocin BM1 to B1 also occurred under the same conditions.

2.3.2. Identification, cloning, and nucleotide sequence of the structural genes of carnobacteriocins BM1 and B2

The structural genes of carnobacteriocins BM1 and B2 (*cbnBM1* and *cbnB2*) were located with the specific oligonucleotide probes by Southern hybridization analysis of restriction digests of the 61-kb plasmid and genomic DNA (Fig. 2.2). A 1.9-kb *Hin*dIII fragment from pCP40 that hybridized with the B2 probe, and a 4.0-kb *Eco*RI-*Pst*I

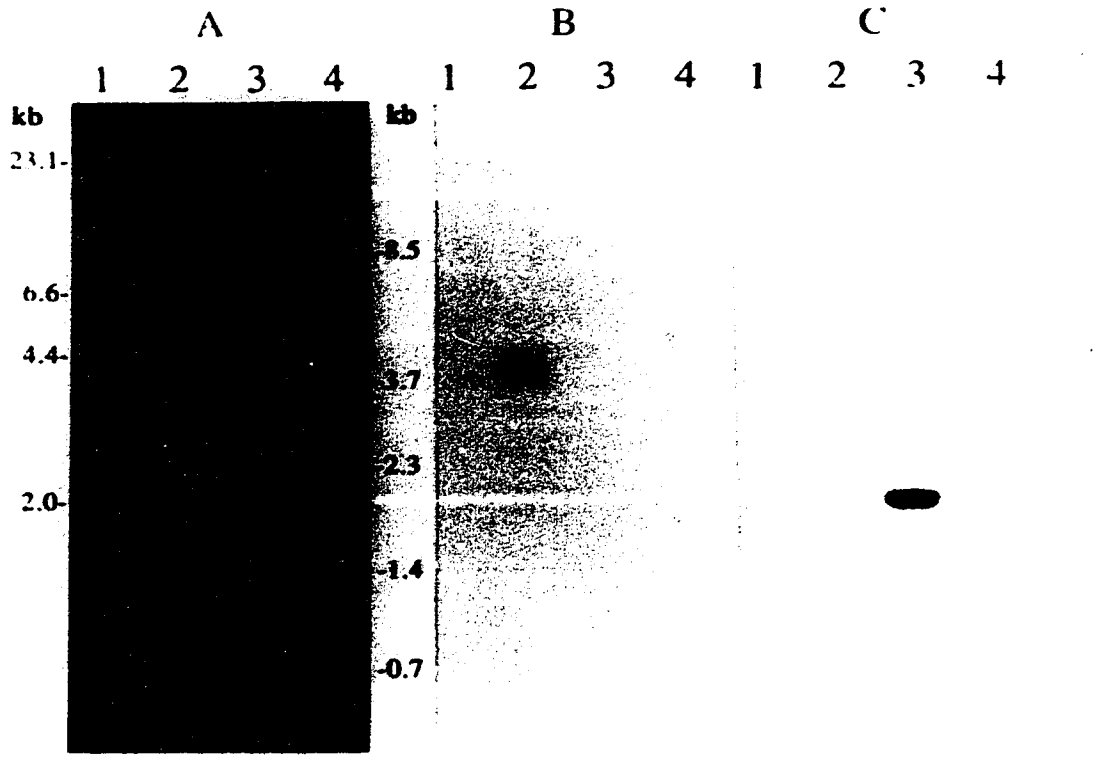

Figure 2.2. Southern hybridization analysis of plasmid and genomic DNA from *C. piscicola* LV17B and LV17C. Panel A, 0.75 % agarose gel electrophoresis. Lanes 1 and 4, phage λ DNA digested with *Hin*dIII and *Bst*EII, respectively; lane 2, genomic DNA from *C. piscicola* LV17C digested with *Eco*RI and *Pst*I; lane 3, pCP40 digested with *Hin*dIII. Panels B and C, Southern hybridization of gel from panel A with BM1 and B2 probes, respectively.

genomic fragment that hybridized with the BM1 probe were cloned into pUC118 to create the plasmids pLQ5.21 and pLQ7.2 (Table 2.1).

A 0.67-kb segment of the 4.0-kb *Eco*RI-*Pst*I genomic fragment that hybridized with the BM1 probe was sequenced. The physical map, sequencing strategy and the nucleotide sequence of this segment are shown in Fig. 2.3. Analysis of the nucleotide sequence revealed the presence of two ORF. ORF-α1 (*cbnBM1*) consisting of 183 bp encoding a 61-amino acid polypeptide (cbnBM1) comprised of an 18-amino acid N-terminal extension followed by the 43-amino acid sequence that matches the sequence of purified carnobacteriocin BM1 determined by Edman degradation. ORF-α2 may encode a polypeptide of 88 amino acids with calculated MW of 10189.20 and isoelectric point of 9.51. Located 9 bp upstream of *cbnBM1* and ORF-α2 there are potential RBS, AGGAG and AGAGG, respectively, resembling the consensus for the short RBS nucleotide sequence (Shine and Delgarno, 1975; Gold, 1988). Downstream of ORF-α2, there is a region of dyad symmetry that could form a stem-loop structure with $\Delta G_{(25\ °C)}$ of -18.3 kcal. This structure could represent a transcriptional terminator for the carnobacteriocin BM1 operon.

The physical map, sequencing strategy and nucleotide sequence of the 1.9-kb *Hin*dIII fragment from pCP40 are shown in Fig. 2.4. The nucleotide sequence analysis indicated the presence of three ORF. ORF-β1 (*cbnB2*) consists of 198 bp encoding a 66-amino acid polypeptide (cbnB2) including an 18-amino acid N-terminal extension followed by a 48-amino acid sequence identical to that of purified carnobacteriocin B2. ORF-β2 and ORF-β3 may encode polypeptides of 111 and 82 amino acids, with MW of 12666.50 and 9070.80 and isoelectric points of 8.91 and 4.49, respectively. Located 9 bp upstream of *cbnB2* and ORF-β2 there are two potential RBS, AGGAG and AGGTGG, respectively. Possible -10 and -35 promoter sequences TATAGT and TTCCAA, respectively, are present upstream of *cbnB2*. These sequences are similar to the

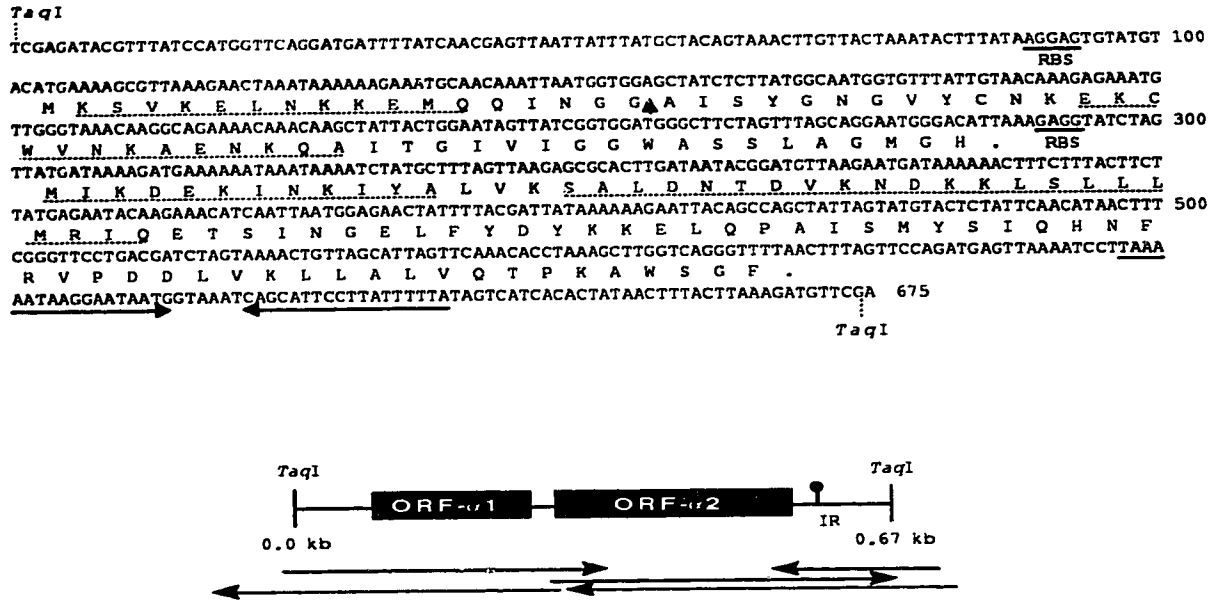

Figure 2.3. Nucleotide sequence, physical map of the sequenced region and sequencing strategy of carnobacteriocin BM1 structural gene (ORF-α1) and adjacent region. The deduced amino acid sequences of *cbnBM1* and ORF-α2 are shown below the DNA sequence. Possible ribosomal binding sites (RBS) and the inverted repeat sequence with the greatest negative free energy are underlined. The vertical arrow in the amino acid sequence of the prebacteriocin indicates the cleavage site. Dotted lines below the amino acid sequence indicate amino acid residues in α-helix conformation.

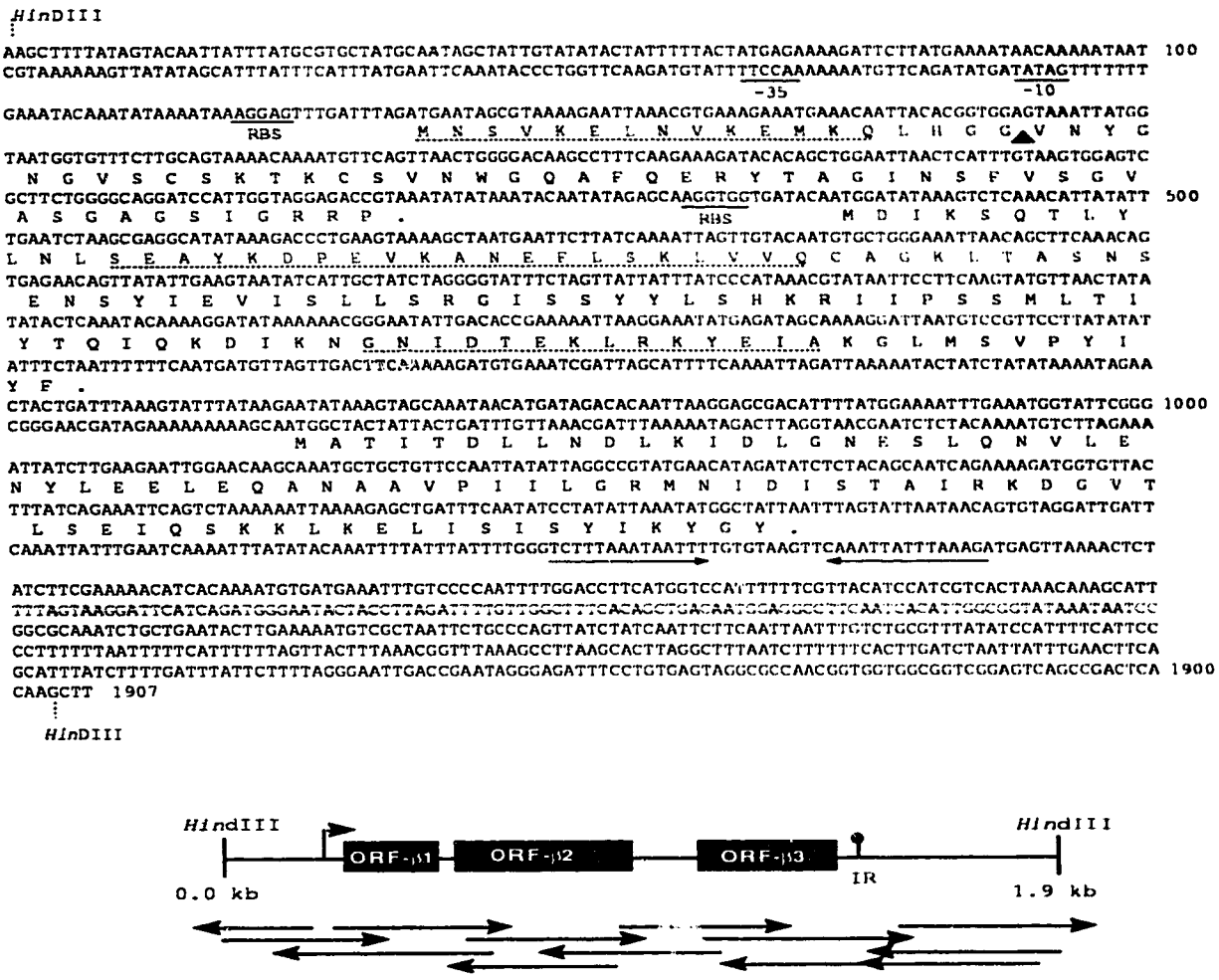

Figure 2.4. Nucleotide sequence, physical map and sequencing strategy of the *Hin*dIII fragment from pLQ5.21 containing carnobacteriocin B2 structural gene (ORF-β1). The deduced amino acid sequences of ***cbnB2***, ORF-β2 and ORF-β3 are shown below the DNA sequence. Possible promoter sequences, ribosomal binding sites (RBS) and the inverted repeat sequence with the greatest negative free energy are underlined. The vertical arrow in the amino acid sequence of the prebacteriocin indicates the cleavage site. Dotted lines below the amino acid sequence indicate amino acid residues in α-helix conformation.

consensus for *E. coli* σ^{70} and *B. subtilis* σ^{43} promoter sequences (Hawley and McClure, 1983; Doi and Wang, 1986). Upstream of the possible -10 sequence, and separated by one nucleotide, a TG dinucleotide is present. This pair is present in the postulated promoter region of the carnobacteriocin A structural gene (Worobo et al., 1994) and is conserved in bacteria such as *Lactoccocus lactis* ssp. *lactis* and *cremoris*, *Bacillus subtilis* and other Gram-positive bacteria (Moran et al., 1982; Graves and Rabinowitz, 1986; Van der Vossen et al., 1987; Koivula et al., 1991). A region of dyad symmetry within the sequenced region that could form a stem-loop structure with negative free energy ΔG(25 °C) of -14.2 kcal is located downstream of ORF-β3. This structure could represent the 3' limit for the polycistronic transcript of the carnobacteriocin B2 operon.

2.3.3. Amino acid homology, prediction of secondary structure and hydropathic profiles

Amino acid comparison between cbnBM1 and cbnB2 showed 72% identity between the leader regions, and 34% between the mature bacteriocins. The identity between the aligned segments of the proteins encoded by ORF-α2 and ORF-β2 is 19%. No similarity was observed between these proteins and the polypeptide encoded by ORF-β3. A search of the protein data banks showed homology between the precarnobacteriocins B and other bacteriocins (see Discussion). No homology was found between ORF-α2, ORF-β2 or ORF-β3 products and reported proteins. The secondary structure predicted using the method of Garnier and Osgothorpe (1978) for cbnBM1, cbnB2 and the translational products of ORF-α2, ORF-β2 and ORF-β3 are shown in Figs. 2.3 and 2.4. The predictions suggest that the leader regions of both precursors adopt α-helix conformation. The products of ORF-α2, ORF-β2 and ORF-β3 also have several regions that could assume α-helix conformation. Similar predictions were obtained with the method of Gascuel and Golmard (1988). Hydropathic profiles of cbnBM1, cbnB2, and ORF-α2, ORF-β2 and ORF-β3 products are shown in Fig. 2.5. The average

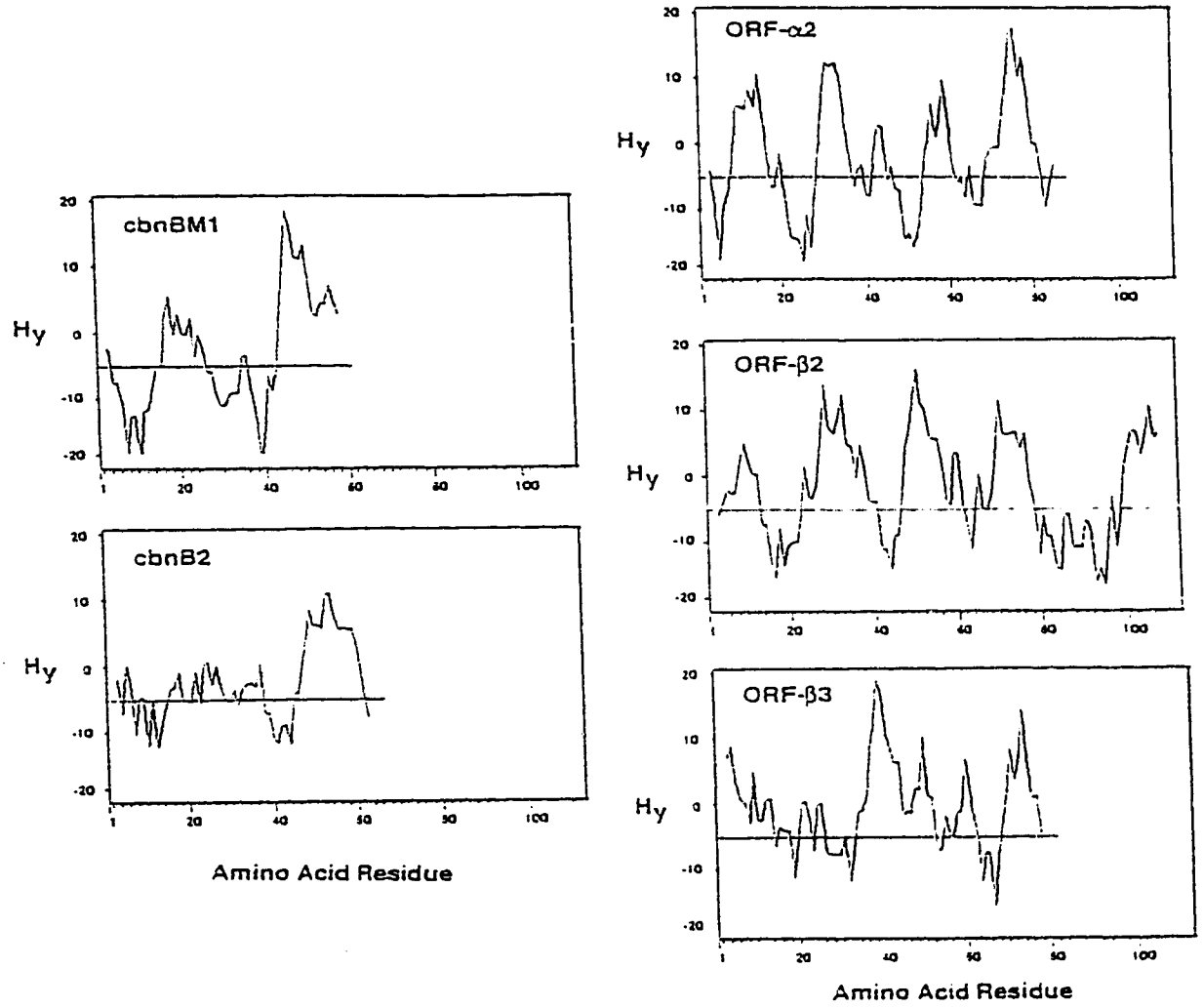

Figure 2.5. Hydropathic profiles of precarnobacteriocins BM1 (cbnBM1) and B2 (cbnB2); possible immunity proteins ORF-α2 and ORF-β2; and ORF-β3. Hydrophobicity is shown as values above and hydrophilicity as values below the universal midline (horizontal line in each graph). The profiles were calculated using the algorithm of Kyte and Doolittle (1982) with a seven residue moving window. Hy = hydropathic index.

hydropathic value for both prebacteriocins is similar: -4.14 for cbnBM1 and -4.17 for cbnB2; and for ORF-α2 and ORF-β2 products are -2.56 and -2.03, respectively.

ORF-β3 product has a hydropathic value of -0.18. Although hydrophobic regions can by identified at the C-terminal segment of the prebacteriocins and in ORF-α2, ORF-β2 and ORF-β3 products (Fig. 2.5), no membrane spanning segments are predicted for any of the polypeptides analyzed (Klein et al., 1985).

2.4. Discussion

The purification and characterization of carnobacteriocins BM1 and B2 adds two new members to the group of class II bacteriocins from lactic acid bacteria (Klaenhammer, 1993). Carnobacteriocin B1, which is an oxidized form of carnobacteriocin BM1, was also purified and characterized. Because the nonenzymatic aerobic conversion of pure carnobacteriocin BM1 to B1 is slow, and the fermentation conditions are relatively anaerobic, the detection of carnobacteriocin B1 at the time of purification suggests that the sulfoxide formation at methionine 41 may be mediated by the organism. Interestingly, oxidation of the sulfur of the Met 41 residue of carnobacteriocin BM1 has an important effect on the antagonistic activity of the bacteriocin, and the resulting carnobacteriocin B1 is less active by a factor of 4 to 8, depending on the indicator strain challenged.

The amino acid composition and the molecular mass of the peptides confirmed the structures and excluded the possibility of extensive posttranslational modification that is characteristic of lantibiotics. The -2 Da difference between the average molecular mass and the calculated molecular weight of carnobacteriocins BM1 and B1, as well as of the N-terminal fragments generated from them by Glu-C protease digestion, indicates the presence of a disulfide bridge between cysteine residues 10 and 15. Pediocin PA-1 and leucocin A also have a disulfide bridge in the same position (Hastings et al., 1991;

Henderson et al., 1992). Although the same two cysteine residues are present in carnobacteriocin B2, a disulfide bridge does not appear to be present. Experiments with reduction of the disulfide bridge in leucocin A suggest that it may not be essential for antimicrobial activity (Hastings et al., 1991), despite the occurrence of these two highly conserved cysteine residues in a number of such bacteriocins.

Comparison of the primary structures of the purified peptides with the predicted translational products (cbnBM1 and cbnB2) of the bacteriocin structural genes indicates that both carnobacteriocins are synthesized as precursors that undergo proteolytic cleavage of an 18-amino acid leader peptide. These bacteriocin leaders differ from those of known signal sequences in the precursors of other secreted proteins (Gierasch, 1989). The function of the leader portions in the bacteriocin precursors is still unclear, but it may be required for recognition by secretion machinery. Proteins which may be implicated in the transport of the class II bacteriocins, lactococcin A and pediocin PA-1, have been identified (Marugg et al., 1992; Stoddard et al., 1992). Another possible function of the leader peptides could be to neutralize the biological activity of the bacteriocin within the cell. In this regard, the precursor of nisin A was shown to be inactive (Van der Meer et al., 1993), but the precursor of lactococcin B showed antimicrobial activity (Van Belkum et al., 1992).

CbnBM1 and cbnB2 have a high degree of homology with other class II bacteriocins and their leader peptides (Fig. 2.6). In the leader peptides a common Gly-Gly motif (positions -2 and -1) is present at the cleavage site. Other conserved sections include: a central region rich in charged and hydrophilic amino acids, but having hydrophobic residues at positions -7 and -12; hydrophobic residues in positions -4 and -15; and polar or positively charged residues close to the N-terminus of the prebacteriocin. In the mature bacteriocins, the homology is greater at the N-terminal region (Lozano et al., 1992). There is a "x_1 YGNGV x_2 C x_3 K/N x_4 x_5 C x_6 V N/D x_7 G x_8 A"

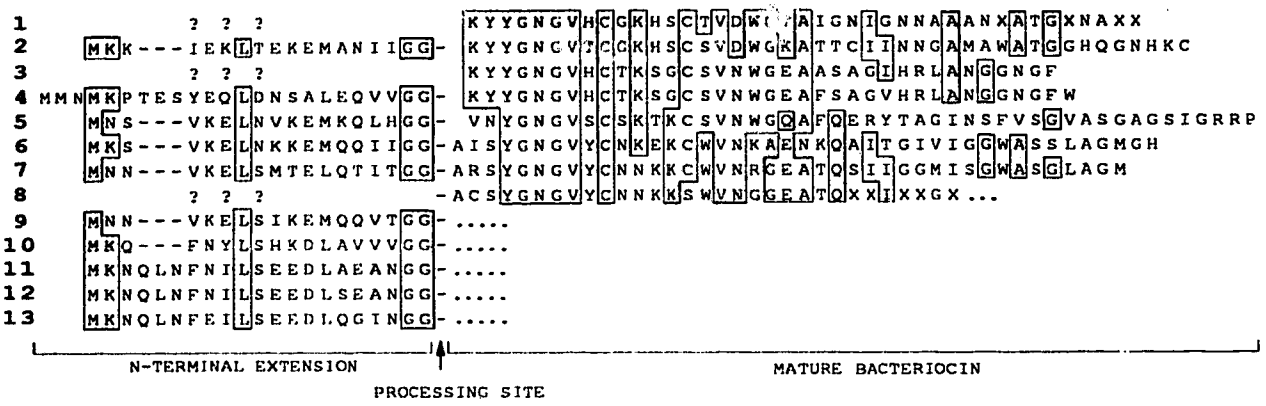

Figure 2.6. Multiple sequence alignment of bacteriocins containing the "YGNGV" amino acid motif and N-terminal extensions of other class II bacteriocins. 1: sakacin P (Tichaczek et al., 1992); 2: pediocin PA-1 (Marugg et al., 1992); 3: mesentericin Y105 (Hechard et al., 1992); 4: leucocin A (Hastings et al., 1991); 5: carnobacteriocin B2 (this study); 6: carnobacteriocin BM1 (this study); 7: sakacin A (Holk et al., 1992); 8: curvacin A (Tichaczek et al., 1992); 9: carnobacteriocin A (Worobo et al., 1994); 10: lactacin F (Muriana and Klaenhammer, 1991); 11: lactococcin B (Van Belkum et al., 1992); 12: lactococcin A (Van Belkum et al., 1991); 13: lactococcin M (Van Belkum et al., 1991). The vertical arrow indicates the cleavage site in the prebacteriocins. The shaded areas indicate identical amino acids in the sequences.

consensus motif, wherein x_1 and x_3 represent polar residues; x_2, x_5 and x_8 represent polar or positively charged residues; x_4 represents a polar or charged residue; x_6 represents a polar or aromatic residue; and x_7 represents an aromatic or positively charged residue. The significance of this arrangement, especially the YGNGV motif, that may identify a family within the class II bacteriocins, is not understood at present.

The organization of the genetic determinants for the B carnobacteriocins, *cbnBM1* in the chromosome and *cbnB2* in the plasmid pCP40 presents an interesting situation. Although the structural gene of cbnBM1 is located in the chromosome, the plasmidless strain *C. piscicola* LV17C does not display bacteriocin activity and is sensitive to both carnobacteriocins BM1 and B2. This indicates that the presence of the 61-kb plasmid is required for the phenotypic expression of the immunity functions and both carnobacteriocins. The nature of the complementing trans-acting factor encoded in the plasmid that is required for the expression of the chromosomal bacteriocin remains unclear. The required function could be part of a transcriptional activation, secretion, or processing system.

The analysis of the nucleotide sequences adjacent to *cbnBM1* and *cbnB2* revealed the presence of one ORF immediately downstream of each bacteriocin gene. The functions of these gene products have not been established, but they could be part of the bacteriocin operons and may be involved in immunity. Proteins responsible for immunity to lactococcins A, B and M are 98, 91 and 154 amino acids, respectively (Van Belkum et al., 1991, 1992), and they are located downstream of the bacteriocin structural genes and form part of the bacteriocin operons.

In preliminary studies to identify the genetic elements located in the plasmid pCP40 that are required for expression of the chromosomally encoded bacteriocin, carnobacteriocin BM1, we recently cloned a 9-kb fragment from pCP40 into the

plasmidless strain *C. piscicola* LV17C and thereby restored the production of both bacteriocins and their immunity functions (unpublished data). On-going studies to identify the genetic determinants and elucidate the control involved in the phenotypic expression of the B carnobacteriocins and immunity to them should provide a more detailed understanding of these natural "biopreservatives". This knowledge will facilitate rational use of bacteriocinogenic strains of lactic acid bacteria to enhance meat safety and preservation.

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3. CHARACTERIZATION OF THE PROTEIN CONFERRING IMMUNITY TO THE ANTIMICROBIAL PEPTIDE CARNOBACTERIOCIN B2 AND EXPRESSION OF CARNOBACTERIOCINS B2 AND BM1[1]

3.1. Introduction

In recent years there has been growing interest in the preservation of food by lactic acid bacteria and their commercial potential has led to the identification and characterization of several bacteriocins produced by this group of organisms. Bacteriocins are peptides or proteins with antimicrobial activity against closely related bacterial strains but they are not active against the producer strain (Klaenhammer, 1993). The best characterized of these compounds is nisin A, an extensively posttranslationally modified lantibiotic which contains 34 amino acids and is produced by *Lactococcus lactis* subsp. *lactis* (Jung, 1991). Its NMR assignment, the genes required for its production, the mode of action, and the protein responsible for nisin immunity have been determined (Kuipers et al., 1993; Sailer et al. 1993; Van der Meer et al., 1993; Engelke et al., 1994). Although available information indicates that the bacteriocins from lactic acid bacteria exert their antimicrobial effects through action at the cell membrane (Gao et al., 1991; Van Belkum et al., 1991; Chikindas et al., 1993; Venema et al., 1993; Abee et al., 1994), the mechanism of immunity that protects the producer organism against its own bacteriocin is not understood. The genetic determinants of proteins that afford protection against the nonlantibiotic bacteriocins, lactococcins A, B and M have been cloned and sequenced (Van Belkum et al., 1991, 1992). More recently, the immunity protein to lactococcin A produced by *Lactococcus lactis* subsp. *cremoris* has been purified and characterized (Nissen-Meyer et al., 1993; Venema, 1994).

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[1] A version of this chapter was previously published: Quadri, L. E. N., M. Sailer, M. R. Terebiznik, K. L. Roy, J. C. Vederas, and M. E. Stiles. 1995. Characterization of the protein conferring immunity to the antimicrobial peptide carnobacteriocin B2 and expression of carnobacteriocins B2 and BM1. J. Bacteriol. 177:1144-1151.

A quite different group of bacteriocins is produced by strains of lactic acid bacteria that prevail in the microflora of chill-stored meats, packaged under vacuum or in modified atmospheres containing elevated levels of carbon dioxide (McMullen and Stiles, 1993). Carnobacteriocins B2 and BM1 (CbnB2 and CbnBM1) are small class II bacteriocins produced by *Carnobacterium piscicola* LV17B that belong to a rapidly growing family of unmodified peptides having a YGNGV sequence motif near the N-terminus (Quadri et al., 1994). Other members of this family include leucocin A (Hastings et al., 1991), mesentericin Y105 (Héchard et al., 1992), sakacins P and A (Holck et al. 1992; Tichaczek et al., 1992), pediocin PA-1/AcH (Marugg et al., 1992; Bukhtiyarova et al., 1994) and curvacin A (Tichaczek et al., 1992). Their antimicrobial spectra include many lactic acid bacteria, as well as strains of potentially pathogenic *Enterococcus* and *Listeria* species. The genetic determinants of CbnB2 and CbnBM1 (*cbnB2* and *cbnBM1*) have been cloned, sequenced, and shown to be located on a 61-kb plasmid (pCP40) and on the chromosome, respectively (Quadri et al., 1994). These carnobacteriocins are initially synthesized as precursors containing 66 and 61 amino acids that undergo posttranslational cleavage of an N-terminal extension of 18 amino acids after a Gly-Gly site to yield the mature active peptides of 48 and 43 amino acids, respectively. The phenotypic expression of both bacteriocins produced by *C. piscicola* LV17B, and the immunity functions conferring protection against them are dependent on the presence of the pCP40 plasmid. Downstream of *cbnB2*, two other ORFs, ORF-β2 (*cbiB2*) and ORF-β3, have been sequenced from a 1.9-kb *Hin*dIII fragment of pCP40 (GenBank™/EMBLData Bank L29059; Quadri et al., 1994). In this study we describe the identification, characterization, heterologous expression and purification of CbiB2, the protein conferring immunity to carnobacteriocin B2. We also report the recovery of the wild type carnobacteriocin (Cbn^+) and immune (Imm^+) phenotype in the cured mutant *C. piscicola* LV17C after transformation with DNA fragments derived from pCP40, and the expression of carnobacteriocin B2 in a heterologous host.

3.2. Materials and methods

3.2.1. Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are described in Table 3.1. *Carnobacterium* strains were grown in APT broth (Difco) as previously described (Ahn and Stiles, 1990). *E. coli* cultures were grown in Luria-Bertani (LB) broth or on LB agar (Sambrook et al., 1989). Media containing ampicillin (100 μg/ml), erythromycin (100 μg/ml for *E coli* and 5 μg/ml for *Carnobacterium* strains) or chloramphenicol (5 μg/ml) were used for growth and selection of transformants. Bacteriocin production was determined by spot-on-lawn test (Ahn and Stiles, 1990). Different strains of lactic acid bacteria were challenged with the heat-treated (65°C, 15 min) culture supernatant of the strains to be tested for bacteriocin production.

3.2.2. Bacteriocin sensitivity assay

The sensitivity of selected strains to carnobacteriocins B2 and BM1 was determined by spot-on-lawn test (spotting 0.2 μg of bacteriocin) or in microtiter plates. In the latter case, the wells were loaded with serial four-fold dilutions of the carnobacteriocin B2 (0.25, 1, 4, and 16 μg/ml) in 150 μl of APT broth inoculated with a fresh culture (1% inoculum). The plates were incubated at 25 °C and absorbance was measured at 590 nm at 30 min intervals. The effect of bacteriocin on cell growth in the presence of purified immunity protein (100 μ/ml) or bovine serum albumin (BSA, 100 μg/ml) was evaluated. The bacteriocins used in the tests were purified by methods previously described (Quadri et al., 1994).

3.2.3. DNA isolation, manipulation and sequencing

Isolation of plasmid DNA from *Carnobacterium* strains and *E. coli* was done according to methods previously described (Sambrook et al., 1989; Ahn and Stiles, 1990). Restriction digestion, exonuclease III treatment, 5'-labeling with γ-[^{32}P]-ATP,

Table 3.1. Bacterial strains and plasmids

Bacterial strain and plasmid	Relevant phenotype and properties	Reference
Bacteria		
C. piscicola		
LV17B	Cbn^+, Cbn^R, containing pCP40	Ahn and Stiles, 1990
LV17C	Cbn^-, Cbn^S plasmidless mutant derived from *C. piscicola* LV17B	Ahn and Stiles, 1990
LV17CD	LV17C containing pCaT	Ahn et al., 1992
UAL26	Cbn^S	Ahn and Stiles, 1990
LQ24; 18; 18E	LV17C containing pLQ24,pLQ18, or pLQ18E, respectively.	This study
LV17Ci	LV17C containing pLQ400i	This study
UAL26i	UAL26 containing pLQ400i	This study
C. divergens		
LV13	Cbn^S	Shaw[a]
LV13pCaT	LV13 containing pCaT	Ahn et al., 1992
LQ24	LV13 containing pLQ24	This study
LV13i	LV13 containing pLQ400i	This study
E. coli		
JM107	Cloning host	Sambrook et al., 1989
LQ400i	*E. coli* JM107 containing pLQ400i	This study
LQ300i	*E. coli* JM107 containing pLQ300i	This study
Plasmids		
pUC118	3.2-kb cloning vector, Amp^R, lacZ'	Vieira and Messing, 1987
pMAL-c	6.1-kb expression vector, Amp^R, lacZ'	New England Biolabs Ltd.
pMG36e	3.4-kb expression vector, Em^R	Van der Guchte et al., 1989
pCaT	8.5-kb cloning vector, Cm^R	Jewell and Collins-Thompson, 1989
pCP40	61-kb plasmid conferring Cbn^+ Imm^+ phenotype	Ahn and Stiles, 1990
pLQ24	pCaT containing a 16-kb fragment from pCP40	This study
pLQ18	pCaT containing a 9.5-kb fragment from pCP40	This study
pLQ18E	pLQ18 with *cbnB2* and *cbiB2* deleted	This study
pLQ300i	pMAL-c containing *malE-cbiB2* fusion	This study
pLQ400i	pMG36e containing 442-bp insert with *cbiB2*	This study
pLQ5.21	pUC118 containing a 1.9-kb *Hin*dIII	Quadri et al., 1994

[a] Supplied by Dr B G. Shaw, Institute of Food Research, Langford, Bristol U. K.
Cbn = carnobacteriocin(s), ^{S} = sensitive

Southern hybridization analyses on nylon membranes and DNA ligation were done by standard procedures (Sambrook et al., 1989). Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase and Exonuclease III were purchased from Boehringer Mannheim (Dorval, PQ). Transformation of *E. coli* competent cells and strains of lactic acid bacteria, selection and screening of transformants were done by established methods (Sambrook et al., 1989; Ahn and Stiles, 1992). DNA was sequenced using *Taq* DNA polymerase and fluorescent dyedeoxy-chain terminators, and analyzed on an Applied Biosystems 373A DNA Sequencer. The recombinant plasmid pLQ300i was used as a template (Table 3.1) and the reactions were primed with forward and reverse primers for pMAL vectors (New England Biolabs, Ltd., Mississauga, ON, Canada).

3.2.4. Subcloning of *cbiB2* and construction of *malE-cbiB2* fusion

A 442-bp *Bam*HI-*Cla*I fragment from pLQ5.21 containing the last 21-bp of *cbnB2*, the intergenic region between *cbnB2* and *cbiB2*, *cbiB2*, and 48-bp downstream *cbiB2* was subcloned into pUC118, excised with *Sac*I-*Hin*dIII and subcloned into pMG36e to create pLQ400i (Table 3.1). pLQ400i was introduced into selected strains of lactic acid bacteria and the sensitivity of the transformants to the carnobacteriocins was studied. For the construction of *malE-cbiB2* fusion, *CbiB2* was amplified by PCR using two specific primers synthesized on an Applied Biosystems 391 PCR MATE DNA synthesizer. ImmF (5' XATGGATATAAAGTCTCAAAC 3', X= phosphate), based on the 5' nucleotide sequence of *cbiB2*, and ImmR (5' CGCTCTAGATTAGAAATATATATAAGGAAC 3') based on the 3' nucleotide sequence of *cbiB2* and containing a 5' overhang of 9 nucleotides with a *Xba*I restriction site were used to prime the forward and reverse reactions, respectively. The amplified fragment was digested with *Xba*I and cloned into pMAL-c linearized with *Stu*I and *Xba*I to create a fusion in the correct translational reading frame between the *E. coli* maltose binding protein gene (*malE*) and *cbiB2* in pLQ300i (Table 3.1).

3.2.5. Immunity protein purification

Carnobacteriocin B2 immunity protein (CbiB2) was purified from 1 liter of *E. coli* LQ300i culture grown in LB broth with 0.2% glucose and 100 μg/ml of ampicillin at 37 °C to an optical density at 600 nm of 0.5. The expression, affinity purification and Factor Xa cleavage of the fusion protein were done as recommended in the Protein Fusion and Purification System instruction manual (New England Biolabs). No denaturation of the fusion protein was done before Factor Xa cleavage. CbiB2 was purified by reversed-phase HPLC using a C8 - VYDEC column (10 x 250 mm, 10 μm particle size, 300 Å pore size) and a gradient from 38.5 to 45.5% of acetonitrile in 0.1% trifluoroacetic acid (flow rate 2.5 ml/min, monitored at 218 nm). Purified samples of CbiB2 were lyophilized and stored at -20 °C. Protein concentration was estimated using a modification of the method of Lowry (Markwell et al., 1978).

3.2.6. N-terminal sequence analysis and mass spectrometry

The N-terminal amino acid sequencing of CbiB2 was done by Edman degradation on an automated gas phase sequencer (Applied Biosystems model 470A) with on-line phenylthiohydantoin derivative identification by reversed-phase HPLC (Applied Biosystems model 120A). The mass spectrometry data for the purified immunity protein was obtained from the positive ion electrospray analysis done at the National Research Council (Canada) Plant Biotechnology Institute (Saskatoon, SK) on a Fisons Trio 2000 mass spectrometer equipped for electrospray. The lyophilized sample was dissolved in 50% acetonitrile: 50% of a 1% formic acid solution and loop injected (10 μl) at a flow rate of 6 μl/min.

3.2.7. Antiserum against carnobacteriocin B2 immunity protein

Two rabbits were injected at 3-week intervals, three times with 200 μg of purified fusion protein and once with 100 μg of purified CbiB2 (final injection). Immunogens

were prepared in Ribi Adjuvant for rabbits as recommended by the suppliers (Immuno Chem Research Inc., Hamilton, Montana, USA). The immunization procedure was done in accordance with the Animal Welfare guidelines of the University of Alberta. The antibodies against CbiB2 in the serum collected after the last injection were purified by affinity binding to an antigen column. To obtain the CbiB2 column, the immunity protein was electroeluted after electrophoresis and covalently linked to Sepharose CL-6B (Pharmacia) using established methods (Jacobs and Clad, 1986; Stults et al., 1989).

3.2.8. Preparation of cytoplasmic, membrane and extracellular protein fractions

Cytoplasmic (soluble) and membrane fractions were prepared based on previously reported methods (Kaback, 1971). Cells from 1-liter cultures of *C. piscicola* LV17B and LV17C were grown to early stationary phase in APT broth and harvested by centrifugation (13,000 x *g*, 10 min). The supernatants were stored at -20°C (extracellular protein fraction). The cells were washed with 150 ml of 10 mM Tris-HCl buffer pH 8 (solution A), harvested by centrifugation, resuspended in 60 ml of the same buffer containing 20% sucrose, 10 mM $MgSO_4$, 4 mg/ml of lysozyme (Sigma, Chemical Company, St. Louis) and 15 µg/ml of mutanolysin (Sigma, solution B) and incubated for 1 h at 37°C. After the incubation the presence of protoplasts (*ca.* 90%) was confirmed by phase contrast microscopy and lysis was achieved by a combination of osmotic shock and treatment in a French pressure cell. Protoplasts harvested by centrifugation (20,000 x *g*, 15 min) were resuspended in 300 ml of 10 mM Tris-HCl buffer pH 7.5 containing 10 mM $MgSO_4$, 5 µg/ml of deoxyribonuclease I (Sigma) and 5 µg/ml of ribonuclease A (ICN Biomedicals Canada Ltd., Mississauga, Ontario; solution C), and the suspension was passed through a French pressure cell at 1,300 p.s.i. The lysates were centrifuged twice at 8,000 x *g* for 15 min, and the resulting supernatants were centrifuged at 100,000 x *g* for 90 min. The cleared supernatants were stored at -20°C (cytoplasmic fraction). The pellets were resuspended in 20 ml of solution C without nucleases and the suspensions

were centrifuged (100,000 x *g* for 90 min). The pellets (membrane fraction) were resuspended in 6 ml of solution C without nucleases and stored at -20°C.

Glucose-6-phosphate dehydrogenase (London, 1990) and NADH oxidase (Condon, 1987) activities in the fractions were determined spectrophotometrically using previously described methods (Bergmeyer et al., 1974). The reaction volume was 300 µl and contained up to 250 µl of sample. Some reactions were performed in the presence of 1% Triton X-100 to solubilize the membrane fraction. The assays were done in triplicate and the standard errors were less than 10% of the triplicate mean.

3.2.9. Washing of protoplasts and membranes with high ionic strength solutions

Protoplasts from 100 ml of culture prepared as described above in solution B were harvested (20,000 x *g*, 15 min) and resuspended in 4 ml of 10 mM Tris-HCl buffer pH 8 containing 20% sucrose, 10 mM $MgSO_4$ and 1 M NaCl. After shaking at 50 rpm for 20 min the suspension was centrifuged and the clear supernatant (protoplast wash) was stored at -20°C. Cell membranes suspended in solution C prepared from 150 ml of culture, as described above, were harvested (100,000 x *g* for 90 min) and resuspended in 4 ml of solution C or in the same solution containing 1 M NaCl. The membranes were harvested and washed in the same way four more times. The washed membranes were stored at -20°C.

3.2.10. Bacteriocin-immunity binding analysis

The binding of the immunity protein (30 µg/ml solution) to microtiter plate wells coated with carnobacteriocin B2 after 1 h of incubation at room temperature was determined by standard indirect enzyme-linked immunosorbent assay (ELISA) using antibodies against CbiB2 as the primary antibody and goat antirabbit peroxidase conjugated antibodies as the detection system. The assays were done by standard procedures (Tijssen, 1985). Similarly, the binding of immunity protein biotinylated via

biotin-N-hydroxysuccinimide in molar ratio 1:3 to polystyrene wells coated with bacteriocin was determined using peroxidase-streptavidin conjugates. Labeling with biotin and detection with peroxidase-streptavidin were done according the supplier's recommendations (ICN Biomedicals).

To determine if the immunity protein was able to inactivate the bacteriocin in solution, 7.5 µg of carnobacteriocin B2 were mixed with 10 or 100 µg of immunity protein in 100 µl of phosphate buffer pH 7. Controls with BSA instead of immunity protein and with bacteriocin alone were also prepared. The inhibitory activity of the samples was determined by spot-on-lawn test before and after incubation at 22°C for 24 h.

3.2.11. Polyacrylamide gel electrophoresis and Western blot analysis

Sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) was performed by standard procedures (Sambrook et al., 1989). Western blot analyses were done using the ECL™ Western Blotting Detection System as recommended by the suppliers (Amersham Canada Ltd., Oakville, Ontario). The proteins were electroblotted onto nitrocellulose membranes (0.2 µm pore size) using carbonate blotting buffer containing: 10 mM $NaHCO_3$, 3 mM Na_2CO_3, pH 9.9, in 20% methanol (Dunn, 1986). The proportion of the immunity protein represented by the signals on the Western blots was estimated using a gel scanner (Pharmacia LKB Biotechnology 2222-020 Ultoscan XL)

3.3. Results

3.3.1. Expression of carnobacteriocins B and identification of carnobacteriocin B2 immunity protein

The plasmid pLQ24 carrying a *PstI* fragment of approximately 16-kb derived from pCP40, and pLQ18 carrying a smaller fragment of approximately 9.5-kb derived from pLQ24 by partial digestion with *ClaI*, restored the production of carnobacteriocins B2 and BM1 and the immune phenotypes in *C. piscicola* LV17C when the plasmids were electrotransformed into the strain. Similarly, introduction of pLQ24 into the heterologous host *C. divergens* LV13, provided the genetic information necessary to give transformants that produced, and showed immunity to, carnobacteriocin B2, but not to carnobacteriocin BM1. *C. piscicola* LQ24 and LQ18 displayed a four-fold increase in inhibitory activity of the supernatant compared with the wild type *C. piscicola* LV17B. Although there is no precise information about the copy number of pCP40 and pCaT, pLQ24 and pLQ18 appear to have a higher copy number than pCP40, judging by the intensity of the bands in agarose gel electrophoresis. This might account for the increase in inhibitory activity by these transformants. *C. piscicola* LV17C carrying pLQ18E, a derivative of pLQ18 with an exonuclease III deletion encompassing *cbnB2* and *cbiB2*, did not produce CbnB2 and was sensitive to it, but it remained immune to, and produced CbnBM1 (Tables 3.2 and 3.3, and Fig. 3.1). The 9.5-kb insert of pLQ18 contains the genetic information necessary to restore the expression of CbnB2 and its immunity protein, and the expression and immunity function of the chromosomally encoded CbnBM1. This suggests that complementing trans-acting factors encoded in the plasmid pCP40 that are required for expression of the chromosomal bacteriocin are encoded upstream of *cbnB2* in the 9.5-kb insert of pLQ18. These results also indicate that CbiB2, a protein of 111 amino acids, could provide immunity to CbnB2. To verify the immunity

Table 3.2. Production of carnobacteriocins B by strains of *C. piscicola*

Indicator strain	Presence of zone of inhibition with indicated producer strain [a]				
	C. piscicola				*C. dive* LQ
	LV17B	LQ24	LQ18	LQ18E	
C. piscicola					
LV17CD	+	+	+	+	nd
LQ24	-	-	-	-	nd
LQ18	-	-	-	-	nd
LQ18E	+	+	+	-	nd
C. divergens					
LV13pCaT	+	+	+	+	+

[a] Antimicrobial activity was determined by spotting 10 µl of heat treated supernatant (65°C, 15 min) of the producer strain culture onto an overlay inoculated (1%) with the specific indicator strain. The presence (+) or absence (-) of zones of inhibition was assessed after 18 h of incubation. nd: Not determined.

Table 3.3. Sensitivity of selected strains to carnobacteriocins B2 and BM1

Indicator strain	Sensitivity to carnobacteriocin[a]:	
	B2	BM1
C. piscicola		
LV17B	-	-
LV17C	+	+
UAL26	+	+
LQ24	-	-
LQ18	-	-
LQ18E	+	-
LV17Ci	-	+
UAL26i	-	+
C. divergens		
LV13	+	+
LQ24	-	+
LV13i	-	+

[a] Sensitivity to carnobacteriocins B2 and BM1 was determined by spotting 10 µl of bacteriocin solution (20 µg/ml in peptone water) onto an overlay inoculated (1%) with the specific indicator strain. The presence (+) or absence (-) of zones of inhibition was assessed after 18 h of incubation.

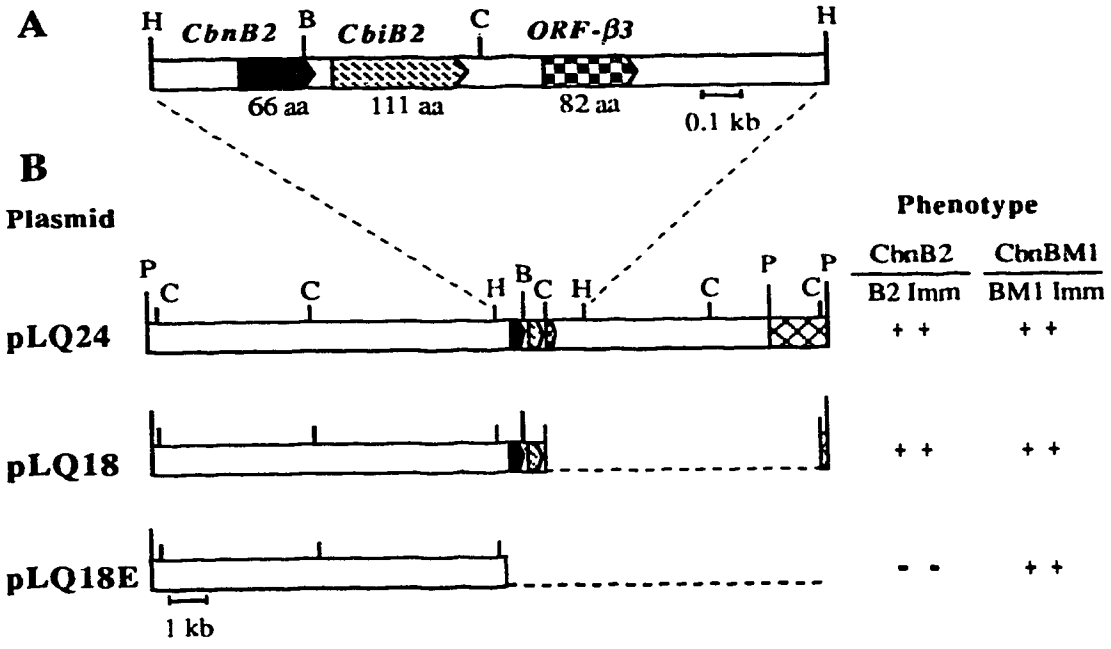

Figure 3.1. Schematic representations and partial restriction maps of the previously sequenced 1.9-kb *Hin*dIII fragment from pCP40 containing *cbnB2* and *cbiB2* (A), and the inserts present in pLQ24, pLQ18 and pLQ18E (B). The phenotype of the *C. piscicola* LV17C hosts is indicated. P, *Pst*I; C, *Cla*I; B, *Bam*HI and H, *Hin*dIII. The dashed lines indicate deleted segments.

function of CbiB2, pLQ400i containing the structural gene of the suspected immunity protein under the control of a constitutive promoter of the shuttle vector pMG36e, was transformed into the homologous host *C. piscicola* LV17C and into the heterologous hosts *C. piscicola* UAL26 and *C. divergens* LV13, all of which are sensitive to carnobacteriocins B2 and BM1. Spot-on-lawn tests showed that the transformants are immune to carnobacteriocin B2; however, they are not immune to carnobacteriocin BM1 (Table 3.3).

The growth of the transformants (LV17Ci, LV13i and UAL26i), the parental strains (LV17C, LV13 and UAL26), and the carnobacteriocin producer strain LV17B (Table 3.1) was evaluated in the presence of several concentrations of carnobacteriocin B2. No difference in growth of the producer strain was observed with the range of bacteriocin concentrations tested (0, 0.25, 1, 4, and 16 µg/ml). However, differences in the level of sensitivity to the bacteriocin were observed between the parental strains and the strains transformed with pLQ400i. *C. divergens* LV13i, *C. piscicola* LV17Ci and UAL26i display increased immunity compared with the parental strains (Fig. 3.2). Although growth of the sensitive strains was reduced or suppressed in the presence of the bacteriocin during the first 20 h of incubation, a final reading of the optical density after 48 h indicated that all of the strains had grown to maximum population (data not shown). To verify the presence of carnobacteriocin B2, the bacteriocin activity was monitored after 30 and 48 h of incubation. The bacteriocin concentration at 30 and 48 h was the same and represented approximately half of the initial concentration. To determine if the presence of purified immunity protein in the medium protected sensitive strains against the bacteriocin, the immunity protein (100 µg/ml) was added to the medium containing carnobacteriocin B2 (8 µg/ml), and growth of the inoculum was monitored. The same experiment was performed using BSA (100 µg/ml) instead of immunity protein, as a control. Neither the addition of the immunity protein nor the BSA protected the sensitive

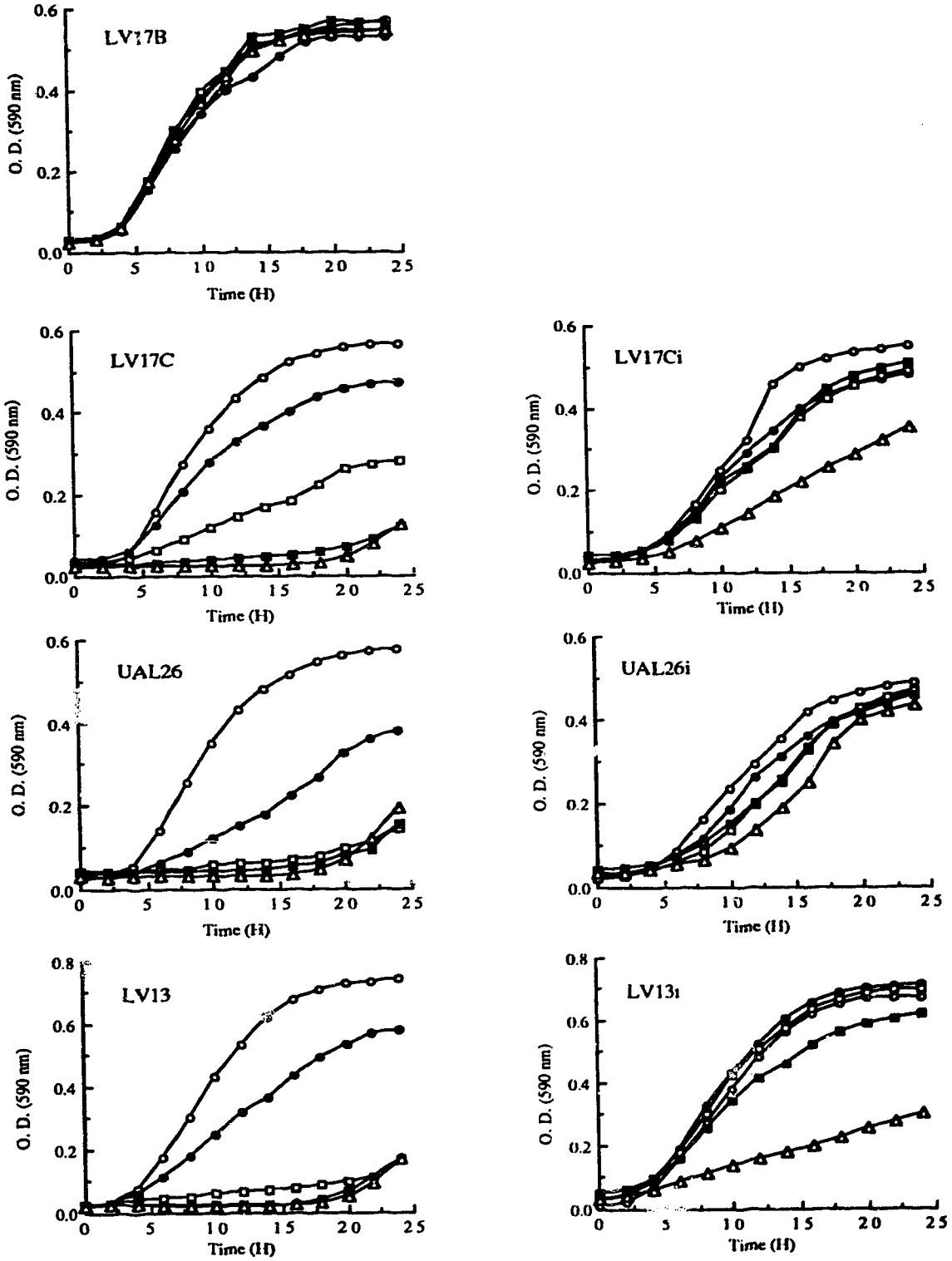

Figure 3.2. Kinetics of growth of selected *Carnobacterium* strains in the presence of carnobacteriocin B2 at 25°C. The plots show the change in optical density of selected cultures in the absence (O) or presence of the bacteriocin (● 0.25, □ 1, ■ 4, and Δ 16 µg/ml).

strains against the bacteriocin in the broth. In all of these cases the same extended lag phase was observed (data not shown). Preincubation for 24 h of bacteriocin alone, with immunity protein or with BSA did not have any effect on the inhibitory activity evaluated by spot-on-lawn test. The inhibitory activity of all of the samples was the same (4,000 AU/ml).

3.3.2. Purification of carnobacteriocin B2 immunity protein

CbiB2 was expressed in *E. coli* as a stable hybrid protein composed of the maltose binding protein encoded on pMAL-c and 111 amino acids of CbiB2. The proper DNA fusion and the nucleotide sequence of the fused *cbiB2* of pLQ300i was confirmed by DNA sequencing. The fusion protein was expressed in, and purified from the cytoplasm of *E. coli* LQ300i, and it represented approximately 7 to 9% of the total cellular protein. After cleavage with Factor Xa, which did not require previous denaturation of the fusion protein, the immunity protein was isolated and purified by reversed-phase HPLC. The retention times of the maltose binding protein, the undigested fusion protein and the immunity protein were 18.9, 21.4 and 23.0 min, respectively. The final yield of CbiB2 was 4 to 5 mg/liter of culture. To verify the proper cleavage with Factor Xa at the N-terminus of CbiB2, the first five amino acids of the protein sequence were determined by Edman degradation. The amino acid sequence Met-Asp-Ile-Lys-Ser- was found, which coincides with the amino acid sequence predicted from *cbiB2*. To ascertain that CbiB2 was fully translated and whether it was posttranslationally modified in *E. coli*, the molecular mass of the purified protein was determined by mass spectrometry. The molecular mass of 12662.2 ± 3.4 (Fig. 3.3) is in agreement with the molecular mass of 12,666.50 predicted from the amino acid sequence deduced from *cbiB2* (Quadri et al., 1994). Experiments were done to detect the binding of the immunity protein or biotinylated immunity protein to microtiter plates coated with carnobacteriocin B2. No

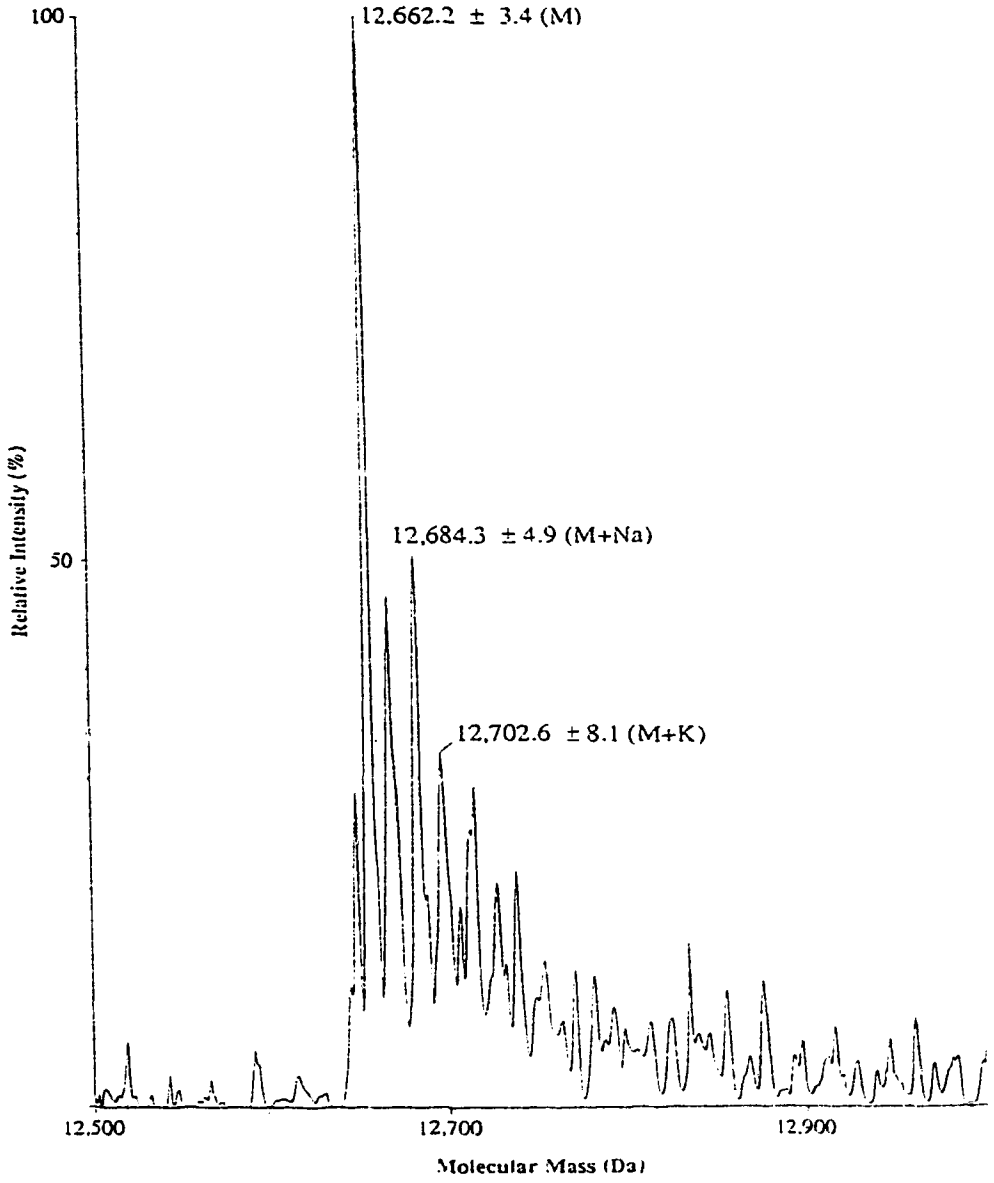

Figure 3.3. Molecular mass of carnobacteriocin B2 immunity protein calculated from the positive ion electrospray mass spectra.

binding was detected in either of two independent experiments under the conditions of the assays (data not shown).

3.3.3. Cellular localization of CbiB2

To establish the cellular localization of CbiB2, the proteins from the cytoplasm and cell membrane of *C. piscicola* LV17B were analyzed by Western blot using purified antibodies against CbiB2. The total proteins in the cytoplasmic and membrane fractions produced with the cell fractionation protocol were approximately 130 and 13 mg, respectively. Cytoplasmic and membrane proteins of *C. piscicola* LV17C were also analyzed as negative controls to determine the specificity of the antibodies. No signal was detected (data not shown). The results revealed the presence of a protein in the cytoplasmic and membrane fractions of *C. piscicola* LV17B with the same electrophoretic mobility as that of the purified immunity protein. The protein had an apparent molecular weight of 9.5 kDa based on SDS-PAGE. The majority of CbiB2 is present in the cytoplasm and a small proportion is present in the membrane fraction of *C. piscicola* LV17B. Scanning of the X-ray film indicated that the proportion of the immunity protein in the cytoplasmic and membrane fractions was 92% and 8%, respectively (Fig. 3.4).

To monitor the possible contamination of the membranes with components from the cytoplasmic fraction, the glucose-6-phosphate dehydrogenase activity of the fractions was evaluated. The specific activities of the cell lysate before ultracentrifugation and that of the cytoplasmic fraction were 10.4 and 10.2 mU/mg, respectively. The presence of Triton X-100 in the reaction mixture did not affect the enzymatic activity of these fractions. No activity was detected in the membrane fraction regardless of the absence or presence of Triton X-100. To confirm the enzymatic activity of the membranes, NADH oxidase activity was also determined. The specific activities of the membrane and cytoplasmic fractions were 71.8 and 26.3. mU/mg, respectively.

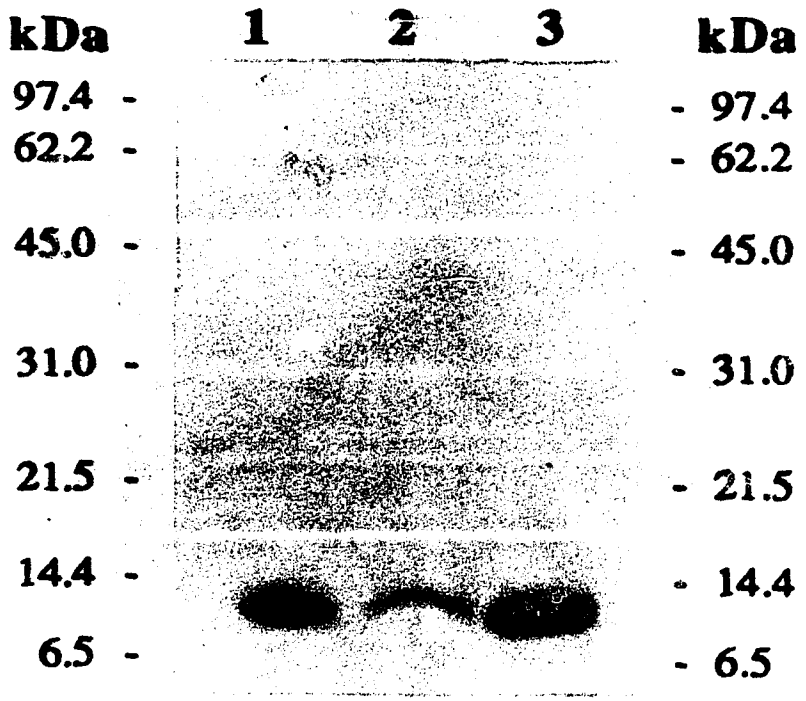

Figure 3.4. Western blot analysis of membrane, cytoplasmic and extracellular proteins from *C. piscicola* LV17B. Lanes 1, 2, and 3 were loaded with approximately 0.1 µg of purified CbiB2, and 90 µg of proteins from *C. piscicola* LV17B membrane and cytoplasmic fractions, respectively.

The presence of the immunity protein was also evaluated in the culture broth, the cell wash (solution A), sucrose solution (solution B), protoplast wash, and membranes washed with solution C with or without NaCl. Traces of the immunity protein were detected in the first four fractions, only by exposing the X-ray film for prolonged periods of time (data not shown). The presence of immunity protein in these fractions is probably due to some cell lysis, as indicated by the glucose-6-phosphate dehydrogenase activity in some of these fractions; 0.04 and 1.4 mU/mg for the sucrose solution and protoplast wash, respectively. The membranes washed with high ionic strength solution (1 M NaCl) contained approximately the same content of immunity protein as those washed with solution C without NaCl. Scanning indicated that the proportion of the immunity protein in the membranes washed with or without NaCl was 62% and 38%, respectively.

3.4. Discussion

C. piscicola LV17B produces two class II bacteriocins, carnobacteriocin B2 encoded on a 61-kb plasmid and carnobacteriocin BM1 encoded on the chromosome (Klaenhammer, 1993; Quadri et al., 1994). A 16-kb fragment from the plasmid has the required genetic information not only to restore the wild type Cbn^+ Imm^+ phenotype in the homologous host, but also to confer $CbnB2^+$ $CbnB2Imm^+$ phenotype to a heterologous host. This fragment encodes proteins that appear to form part of a secretion, transcriptional activation or processing system required for Cbn^+ Imm^+ phenotype. Specific proteins that belong to the HlyB family of ATP-dependent translocators (Blight and Holland, 1990) that may be involved in the transport of the class II bacteriocins lactococcin A and pediocin PA-1/AcH have been identified (Marugg et al., 1992, Stoddard et al., 1992; Bukhtiyarova et al., 1994) and proteins with homology to the elements of the two-component signal transduction system (Stock et al., 1989) required for the production of the class II bacteriocins sakacin A and plantaricin A have also been

reported (Axelsson et al., 1993; Diep et al., 1994). Preliminary results have revealed the presence of several ORFs upstream of *cbnB2* and one of them encodes a protein of 965 amino acids with homology to ATP-dependent transmembrane translocators. The possible function of this ORF in bacteriocin secretion is under investigation.

Deletion and subcloning experiments allowed the identification of *cbiB2* as the gene encoding the protein that confers immunity against carnobacteriocin B2. CbiB2 is a protein consisting of 111 amino acids with a calculated pI of 9.31 and no homology to other proteins found in data banks (Quadri et al., 1994). Proteins that confer immunity to lactococcins A, B and M are also relatively small, containing 98, 91 and 154 amino acids, respectively; and they do not have homology with other reported proteins (Van Belkum et al., 1991, 1992). CbiB2 is able to protect homologous and heterologous hosts against the activity of carnobacteriocin B2. However, the level of immunity of the transformed strains expressing CbiB2 is not as great as that of the wild type strain. This could be due to poor levels of transcription of *cbiB2* under the control of the pMG36e promoter or it might indicate that another gene product, in addition to CbiB2, is required for full immunity. The nature of the resistance to carnobacteriocin B2 manifested by the sensitive strains after 20 hours of incubation in the presence of bacteriocin is unknown. The resistance is [illegible] by homologous and heterologous strains, indicating that this mechanism of resistance is not exclusive to the carnobacteriocin producer strain and it is unrelated to the immunity conferred by CbiB2. The occurrence of resistant strains within a sensitive bacterial population is a common phenomenon for bacteriocins produced by lactic acid bacteria (Rekhif et al., 1984; Harris et al., 1991; Noerlis and Ray, 1994).

Although carnobacteriocins B2 and BM1 have significant amino acid homology (34% identity) and a similar spectrum of antimicrobial activity (Quadri et al., 1994), CbiB2 does not confer immunity to carnobacteriocin BM1. Interestingly, *cbiBM1*, located downstream of *cbnBM1*, encodes a basic protein (pI 9.51) of 88 amino acids with

19% identity with CbiB2 (Quadri et al., 1994). The possible immunity function of CbiBM1 against CbnBM1 is under investigation. The gene *cbiB2* is located downstream of *cbnB2*. This genetic arrangement is the same as that found for the genes responsible for immunity to lactococcins A, B and M, which are also located downstream of the bacteriocin structural gene and form part of the bacteriocin operons (Van Belkum et al., 1991, 1992). Likewise, downstream of the genes for the class II bacteriocins leucocin A and pediocin PA-1/AcH there are ORFs encoding basic proteins of 113 and 112 amino acids, respectively, with limited amino acid homology to CbiB2 (12% identity) that have been reported as possible immunity proteins (Hastings et al., 1991; Marugg et al., 1992; Bukhtiyarova et al.; Marugg et al., 1992, 1994). However, the function of these proteins has not been established.

The lack of a specific assay or known biological activity in vitro that would allow the direct detection of immunity proteins makes their purification particularly difficult. In the case of the carnobacteriocin B2 immunity protein, its expression in *E. coli* as a fusion protein proved to be an effective method of purification. Furthermore, the expression in *E. coli* should allow facile isotopic labeling of CbiB2 for study of the solution structure of the protein by NMR.

The majority of the intracellular pool of CbiB2 is located in the cytoplasm and a small proportion is associated with the membrane. Considering the result of the Western blot analysis and the protein content of the cytoplasmic and membrane fractions, the immunity protein present in the membranes represents no more than 1% of the cellular pool. This result contrasts with the results obtained for the lactococcin A immunity protein which was found in approximately the same proportions in the cytoplasmic and membrane fractions of immune strains (Nissen-Meyer et al., 1993; Venema, 1994). Although the immunity protein could have some affinity for the membrane surface because of the protein's positive charge at physiological pH, the nature of the interaction

of CbiB2 with the membrane in unclear. The membranes had similar amounts of immunity protein after several washes with high or low ionic strength solutions. This result could be explained by assuming that the protein is trapped within the membrane vesicles. During protoplast lysis, the protein might be selectively retained inside of the vesicles due to some affinity for membrane components instead of being removed with the rest of the cytoplasmic components. This result could also be explained if a small fraction of immunity molecules is embedded in the membrane. Although several hydrophobic segments of CbiB2 can be identified, no apparent transmembrane spanning domains (Quadri et al., 1994) can be predicted from the amino acid sequence. More recently, lactococcin A immunity protein, which has no transmembrane spanning segment predicted from its sequence, has been shown to have its C-terminus located on the outside of the cell and a possible transmembrane α-amphiphilic helix from residues 27 to 49 that could be responsible for anchoring the protein to the membrane (Venema, 1994). Carnobacteriocin B2 immunity protein is not an extracellular protein, no signal sequence can be predicted from its amino acid sequence, and it cannot be removed from protoplasts with high ionic strength solutions.

Carnobacteriocin B2, like other bacteriocins, targets the cytoplasmic membrane of sensitive cells and it causes dissipation of the proton motive force with leakage of intracellular components through the probable formation of pores (Van Belkum, 1994). Expression of CbiB2 within the bacterial cell protects against the antimicrobial action of CbnB2 applied externally, but the addition of purified CbiB2 to the medium does not protect the sensitive strains. This is in agreement with the cellular localization of CbiB2. These results, together with the apparent low binding affinity of CbiB2 with the bacteriocin, and the inability of CbiB2 to inactivate the bacteriocin in solution suggest that the immunity protein and the bacteriocin do not interact directly in aqueous solution. However, the possibility that the purified immunity protein is not in its native conformation cannot be eliminated. It seems unlikely that the immunity protein directly

inhibits the interaction of the extracellular bacteriocin with the membrane. Rather, CbiB2 may interfere with the formation of a functional pore complex in the membrane, or it may block the functional pore to prevent the efflux of intracellular components by interacting with the portion of the pore structure facing the cytoplasm. A key feature of the mode of action of the immunity protein might be its interaction with a receptor protein that binds both the bacteriocin and its immunity protein, as might be the case for the lactococcin A immunity protein (Venema, 1994). Current studies on the interaction of CbnB2 and CbiB2 in membrane environments and the manipulation of their genes to identify key residues involved in biological activity will facilitate understanding of the mechanism of antimicrobial action as well as immunity.

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4. CHARACTERIZATION OF FOUR GENES INVOLVED IN PRODUCTION OF ANTIMICROBIAL PEPTIDES BY*CARNOBACTERIUM PISCICOLA* LV17B[1]

4.1. Introduction

The potential of antimicrobial peptides (bacteriocins) or bacteriocinogenic lactic acid bacteria to reduce the growth of spoilage and pathogenic bacteria in foods has stimulated further study of these compounds. The best known bacteriocin produced by lactic acid bacteria is nisin A, a lantibiotic produced by strains of *Lactococcus lactis* and approved for use as a food additive in more than 45 countries (Delves-Broughton, 1990). Nisin is ribosomally synthesized as a precursor peptide that contains 57 amino acids. It undergoes extensive posttranslational modification that involves cleavage of 23 amino acids at the N-terminus and formation of dehydrated amino acid residues and lanthionine and ß-methyllanthionine residues that form several intramolecular thioether bridges (Gross and Morell, 1971; Buchman et al., 1988). The genes required for production of, and immunity to, nisin have been identified and they are clustered on a 15-kb DNA fragment that is located on the nisin-sucrose transposon Tn*5276* (Horn et al., 1991; Kuipers et al., 1993; Van der Meer et al., 1993; Engelke et al., 1994; Siegers and Entian, 1995).

Class II bacteriocins produced by lactic acid bacteria are characterized as small, heat stable antimicrobial peptides without unusual amino acids (Klaenhammer, 1993). Although several bacteriocins of this class have been identified, only a few have been extensively characterized. Carnobacteriocins B2 and BM1 (CbnB2 and CbnBM1) are produced by *Carnobacterium piscicola* LV17B, a new genus of lactic acid bacteria associated with chill stored, vacuum-packaged meats (Collins et al., 1987; Quadri et al.,

[1] A version of this chapter was been submitted for publication: Quadri, L. E. N., K. L. Roy, J. C. Vederas, and M. E. Stiles. 1996. Characterization of Four Genes Involved in Production of Antimicrobial Peptides by *Carnobacterium piscicola* LV17B J. Bacteriol.

1994). Their antimicrobial spectra include many lactic acid bacteria, as well as strains of potentially pathogenic *Enterococcus* and *Listeria* species. The carnobacteriocins exert their antimicrobial activity by dissipating the proton motive force and allowing leakage of intracellular components through the formation of pores in the cytoplasmic membrane of sensitive cells (Van Belkum, 1995). The phenotypic expression of both carnobacteriocins and the immunity functions are dependent of the presence of a 61-kb plasmid (pCP40) in *C. piscicola* LV17B. The cured strain *C. piscicola* LV17C does not produce bacteriocins and shows sensitivity to CbnBM1 and CbnB2 (Ahn and Stiles, 1992). Both bacteriocins have been purified and characterized and their genetic determinants have been identified (Quadri et al., 1994). The genetic determinants of CbnB2 (*cbnB2*) and its immunity protein (*cbiB2*) are located on the 61-kb plasmid. A 1.9-kb *Hin*dIII fragment from pCP40 containing *cbnB2*, *cbiB2*, and a third open reading frame (*orf-ß3*), has been cloned and sequenced (Quadri et al., 1994). The genetic determinants of carnobacteriocin BM1 (*cbnBM1*) and its possible immunity protein (*cbiBM1*) are located on the chromosome, as revealed by the nucleotide sequence analysis of a 0.67-kb *Taq*I genomic fragment containing *cbnBM1* and *cbiBM1* (Quadri et al., 1994).

Carnobacteriocins B2 and BM1 are ribosomally synthesized as precursors containing 66 and 61 amino acids that undergo posttranslational cleavage of an N-terminal extension of 18 amino acids to yield the mature active peptides of 48 and 43 amino acids, respectively. The recovery of bacteriocin production and the immunity functions following the introduction of fragments derived from pCP40 into *C. piscicola* LV17C, and the expression of carnobacteriocins in a heterologous host has already been accomplished (Quadri et al., 1995). These fragments contain sufficient genetic information to allow expression of carnobacteriocin B2 and its immunity in homologous and heterologous hosts, and to restore production of, and immunity to, the chromosomal bacteriocin CbnBM1. The results indicated that complementing trans-acting factors

encoded on pCP40 required for expression of the chromosomal bacteriocin CbnBM1 and the immunity protein are located on the cloned fragments.

In this study we report the nucleotide sequence of the fragment of DNA from the plasmid pCP40 required for bacteriocin production and immunity in *C. piscicola* LV17B. We present results indicating that the carnobacteriocins produced by *C. piscicola* LV17B are exported by a dedicated signal-sequence independent secretion machinery, and that production of, and immunity to, the carnobacteriocins are regulated by a two-component signal transduction system that could be involved in a bacteriocin autoregulation phenomenon in which the bacteriocins provide the signals that trigger their own production and synthesis of the immunity proteins.

4.2. Materials ans methods

4.2.1. Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are described in Table 4.1. *Carnobacterium* cultures were grown in APT broth (Difco Laboratories Inc., Detroit, MI) and *Escherichia coli* were grown in Luria-Bertani (LB) broth or on LB agar (Ahn and Stiles, 1990; Sambrook et al., 1989). Media containing ampicillin (100 µg/ml) or chloramphenicol (5 µg/ml) were used for growth and selection of transformants.

4.2.2. Bacteriocin production and sensitivity assay

Production of bacteriocin was detected by deferred inhibition or by spot-on-lawn tests (Ahn and Stiles, 1990). The effect of inoculum size on the ability of selected strains to produce bacteriocin was also evaluated. For this experiment, the transformants were serially diluted (ten-fold dilutions) in sterile, fresh APT broth until no growth occurred after three days of incubation at 25°C. The presence of inhibitory activity in the culture supernatant was evaluated when the cultures reached the stationary phase of growth.

Table 4.1. Bacterial strains and plasmids

Strains and plasmids	Relevant phenotype and properties	Reference
Bacteria		
C. piscicola		
LV17B	Cbn$^+$ Imm$^+$, containing pCP40	Ahn and Stiles, 1992
LV17C	Cbn$^-$ Imm$^-$, plasmidless strain derived from *C. piscicola* LV17	Ahn and Stiles, 1992
C. divergens		
LV13	Cbn$^-$ Imm$^-$	Shaw[a]
E. coli		
JM107	Cloning host	Sambrook et al., 1989
Plasmids		
pCaT	8.5-kb cloning vector, Cm^R	Jewell and Collins-Thompson, 1989
pCP40	61-kb plasmid conferring Cbn$^+$ Imm$^+$ phenotype	Ahn and Stiles, 1992
pLQ24	pCaT containing a 17.2-kb fragment from pCP40	Quadri et al., 1995
pLQ18	pCaT containing a 10-kb fragment from pCP40	Quadri et al., 1995
pLQ18'	pCaT containing the same insert as in pLQ18, but in opposite orientation	This study
pLQ18E	pLQ18 with *cbnB2* and *cbiB2* deleted	Quadri et al., 1995
pLQ18B	pLQ18 with CbnB2 inactivated	This study
pLQ11/12/15	deletion derivatives of pLQ18	This study
pLQ18K	pLQ18 with CbnK inactivated	This study
pLQ18R	pLQ18 with CbnR inactivated	This study
pLQ18T	pLQ18 with CbnT inactivated	This study
pLQ18D	pLQ18 with CbnD inactivated	This study
pUC118	3.2-kb cloning vector, Amp^R, lacZ'	Vieira and Messing, 1987

[a] Supplied by B. G. Shaw, Institute of Food Research, Langford, Bristol U. K.
Cbn: carnobacteriocin(s), Imm: carnobacteriocin immunity, ^{R} resistant.

Sensitivity of selected strains to carnobacteriocins B2 and BM1 was determined by spot-on-lawn test with 10 arbitrary units (AU) of bacteriocin (one AU is the minimun amount of bacteriocin that inhibits the indicator strain *C. divergens* LV13 in the spot-on-lawn test), or with a microtiter plate reader (THERMOmax Microplate Reader, Molecular Devices Corp., Merlo Park, Calif). In the microtiter plates, wells were loaded with serial, four-fold dilutions of the carnobacteriocins B2 or BM1 (4000, 1000, 250, 64, 16, 4 and 0 AU/ml) in 200 µl of APT broth inoculated with a fresh culture (2.5% inoculum). Inoculated plates were incubated at 25°C and absorbance at 650 nm was measured at 30 min intervals. Bacteriocins used in the tests were purified by methods previously described (Quadri et al., 1994). The data were analyzed using the software SOFTmax version 2.3 (Molecular Devices Corp., Merlo Park, Calif). The maximum growth rates in the presence or absence of purified carnobacteriocins were calculated using six consecutive measurements. Correlation coefficients ranged between 0.98 and 1.00. The immunity of the clones was expressed as a percentage of the rate of growth in the presence of the bacteriocin relative to the rate of growth in the absence of the bacteriocin. Deferred inhibition tests were also used to determine the sensitivity of selected strains.

4.2.3. DNA isolation, manipulation and sequencing

Isolation of plasmid DNA from *Carnobacterium* strains and *E. coli* was done according to methods previously described (Ahn and Stiles, 1990; Sambrook et al., 1989). Full and partial restriction digestion, agarose gel electrophoresis, exonuclease III treatment, blunt end creation using Klenow fragment, and DNA ligation were done by standard procedures (Sambrook et al., 1989). Restriction enzymes, the Klenow fragment of the DNA polymerase I from *E coli*, T4 DNA ligase and Exonuclease III were purchased from Boehringer Mannheim (Dorval, Quebec, Canada). Transformation of lactic acid bacteria and *E. coli* strains, and selection and screening of transformants were done by established methods (Ahn and Stiles, 1990; Sambrook et al., 1989).

4.2.4. Inactivation of *cbnK*, *cbnR*, *cbnT*, *cbnD* and *cbnB2*

Inactivation was achieved by introducing a frame-shift mutation at a specific restriction site within each gene. For inactivation of *cbnK*, *cbnR*, *cbnT* and *cbnD*, the plasmid pLQ18 was partially digested with *Sph*I, *Bst*NI, *Nhe*I or *Ban*II, respectively. The digests were resolved by agarose gel electrophoresis and the linear forms of pLQ18 (one cut per plasmid) were recovered by electroelution. The linear plasmids were blunt-ended, religated and electrotransformed into *C. piscicola* LV17C. Transformants were screened for bacteriocin production. Plasmid DNA from Bac^+ and Bac^- transformants was isolated and screened for the loss of the specific restriction site (*Sph*I, *Bst*NI or *Ban*II). Changes at the specific positions were confirmed by sequencing the DNA over the restriction site. In the case of the frame-shift mutation introduced at the *Nhe*I site of pLQ18T, plasmid DNA was isolated and directly screened for the modification at the specific site by DNA sequencing. CbnB2 was inactivated by creating a deletion in *cbnB2* with exonuclease III after digestion of pLQ18 with *Bam*HI. The extent of the deletion was determined by DNA sequencing.

4.2.5. DNA sequencing and synthesis of oligonucleotides

DNA was sequenced bidirectionally using *Taq* DNA polymerase and fluorescent dyedeoxy-chain terminators, and analyzed on a DNA Sequencer (Applied Biosystems 373A). The reactions were primed with specific oligonucleotides synthesized on a DNA Synthesizer (Applied Biosystems 391 PCR MATE) or with forward and reverse universal primers for the pUC plasmid series. The plasmids used as templates were pCaT and pUC118-based constructs containing fragments from the insert of pLQ18 (Table 4.1). Overlapping sequences were assembled, scanned for the presence of open reading frames (ORF) and interpreted using the DNA Star program (Madison, Wisc). The nucleotide sequence was submitted to GenBank (Los Alamos, NM) and was given the accession number L47121

4.2.6. Protein sequence analysis

Hydropathic profiles of the possible translational products of *cbnK*, *cbnR*, *cbnT* and *cbnD* were determined with the algorithm of Kyte and Doolittle (1982). Protein alignment was done with GeneWorks 2.3 software (IntelliGenetics Inc., El Camino Real, Mountain View, Calif). Search for homology with other proteins was done in the following data bases: Brookhaven Protein Data Bank, April 1995 release; SWISS-PROT release 31.0, March 1995; PIR release 44.0 (complete), March 31, 1995; GenBank release 88.0, April 1995. The computation was performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service.

4.3. Results

4.3.1. Identification of the region required for bacteriocin production

The region involved in bacteriocin production and immunity was identified by cloning several fragments from pCP40 into *C. piscicola* LV17C and into the heterologous host *C. divergens* LV13. The ability of the clones to produce bacteriocin was evaluated by spot-on-lawn test against *C. divergens* LV13 containing pCaT. Immunity to carnobacteriocins BM1 and B2 was evaluated by spotting the bacteriocins onto a lawn of the specific clones. The plasmid constructs and the phenotypes of the clones are shown in Fig. 4.1.

The plasmids pLQ24, pLQ18 and pLQ18′ (same insert as pLQ18, but in the opposite orientation) restored bacteriocin production and immunity to carnobacteriocins B2 and BM1 in *C. piscicola* LV17C. When *C. divergens* LV13 was used as the host strain, bacteriocin production was observed, but immunity to carnobacteriocin BM1 was not acquired by the transformants (Fig. 4.1). Similar results were obtained when pLQ18B was electrotransformed into *C. piscicola* LV17C and *C. divergens* LV13.

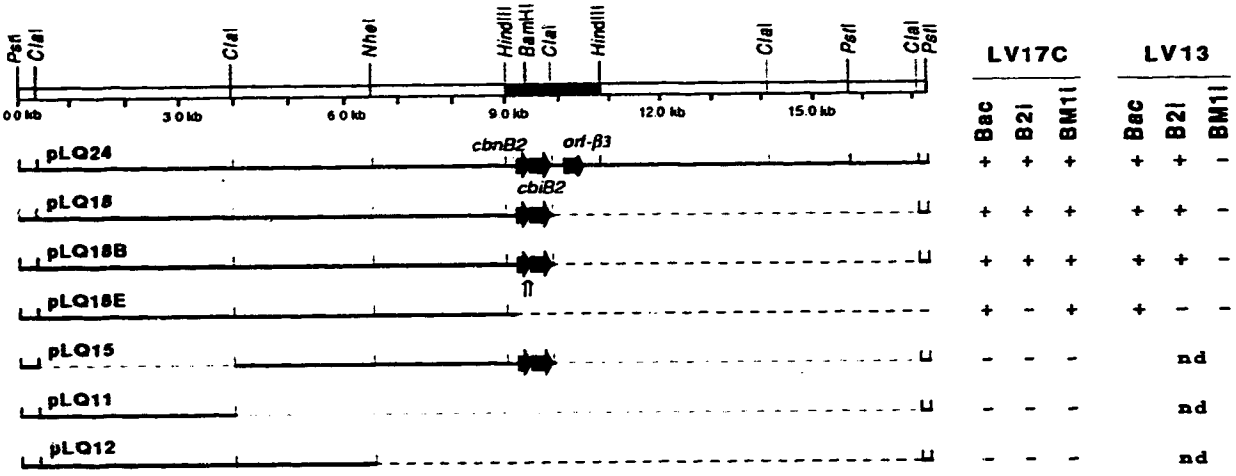

Figure 4.1. Schematic representation of DNA fragments cloned into pCaT. The previously sequenced *Hin*dIII fragment containing *cbnB2*, *cbiB2* and *orf-β3* is indicated by the solid bar. The vertical arrow in pLQ18B indicates the site of a small deletion in *cbnB2*. The dotted lines represent deleted segments of DNA. Relevant restriction sites are indicated. The bacteriocin and immunity phenotypes are shown for each clone in *C. piscicola* LV17C and *C. divergens* LV13: the first column (Bac) indicates the presence (+) or absence (–) of bacteriocin activity in the culture supernatant; and the second and third columns (B2i, BM1i) indicate the presence (+) or absence (–) of immunity to carnobacteriocins B2 and BM1, respectively. nd, not determined.

pLQ18B has a 31-bp exonuclease III deletion at the C-terminus of CbnB2 (Fig. 4.1). The deletion eliminated the last 12 amino acids of CbnB2 and introduced a shift in the *cbnB2* reading frame that resulted in the addition of eight different amino acids, rendering the peptide inactive (see below). The strains *C. piscicola* LV17C and *C. divergens* LV13 transformed with pLQ18E that contains an exonuclease III deletion encompassing *cbnB2* and *cbiB2* produced bacteriocin and they were sensitive to carnobacteriocin B2. In the case of *C. piscicola* LV17C containing pLQ15, the transformant did not produce bacteriocin and was sensitive to carnobacteriocins BM1 and B2, despite the presence of *cbnB2* and *cbiB2*. Similarly, bacteriocin production or immunity was not detected in *C. piscicola* LV17C transformed with pLQ11 and pLQ12 (Fig. 4.1). Bacteriocin production was also evaluated by deferred inhibition test. Results were in agreement with the spot-on-lawn test. Zones of inhibition produced against *C. divergens* LV13 containing pCaT are shown in Fig. 4.2. To verify the production of carnobacteriocin B2 and the expression of a chromosomal bacteriocin in the homologous host, bacteriocin production of *C. piscicola* LV17C transformed with pLQ18, pLQ18B or pLQ18E was determined against *C. piscicola* LV17C transformed with pLQ18B or pLQ18E, and *C. divergens* LV13 transformed with pLQ18 (Table 4.2). *C. piscicola* LV17C containing pLQ18 was active against *C. divergens* LV13 containing pLQ18 suggesting that the chromosomally encoded carnobacteriocin BM1 was being produced. *C. piscicola* LV17C with pLQ18 was also active against the carnobacteriocin B2 sensitive indicator strain *C. piscicola* LV17C containing pLQ18E, but *C. piscicola* LV17C transformed with pLQ18B showed no activity against this strain (Table 4.2). This suggested that carnobacteriocin B2 was being produced and confirmed that carnobacteriocin B2 in pLQ18B had been inactivated. Production of bacteriocin by *C. divergens* LV13 transformed with pLQ18, pLQ18B or pLQ18E was also tested against *C. divergens* LV13 transformed with pCaT or pLQ18E. The results are shown in Table 4.2 and they indicated that carnobacteriocin B2 was produced in this host and that at least one other bacteriocin responsible for the inhibition

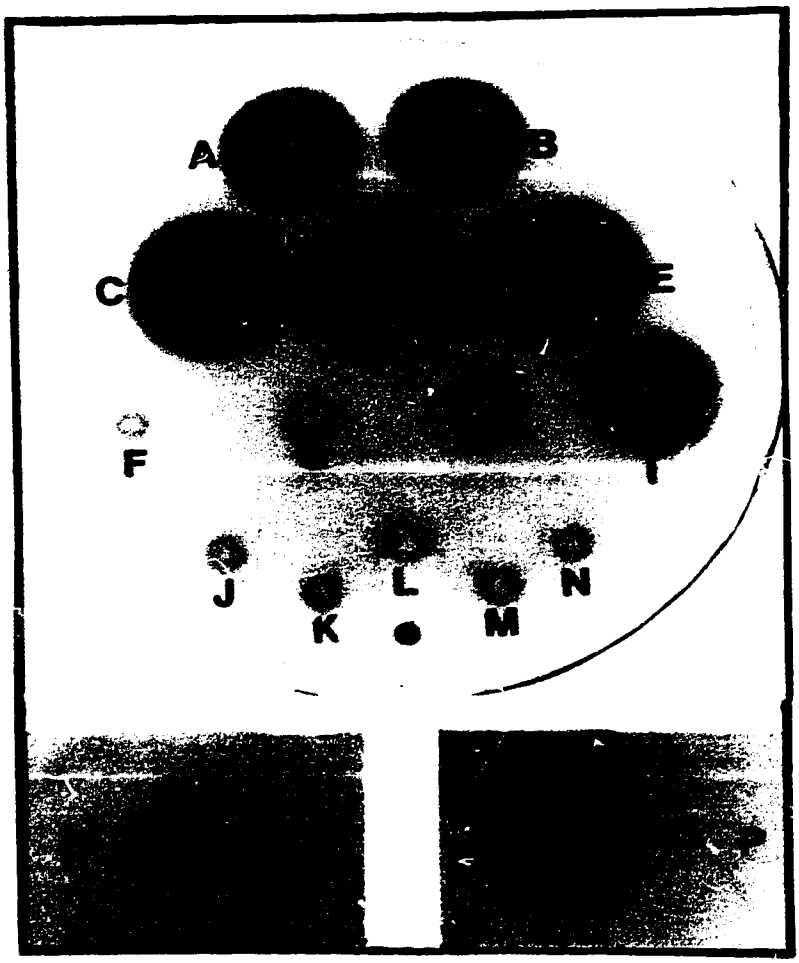

Figure 4.2. Assay for bacteriocin production and sensitivity. The strains were inoculated onto plates, and after incubation at 25 °C for 20 h they were overlayered with soft agar containing a 1% inoculum of the sensitive indicator strain *C. divergens* LV13 transformed with pCaT (A to N). The plates were incubated at 25°C for 16 h before measuring the size of the zones of inhibition. Inocula A to L indicate the positions of *C. piscicola* LV17C transformants containing pLQ18B, pLQ18E, pLQ18′, pLQ18, pLQ24, pLQ18D, pLQ18T, pLQ18R, pLQ18K, pLQ15, pLQ12, and pLQ11, respectively. M and N indicate the positions of *C. piscicola* LV17C and *C. divergens* LV13 transformed with pCaT, respectively. Inocula O and P indicate positions of *C. piscicola* LV17C containing pLQ18 overlayered with *C. piscicola* LV17C containing pLQ18T or pLQ18D respectively.

Table 4.2. Bacteriocin production by transformants of *C. piscicola* LV17C and *C. divergens* LV13

Indicator strains	Producer strain					
	C. piscicola			*C. divergens*		
	pLQ18	pLQ18B	pLQ18E	pLQ18	pLQ18B	pLQ18E
	Diameter of zone of inhibition (mm)[a]					
C. divergens						
pCaT	20	18	18	19	10	10
pLQ18E	19	19	19	19	-	-
pLQ18	19	19	19	-	-	-
C. piscicola						
pLQ18B	-	-	-	nd	nd	nd
pLQ18E	16	-	-	nd	nd	nd

[a] Production of bacteriocin was detected by deferred inhibition test. -: no zone of inibition; nd: not determined.

of *C. divergens* LV13 containing pCaT by *C. divergens* LV13 transformed with pLQ18B or pLQ18E was encoded on the pCP40 fragment present in pLQ18E.

Production of bacteriocin by the strain *C. piscicola* LV17C transformed with pLQ24, pLQ18 or pLQ18' was not affected by inoculum size. These transformants did not display the same properties as the parental strain of *C. piscicola* LV17B (see Discussion). The transformants were serially diluted (ten-fold dilutions) in fresh medium until no growth occurred and bacteriocin activity was detected in the greatest dilution in which bacterial growth was observed. The plasmids pLQ24, pLQ18, pLQ18' and pCaT were also electroporated into the parental strain *C. piscicola* LV17B. Production of bacteriocin by *C. piscicola* LV17B transformants containing pLQ plasmids was not affected by inoculum size; however, production of bacteriocin by *C. piscicola* LV17B containing the plasmid vector pCaT was affected by inoculum size. As previously reported for *C. piscicola* LV17B (Saucier et al., 1995), no bacteriocin was detected in the supernatant when a culture of *C. piscicola* LV17B transformed with pCaT was inoculated at or below $1x10^4$ colony forming units per ml (cfu ml^{-1}). Furthermore, when a nonproducing culture of this strain was challenged with carnobacteriocins BM1 and B2 in a spot-on-lawn test, the culture was sensitive to both bacteriocins. This indicated that both bacteriocin and immunity protein production in the parental strain were affected by inoculum size.

4.3.2. Nucleotide sequence analysis

The nucleotide sequence of the 10-kb fragment and the translation products of the identified open reading frames are shown in Fig. 4.3. The positions and the direction of transcription of each ORF identified in the sequenced region are shown in Fig. 4.4A. Possible ribosome binding sites (Shine and Delgarno, 1975) were located upstream of each ORF except for orf-1 for which only the 3' end has been sequenced. Potential -35

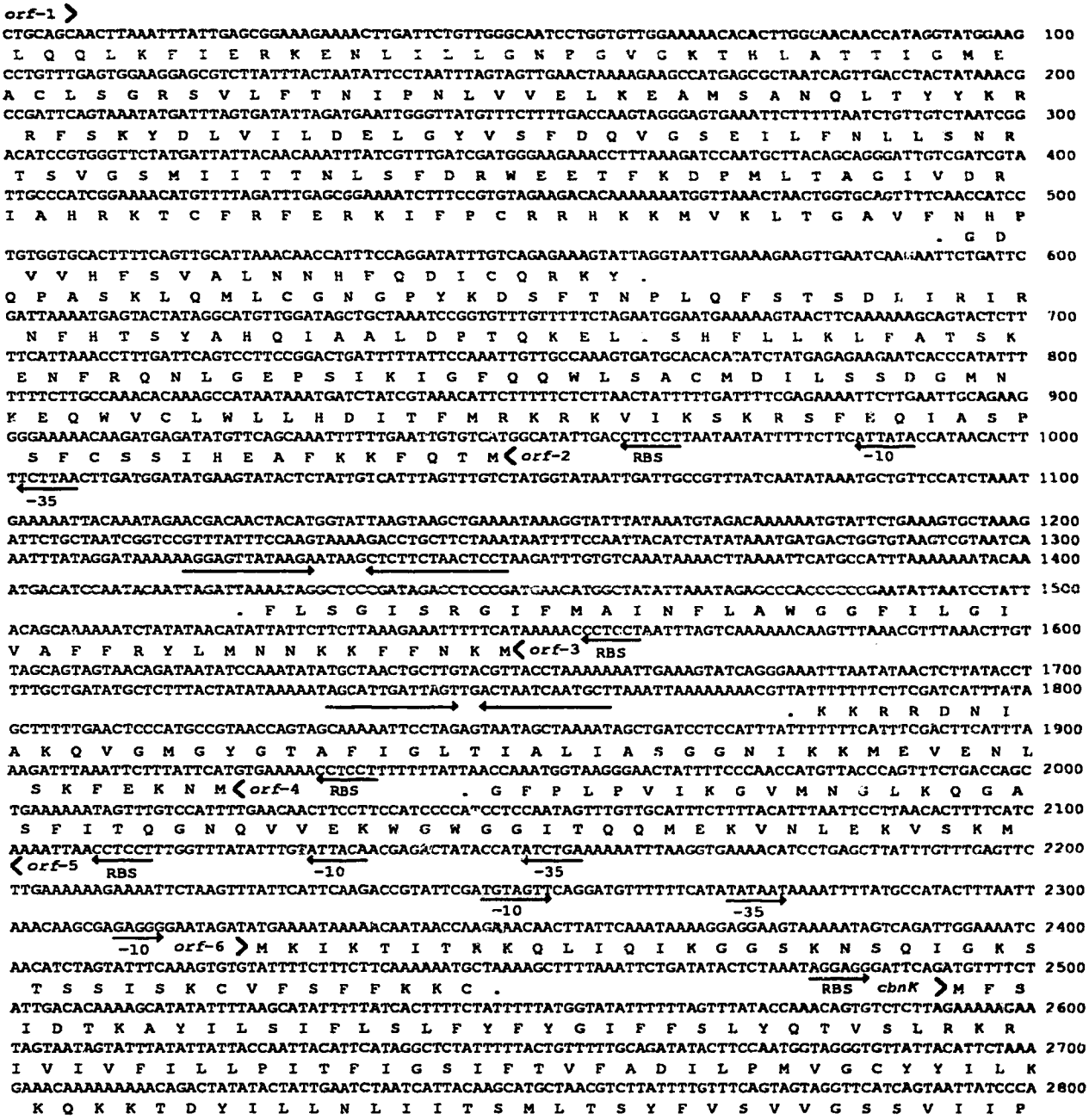

Figure 4.3. Nucleotide sequence of the 10,122-bp DNA fragment of pCP40 from *Carnobacterium piscicola* LV17B. The deduced amino acid sequences from the identified open reading frames are shown below the DNA sequence. Possible ribosome binding sites, promoter sequences and inverted repeat sequences are underlined. The boxed amino acids in CbnT are the conserved key residues for the so-called Gly-Gly leader peptidase family.

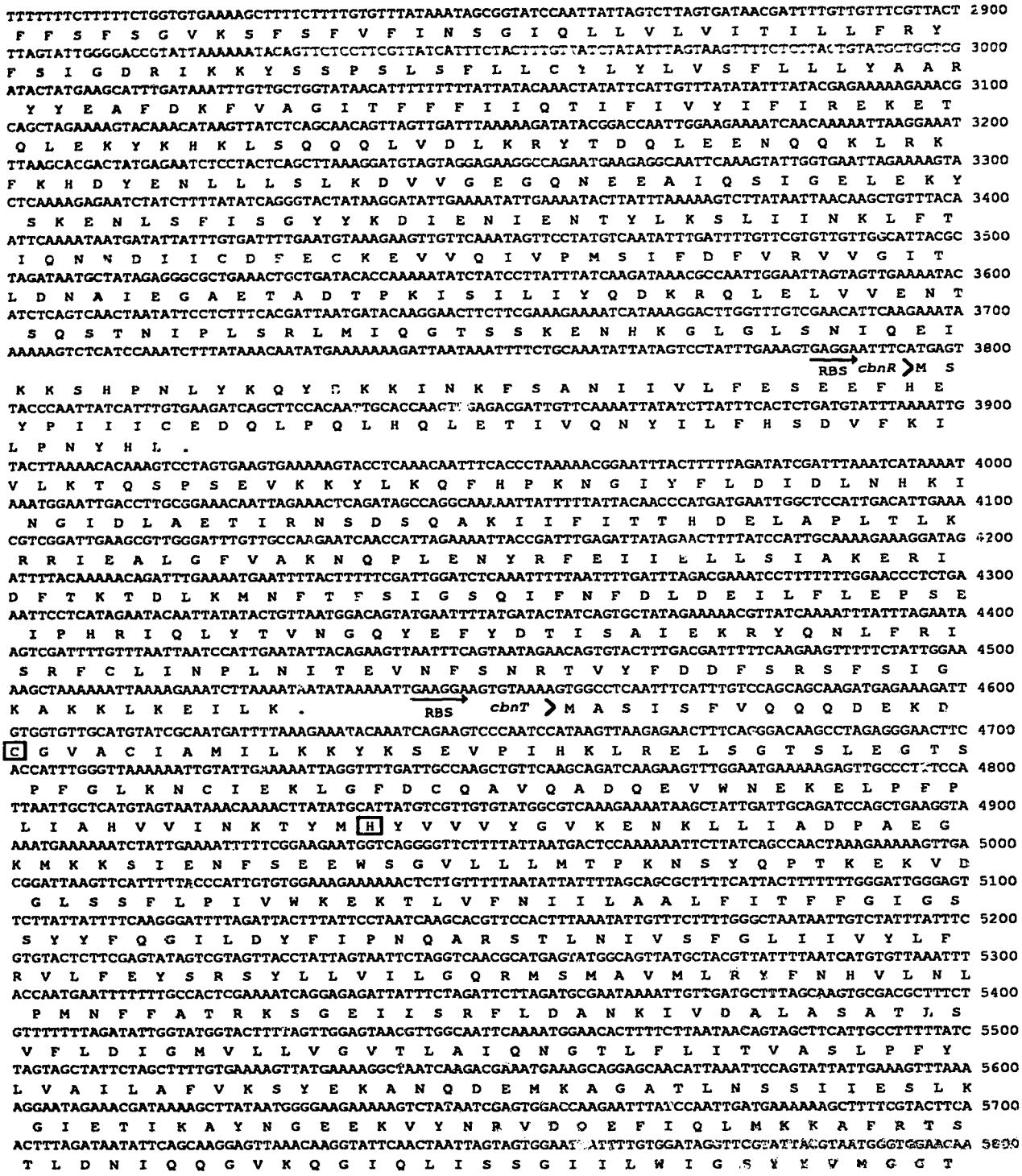

Figure 4.3. Continuation

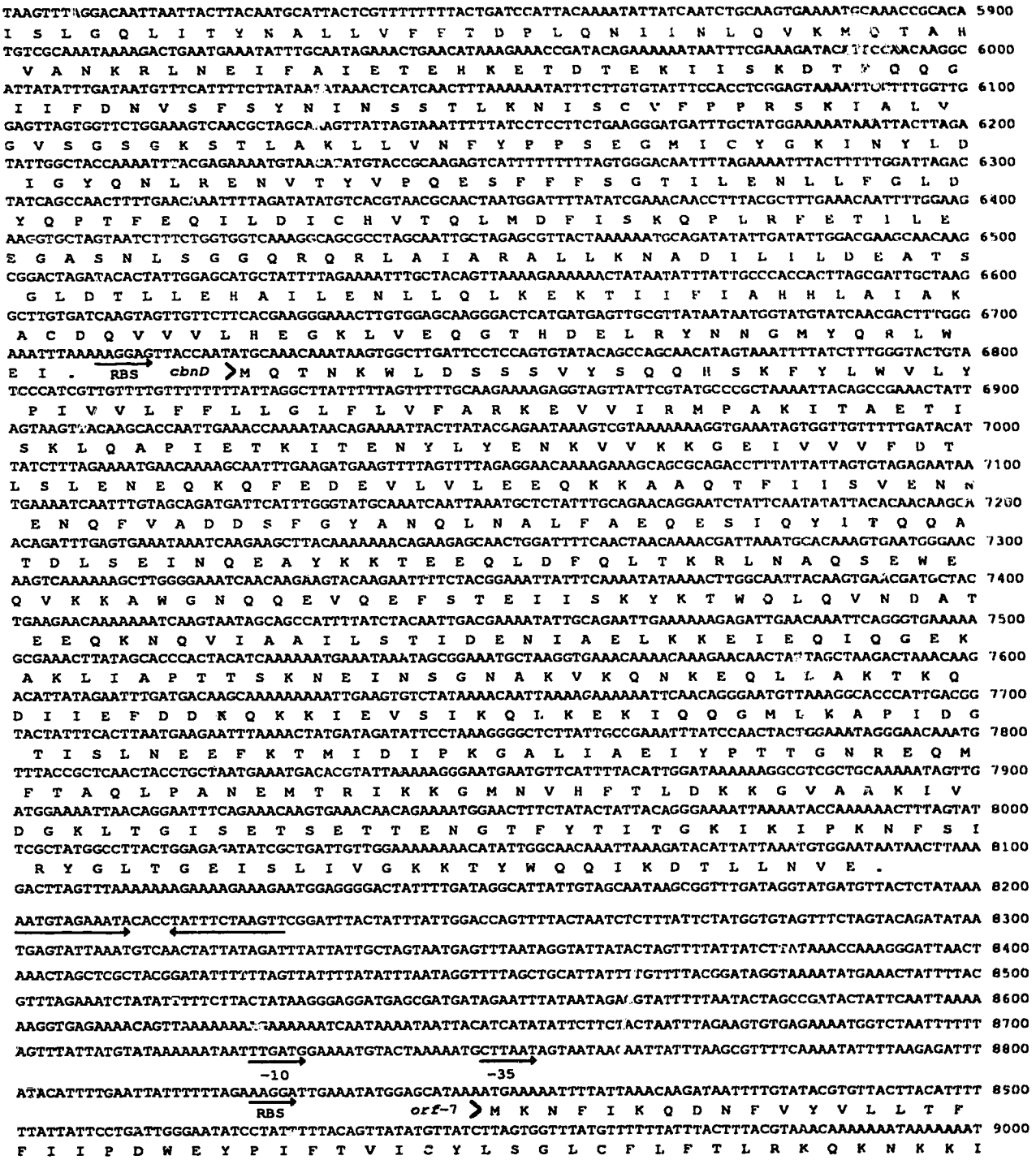

Figure 4.3. Continuation

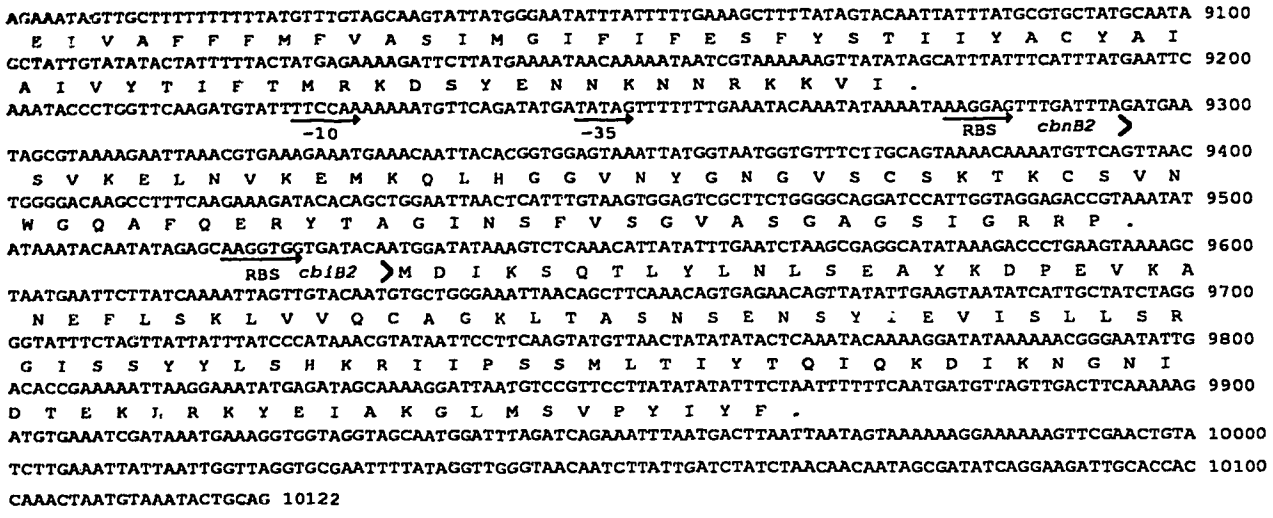

Figure 4.3. Continuation

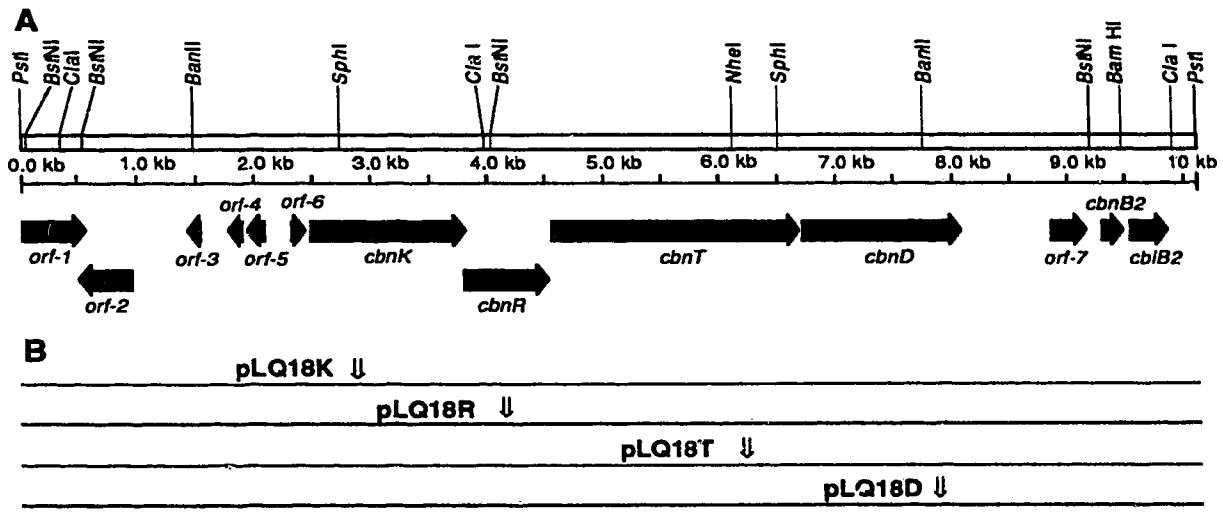

Figure 4.4. Schematic representation of the DNA insert from pLQ18 showing: (A) organization of the genes identified within the sequenced region and relevant restriction sites; and (B) positions of the frame shift mutations in pLQ18K, pLQ18R, pLQ18T, and pLQ18D. The vertical arrows indicate the modified restriction sites for *Sph*I, *Bst*NI, *Nhe*I and *Ban*II.

and -10 promoter sequences (Doi and Wang, 1986; Hawley and McClure, 1983) and transcriptional terminators were identified upstream of *orf-1*, *orf-5*, *orf-6* and *orf-7*, and downstream *orf-4*, *orf-3* and *cbnD*, respectively (Fig. 4.3). Analysis of the nucleotide sequence of the region upstream of the previously sequenced *cbnB2* revealed the presence of several ORFs: *orf-1* (187 codons), *orf-2* (152 codons), *orf-3* (42 codons), *orf-4* (48 codons), *orf-5* (51 codons), *orf-6* (41 codons), *orf-7* (113 codons), *cbnK* (442 codons), *cbnR* (245 codons), *cbnT* (716 codons) and *cbnD* (455 codons).

4.3.3. Amino acid homology and hydropathic profiles

The possible translational products of the ORFs identified in the sequenced region were compared with proteins recorded in the data banks. The amino acid sequences deduced from the *orf-1* and *orf-2* (187 and 152 aa, respectively) have sequence similarity to several transposases. The 187 aa protein encoded by *orf-1* showed the highest amino acid sequence identity (33%) to the last 190 aa at the C-terminus of IstB (265 aa), one of the two transposases required for the transposition of the insertion sequence IS*21* (Reimmann et al., 1989). The 152 aa protein encoded by *orf-2* showed the highest amino acid sequence identity (36%) to the first 150 aa of the 477-aa transposase of IS*231*F (Rezsöhazy et al., 1992). The N-termini of the peptides deduced from the *orf-4* and *orf-5* (48 aa and 51 aa, respectively) have sequence similarity to the N-terminal extensions of CbnBM1 and CbnB2 (Fig. 4.5A). However, no sequence similarity was observed between the C-termini of these peptides and the mature carnobacteriocins B2 and BM1 or any other proteins in the data banks. The possible translational product of *orf-6* (41 aa) showed the highest sequence identity (40%) with a 45 aa peptide encoded by *orf-4* located upstream of *sapK*, the histidine protein kinase involved in the production of sakacin A (see below). The N-terminal region of this 41 aa peptide, as is the case with the 45 aa peptide encoded upstream of *sapK*, has features that resemble the N-terminal extensions of the class II bacteriocins with the Gly-Gly motif at the cleavage site (Fig.

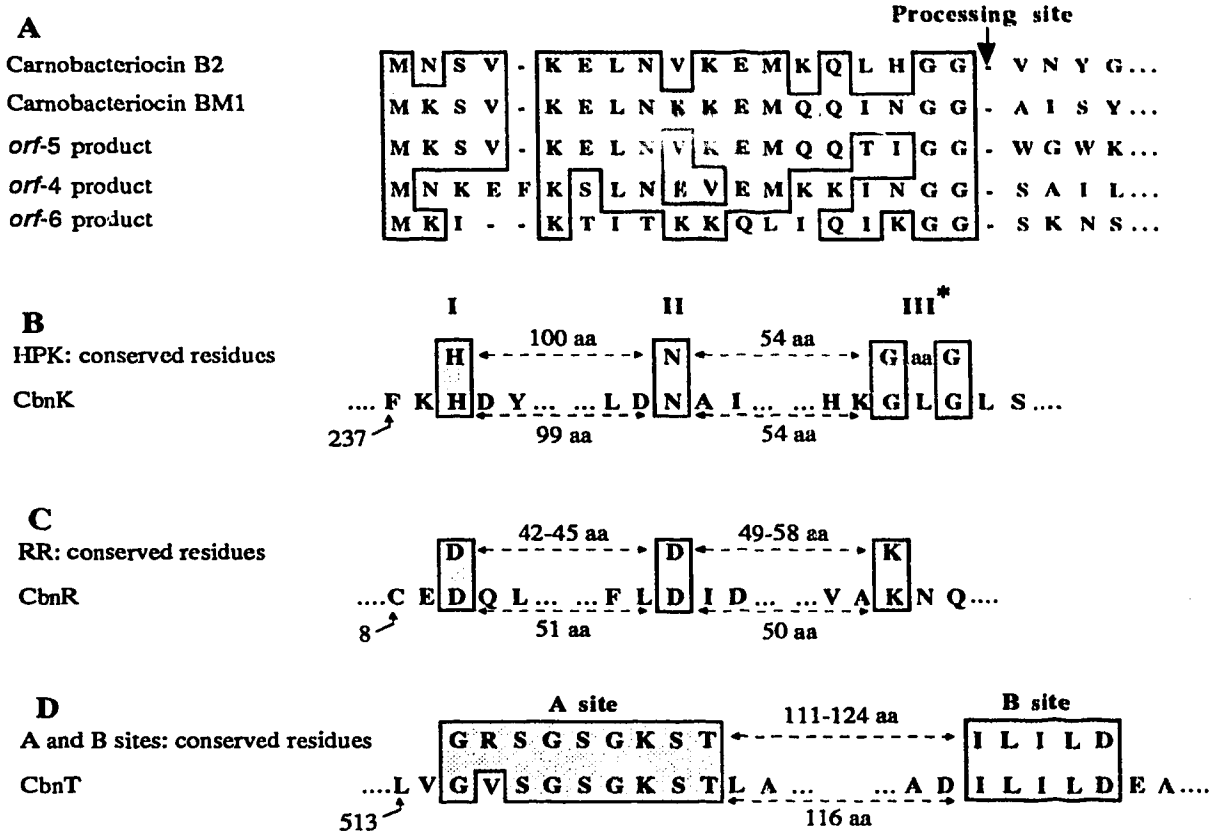

Figure 4.5. Alignment of the N-terminal extensions of the precarnobacteriocins B2 and BM1, and the N-terminal regions of *orf-4*, *orf-5* and *orf-6* gene products (A); schematic diagram of the amino acid alignment between the conserved amino acid residues of the C-terminal conserved domains I, II and III of histidine protein kinase proteins and CbnK (B); the N-terminal half of response regulator proteins and CbnR (C); and the A and B sites at the C-terminus of the ABC exporter proteins and CbnT (D). * The conserved residues in domain III are based on the amino acid sequence of AgrB (see Table 3). The spacing between the residues is indicated (sections B, C and D) and the conserved residues are boxed.

4.5A). The possible translation products of *orf-5* (42 aa) and *orf-7* (113 aa) did not have homology with known sequences.

CbnK and CbnR have sequence homology to histidine protein kinases (HPK) and response regulators (RR) of the bacterial two-component signal transduction system (Stock et al., 1989), as shown in Figs. 4.5B and 4.5C. These proteins showed the highest amino acid sequence identity (18% to 57%) to proteins involved in regulation of bacteriocins or bacteriocin-like peptides produced by several strains of lactic acid bacteria. The percentage of amino acid identity between CbnK and CbnR, and other HPKs and RRs is summarized in Table 4.3. They also have homology to the *agr*-regulatory system that controls the expression of many extracellular and cell wall-associated proteins in *Staphylococcus aureus* (Table 4.3). The hydropathic profile of CbnK (Fig. 4.6A) indicates that this protein, like other HPKs, has six potential membrane-spanning segments located at the N-terminus of the protein (Diep et al, 1994; Vandenesch et al., 1991). The other element of the carnobacteriocin regulatory system (CbnR) does not contain potential membrane-spanning segments indicating that it is probably a cytoplasmic protein (Fig. 4.6B).

CbnT and CbnD have sequence homology to proteins of a bacterial signal-sequence independent translocation system. CbnT and CbnD resemble the ATP dependent translocator and the accessory protein of the bacterial ABC export system, respectively (Fath and Kolter, 1993). The comparison of these proteins with proteins from the data banks revealed that they have the highest amino acid sequence identity (22% to 48%) to proteins involved in the secretion of bacteriocins produced by strains of lactic acid bacteria. The percentage of amino acid identity between CbnT and CbnD, and other members of the bacterial ABC export system is shown in Table 4.3. They also have homology with ABC exporter and accessory proteins that are believed to be involved in the secretion of the competence factor in *Streptococcus pneumoniae* (Table 4.3). The

Table 4.3. Percentage of amino acid identity between proteins of the carnobacteriocin gene cluster and gene products reported in the data banks*

HPK	RR	ABC Exporter	Accessory protein	Source
CbnK	CbnR	CbnT	CbnD	Carnobacteriocin B2 Clusterin *C. piscicola* (this study)
SapK 36%	SapR 57%	SapT 47%	SapE 23%	Sakacin A clusterin *L. sake* (Axelsson and Holck, 1995)
SppK 20%	SppR 29%	SppT 48%	SppE 23%	Sakacin P cluster in *L. sake* (Huehne et al., 1995)
ArgB 20%	ArgA 31%			*agr*-regulatory system in *S. aureus* (Vandenesch et al., 1991)
PlnB 18%	PlnD/C 28%/27%			Plantaricin A regulatory system in *L. plantarum* (Diep et al., 1994)
		LcaC 46%	LcaD 23%	Leucocin A secretion in *L. gelidum* (Van Belkum and Stiles, 1995)
		ComA 46%	ComB 23%	Competence factor secretion in *S. pneumoniae* (Hui and Morrison, 1991; Hui et al., 1995)
		LcnC 44%	LcnD 22%	Lactococcin A secretion in *L. lactis* subsp. *lactis* (Stoddard et al., 1992)
		MesI 46%	MesY 23%	Mesentericin Y105 secretion in *L. mesenteroides* (Fremaux et al., 1995)
		HlyB 27%	HlyD 14%	hemolysin A secretion in *E. coli* (Felmlee et al., 1985)
		PedD 43%		Pediocin PA-1/AcH secretion in *P. acidilactici* (Marugg at al., 1992; Bukhtiyarova et al., 1994)

* Percentages were calculated by aligning the full length of the proteins using the software GeneWorks. HPK: Histidine protein kinase , RR: Response regulator, ABC: ATP binding cassette.

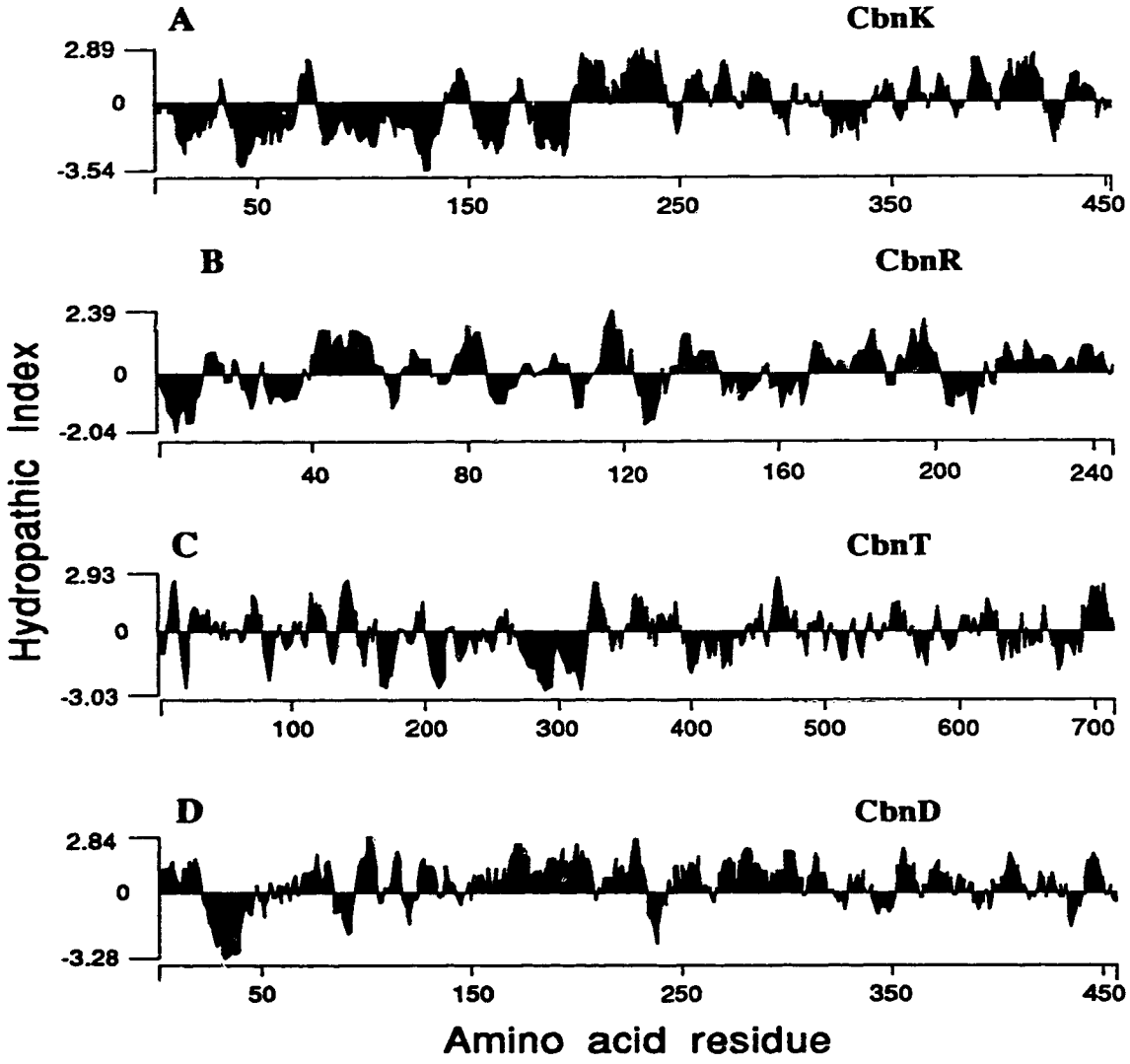

Figure 4.6. Hydropathic profiles of CbnK (A), CbnR (B), CbnT (C), and CbnD (D). Hydrophilicity and hydrophobicity are presented as positive and negative values on the hydropathic index scale, respectively.

two conserved amino acids, cysteine and histidine, postulated as the key residues of the active site at the N-terminal proteolytic domains of the ABC export proteins involved in the secretion and processing of lactococcin G and pediocin PA-1/AcH (Håvarstein, et al., 1995; Venema et al., 1995), are present in CbnT (see boxed residues in Fig. 4.3). This suggests a peptidase activity for this ABC export protein. The hydropathic profiles of CbnT and CbnD (Fig. 4.6C and 4.6D) indicated that CbnT has several membrane-spanning domains, and that CbnD is a hydrophilic protein with the exception of a potential membrane-spanning segment located at the N-terminus that can be predicted for several of the other accessory proteins included in Table 4.3.

4.3.4. Inactivation of CbnK, CbnR, CbnT, and CbnD

The proteins were individually inactivated by creating frame shift mutations in each of the genes in pLQ18 to generate the constructs pLQ18K, 18R, 18T, and 18D (Table 4.1, Fig. 4.4B). The restriction pattern of the mutants digested with other restriction enzymes was analyzed and compared with the restriction pattern of pLQ18. This indicated that the only modifications in the mutants were those introduced at the specific restriction site. The ability of *C. piscicola* LV17C transformed with these pLQ18 derivatives to produce bacteriocin was analyzed by deferred inhibition test using *C. divergens* LV13 transformed with pCaT as the indicator strain (Fig. 4.2). Transformants containing pLQ18R, 18T, or 18D did not produce bacteriocin. The strain containing pLQ18K produced bacteriocin; however, the zone of inhibition was smaller compared with that produced by the strain containing pLQ18 (Fig. 4.2, zones D and I). The sensitivity of *C. piscicola* LV17C transformants containing pLQ18K, 18R, 18T, or 18D to *C. piscicola* LV17C transformed with pLQ18 was evaluated by deferred inhibition test. Although all of the clones were sensitive, those with mutations in *cbnT* and *cbnD* showed some growth, producing a "cloudy" zone of inhibition (Fig. 4.2, zones O and P).

The effect of several concentrations of carnobacteriocins BM1 and B2 on growth of these mutants and on growth of the rest of the *C. piscicola* LV17C transformants is shown in Fig. 4.7. Strains showing similar behavior were grouped. Two different behavior patterns were identified among the strains growing in the presence of carnobacteriocin BM1: those that behaved like the parental strain *C. piscicola* LV17C transformed with pLQ18 (Fig. 4.7A) and those that behaved like the plasmidless mutant *C. piscicola* LV17C containing only the vector pCaT (Fig. 4.7B). In the latter group, deletions or frame-shift mutations introduced in pLQ18 reduced the level of immunity to that of the sensitive strain *C. piscicola* LV17C. Among the strains growing in the presence of carnobacteriocin B2, four major behaviors were identified: a group that behaved like the parental strain containing pLQ18 (Fig. 4.7C); a group that behaved like the plasmidless strain *C. piscicola* LV17C (Fig. 4.7B); and two other groups showing an intermediate behavior between that of the parental strain containing pLQ18 and that of the sensitive strain *C. piscicola* LV17C (Fig. 4.7D and 4.7F).

4.4. Discussion

Two class II bacteriocins produced by *C. piscicola* LV17B were previously purified and characterized: carnobacteriocin B2 which is encoded on a 61-kb plasmid and carnobacteriocin BM1 which is encoded on the chromosome (Quadri et al., 1994). A 10,122-bp fragment from the 61-kb plasmid of *C. piscicola* LV17B contained the genetic information necessary to restore production of, and immunity to, both of these bacteriocins in the homologous host *C. piscicola* LV17C. Deletion analysis of this fragment indicated that the region upstream of *cbnB2* was essential for bacteriocin production and immunity and that complementing trans-acting factors encoded on pCP40 required for expression of the chromosomal bacteriocin CbnBM1 and its immunity were also located on the cloned fragment. The same fragment allowed expression of the plasmid-encoded carnobacteriocin B2 and its immunity protein in the heterologous host

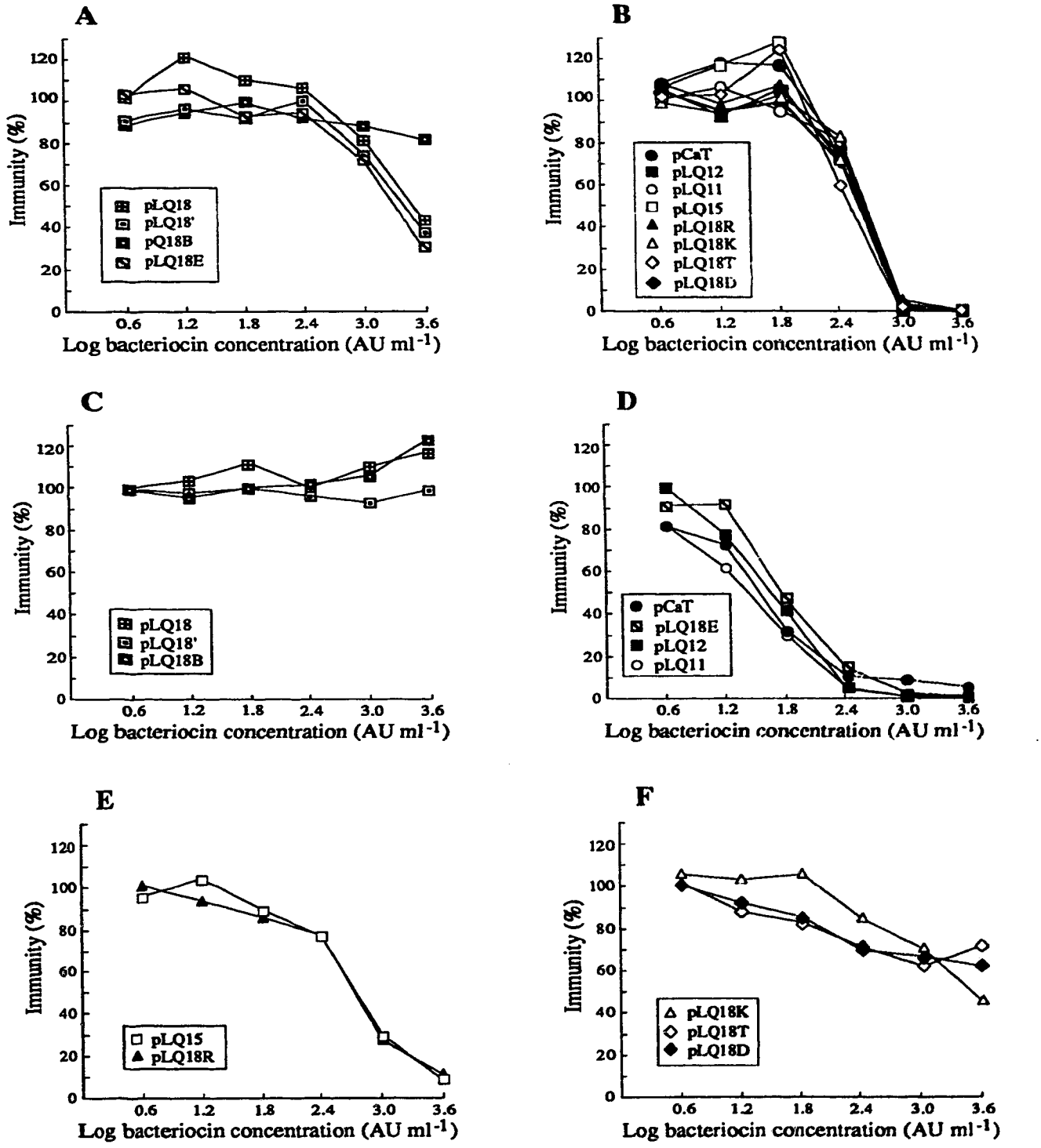

Figure 4.7. Immunity to carnobacteriocins BM1 (plots A and B) and B2 (plots C to F) of selected transformants of *Carnobacterium piscicola* LV17C. Immunity is expressed as the percentage of the growth rate in the presence of bacteriocin relative to the growth rate in the absence of bacteriocin.

C. divergens LV13. Expression studies in *C. divergens* LV13 indicated the presence of at least one other unidentified bacteriocin encoded on the 10-kb fragment. Because *C. divergens* LV13 is sensitive to the bacteriocin(s) encoded in pLQ18E, and it becomes immune following transformation with this plasmid, the presence of a specific immunity function encoded in pLQ18E that protects *C. divergens* LV13 against the unidentified bacteriocin(s) can be predicted. The probable translation products of *orf-4*, *orf-5* and(or) *orf-6* could be responsible for the inhibitory activity detected in *C. divergens* LV13 transformed with pLQ18B or pLQ18E. These small peptides have N-terminal regions similar to those of CbnB2 and CbnBM1, including the common Gly-Gly motif present at the cleavage sites of the precarnobacteriocins and they could be substrates of the processing and translocation machinery formed by CbnT and CbnD (see below).

The presence of two open reading frames encoding potential transposases, indicating the presence of a transposable element located at the 5' end of the cloned fragment, is the first report of an insertion sequence-type element in *Carnobacterium piscicola*. Moreover, the presence of transposable elements in this strain could explain the plasmid rearrangements involving pCP40 previously reported (Ahn and Stiles, 1992) and it could have contributed to the development of multiple copies of an ancestral bacteriocin that evolved to produce the different bacteriocin genes present in *C. piscicola* LV17B.

Involvement of the four largest genes located on the sequenced fragment in bacteriocin production and immunity was studied and they were shown to be required for the wild type phenotype. Mutational analysis indicated that CbnK and CbnR are involved in regulation of production of carnobacteriocins and their immunity proteins. The HPK encoded by *cbnK* could be involved in sensing of an environmental signal. Interaction between the sensing domain of CbnK with the specific ligand would promote autophosphorylation of the cytoplasmic domain of CbnK. The phosphate group could

then be transferred to the response regulator CbnR which would control transcription of genes required for bacteriocin production and immunity. Inactivation of CbnR eliminated bacteriocin production and caused an important reduction in the level of immunity. However, inactivation of CbnK yielded mutants with reduced bacteriocin production and a less severe decrease in immunity compared with that of the CbnR mutant. The phenotype of the CbnK mutant, could indicate that another HPK present in the host strain is able to 'cross talk' and phosphorylate CbnR. The signal recognized by CbnK could be one of the bacteriocins produced by *C. piscicola* LV17B. The behavior of the parental strain *C. piscicola* LV17B indicated that the carnobacteriocins are signals that regulate their own production showing an autoregulation similar to that present in the nisin A and plantaricin A systems (Engelke et al., 1994; Kuipers et al., 1993, 1995; Diep et al., 1995). In this strain, production of bacteriocins can be induced by carnobacteriocin B2. Bacteriocin production by the parental strain *C. piscicola* LV17B is lost when the culture is inoculated below $1x10^4$ cfu ml^{-1} and bacteriocin production of a nonproducing culture is restored by adding sterile supernatant from a bacteriocin-producing culture, or purified carnobacteriocin B2 (Saucier et al., 1995). We have established that the inoculation level also affects immunity to carnobacteriocins BM1 and B2, indicating a common regulation for the production of bacteriocins and immunity proteins. Interestingly, *C. piscicola* LV17B and LV17C transformed with the plasmids pLQ24, pLQ18, pLQ18′ or pLQ18E did not behave in the same manner as *C. piscicola* LV17B; in these transformants bacteriocin production was not affected by inoculum size. The difference in behavior between the *C. piscicola* transformants and the parental strain may be due to increased number of copies of some genes within the cloned region in pLQ18.

The other two large genes in the cluster, *cbnT* and *cbnD*, are essential for bacteriocin production and for full immunity to carnobacteriocins B2 and BM1. The results indicate that CbnT and CbnD are involved in the secretion of the bacteriocins. CbnT could also provide the peptidase activity that cleaves the N-terminal extension of

the prebacteriocins as reported for the ABC export proteins involved in the secretion and processing of lactococcin G and pediocin PA-1 (Håvarstein et al., 1995; Venema et al., 1995). Mutations in *cbnT* and *cbnD* affected the levels of immunity. Because bacteriocin production and full immunity require induction by a bacteriocin(s), and mutations in *cbnT* or in *cbnD* prevent the secretion of the bacteriocin, the immunity of these mutants will be reduced. Similarly, in the autoregulation of nisin, which is comparable to that of the carnobacteriocin system, mutants that produce only the nisin precursor or that have only the *nisA* gene disrupted, also showed decreased levels of immunity to externally added bacteriocin (Kuipers et al., 1993). The production of unusual cloudy zones of inhibition when mutants with *cbnT* or *cbnD* disrupted were exposed to the bacteriocin-producing organisms might be due to the accumulation of bacteriocin precursor and (or) processed molecules within the cells. Exposure of the mutants to the bacteriocin on the plate will trigger production of bacteriocin precursor and immunity. Eventually, the accumulation of bacteriocin precursor and (or) processed molecules within the cells with a nonfunctional secretion system could become deleterious and reduce or stop the growth of the organisms. Intracellular accumulation of precursor, or the precursor and the processed molecule, have already been reported for the bacteriocin pediocin PA-1 in strains missing either PedD or PedC, respectively (Venema et al., 1995).

Our results show that production of bacteriocins in *C. piscicola* LV17B requires a specific signal-sequence independent secretion machinery composed by CbnT and CbnD and that CbnT is probably the peptidase involved in the cleavage of the bacteriocins N-terminal extensions. The results also indicate that production of, and immunity to, the carnobacteriocins are regulated by CbnK and CbnR, a two-component signal transduction system that could be involved in a bacteriocin autoregulation phenomenon in which the bacteriocins provide the signals that trigger their own production and synthesis of the immunity proteins. This complex autoregulation phenomenon is under investigation.

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5. GENERAL CONCLUSIONS

The potential to use bacteriocins or bacteriocinogenic strains of the genus *Carnobacterium* to suppress or reduce the growth of spoilage and pathogenic bacteria in meat systems stimulated our studies on the biochemistry and the genetics of bacteriocins produced by strains of this genus. Strains of the genus *Carnobacterium* form an important component of the population of lactic acid bacteria that develops and prevails on refrigerated raw and processed meat products packaged under modified atmosphere with elevated levels of carbon dioxide, including vacuum packaged products (McMullen and Stiles, 1995).

Carnobacterium piscicola LV17 is a bacteriocinogenic organism isolated from chill-stored vacuum packaged meat (Ahn and Stiles, 1990a). *C. piscicola* LV17 contains three plasmids: pCP49, pCP40 and pCP9, of which only pCP49 and pCP40 are involved in the production of different bacteriocins (Ahn and Stiles, 1990b). The class II bacteriocin carnobacteriocin A from *C. piscicola* LV17A (Worobo et al., 1994), encoded on pCP49, has been purified and characterized. *C. piscicola* LV17A contains only the plasmid pCP49 and it was derived from *C. piscicola* LV17 by Ahn and Stiles (1990b).

The study of the bacteriocins produced by *C. piscicola* LV17B (Ahn and Stiles, 1990b), another strain derived from *C. piscicola* LV17 that contains only the plasmid pCP40, is the subject of this thesis.

5.1. Carnobacteriocins B2 and BM1: The Peptides

C. piscicola LV17B produces at least two class II bacteriocins: carnobacteriocins B2 and BM1. These bacteriocins are ribosomally synthesized as precursors that undergo proteolytic cleavage of an 18-amino acid leader peptide to yield the mature and fully active bacteriocins. The leader peptides contain two Gly residues (positions -1 and -2) at

the cleavage site. These leaders have the characteristics of the so-called "Gly-Gly" leader peptides (Håvarstein et al., 1995).

The mature portion of carnobacteriocins B2 and BM1 have significant amino acid sequence homology between themselves and with other class II bacteriocins. The sequence motif 'Tyr-Gly-Asn-Gly-Val' (YGNGV), located near the N-terminus of the mature carnobacteriocins, is present in several other bacteriocins. Interestingly, this sequence motif is not present in carnobacteriocin A, the third bacteriocin produced by *C. piscicola* LV17. The conservation of the sequence motif YGNGV suggests a key role for this sequence in the biological activity of the bacteriocins. Furthermore, an amino acid substitution that replaced the conserved Tyr by a Phe residue at position 3 in the amino acid sequence of carnobacteriocin B2 produced a peptide without antimicrobial activity (Quadri, 1995). Although this result shows the importance of this amino acid recidue, its role in biological activity is not known.

5. 2. Carnobacteriocins B2 and BM1: Genetic Organization

The lack of production of bacteriocins and the sensitivity to carnobacteriocins B2 and BM1 of the plasmidless cured strain *C. piscicola* LV17C indicated that the plasmid pCP40 is required for bacteriocin production and immunity. Surprisingly, only the genetic determinant of carnobacteriocin B2 and its immunity protein are located on the plasmid; the genetic determinant of carnobacteriocin BM1 and its putative immunity protein are located on the chromosome of *C. piscicola* LV17B. This genetic arrangement is unique for bacteriocins from LAB and indicates the presence of complementing trans-acting factor(s) encoded on the plasmid that are required for the expression of the chromosomal bacteriocin.

In addition to the bacteriocin and immunity genes, production of, and immunity to, most class II bacteriocins produced by lactic acid bacteria require other genetic

determinants. Dedicated secretion machinery consisting of an ABC exporter and an accessory factor of the bacterial *sec*-independent secretion system are usualy required for bacteriocin secretion (Fath and Kolter, 1993). The ABC exporters involved in the secretion of lactococcin G and pediocin PA-1 have a dual function: translocation of the bacteriocins and cleavage of the leader peptides (Håvarstein et al., 1995; Venema et al., 1995). In the cases of nisin A, carnobacteriocin A and sakacins A and P, an histidine protein kinase and a response regulator of the bacterial two-component signal transduction system are required for regulation of bacteriocin production and immunity (Axelsson and Holck, 1995; Huehne et al., 1995; Kuipers et al., 1995). The genetic determinants necessary for these elements are usually clustered and located in the proximity of the gene encoding the bacteriocins. In the case of carnobacteriocins B2 and BM1, the genetic information necessary to restore production of, and immunity to, both bacteriocins in the homologous host, is located on a 10,122-bp fragment of the plasmid pCP40. The same fragment contains sufficient genetic information to allow the expression of the plasmid-encoded carnobacteriocin B2 and its immunity protein in a heterologous host.

In addition to the genes encoding carnobacteriocin B2 and its immunity protein, the cluster includes at least four other genes that are required for full bacteriocin production and immunity. These genes specify proteins that are involved in secretion of the carnobacteriocins and in regulation of gene expression by signal transduction. Several other genes are present in this fragment and some of them might encode inducing factors (see later) or peptides with antimicrobial activity. Bacteriocin activity can be detected from *C. divergens* containing the plasmid pLQ18E with a deletion of carnobateriocin B2 gene against *C. divergens*. This indicates the presence of at least one other unidentified bacteriocin and its specific immunity protein encoded on the 10-kb fragment from pCP40. The purification and characterization of this compound(s) is currently been conducted.

Three clusters with gene organization similar to the one found in carnobacteriocin B2 gene cluster have been recently identified: sakacin A and sakacin P gene clusters and carnobacteriocin A gene cluster (Axelsson and Holck, 1995; Huehne et al., 1995; Worobo et al., 1995). Each of these clusters has genes encoding an ABC exporter, an accessory factor, a histidine protein kinase and a response regulator. Although all of these genes are probably involved in bacteriocin production, the genes from the sakacin P cluster are the only ones that have been inactivated and proved to be required for bacteriocin production. The gene products believed to be involved in the secretion and regulation of sakacins A and P, and carnobacteriocin A have important amino acid homology to those in the carnobacteriocin B2 gene cluster. The homology is particularly high between the gene products of carnobacteriocins A and B2 clusters (over 90 %). Carnobacteriocin A cluster was cloned from the plasmid pCP49 of *C. piscicola* LV17, the same strain that contains pCP40 with the carnobacteriocin B2 gene cluster (Worobo et al., 1995). The exceptionally high homology between these two systems suggests that one originated from the other by gene duplication. It is possible that transposition events played an important role in the development of multiple copies of an ancestral bacteriocin gene cluster that evolved to produce the different gene clusters present in *C. piscicola* LV17. An insertion-sequence like element is located upstream of carnobacteriocin B2 gene cluster. Although it is not known if this represents an active transposable unit, the formation of a cointegrate between pCP40 and pCP49 previously reported, suggests the presence of active transposable elements in this strain (Ahn and Stiles, 1992). Furthermore, another insertion sequence is located between the two operons of the sakacin A gene cluster. The close association between these bacteriocin clusters and transposable elements might indicate that transposition events play an important role in horizontal genetic trasnfer and spreading of the genes required for bacteriocin production among strains of LAB.

5.3. Carnobacteriocins B2 and BM1: Regulation by signal transduction

CbnK and CbnR have homology with elements of bacterial two-component signal transduction systems. They could be involved in a bacteriocin autoregulation phenomenon in which the bacteriocin(s) provide the signals that trigger their own production and synthesis of the immunity proteins. This hypothesis is based on the observation that purified carnobacteriocin B2 and an oxidized form of carnobacteriocin A but not carnobacteriocins BM1 or A are able to induce the production of bacteriocin in *C. piscicola* LV17B (Saucier et al., 1995). However, carnobacteriocin B2 is not the only signal capable of inducing bacteriocin production in *C. piscicola* LV17B. The supernatant of *C. divergens* containing the plasmid pLQ18E induces bacteriocin production in *C. piscicola* LV17B. Because the structural gene of carnobacteriocin B2 is deleted in pLQ18E, the cloned fragment must encode another inducer molecule. Recently, an extracellular peptide of 19 amino acid was shown to induce sakacin P production (Eijsink et al., 1995). The inducer peptide is synthesized as a precursor containing a leader peptide similar to those present in class II bacteriocins. The genetic determinant of this peptide is located upstream of a histidine protein kinase gene that is located in sakacin P gene cluster. A small peptide with characteristics similar to those of the sakacin P inducer is encoded by *orf-6*, located upstream of *cbnK* in the carnobacteriocin B2 gene cluster. Given the similar organization of carnobacteriocin B2 and sakacin P gene clusters, and the similarities of the small peptides encoded upstream of both histidine protein kinase genes, it is possible that the peptide encoded by *orf-6* is an inducer for the carnobacteriocins system. This hypothesis is under investigation. Open reading frames encoding small peptides with homology to the *orf-6* product are also located upstream of the recently sequenced histidine protein kinase genes of the carnobacteriocin A and sakacin A clusters. Interestingly, production of carnobacteriocin A by *C. piscicola* LV17A is an inducible process. An oxidized form of carnobacteriocin

A and carnobacteriocins B2 and BM1, but not carnobacteiocin A, are able to stimulate bacteriocin production *C. piscicola* LV17A (Saucier et al., 1995).

The carnobacteriocins A and B, and sakacins A and P, together with the plantaricin A system could follow a similar regulation strategy whereby bacteriocin production and immunity are regulated by signal transduction. In all of these cases, the main environmental signal would be a small peptide synthesized as a prebacteriocin-type precursor rather than the bacteriocins themselves. The leader peptides of the inducer precursors show homology with leader peptides of the "Gly-Gly" type present in most of the class II bacteriocins. Given this similarity, it is possible that these precursors are processed and secreted by the same dedicated machinery as the bacteriocins themselves. Furthermore, in the plantaricin A system, the inducer peptide is plantaricin A and it induces not only bacteriocin production, but its own synthesis as well (Diep et al., 1995). This could also be the case for the carnobacteriocins A nnd B and sakacins A and P systems.

This complex regulatory strategy appears to be slightly different to the one in lantibiotics. In the case of the nisin A system, nisin A itself is the signal that triggers its own synthesis (Kuipers et al., 1995). In this system no other specific inducer peptide has been found. Further study of these signal transduction and regulation pathways is required. The understanding of these regulatory pathways would provide information that could be used to implement systems to control the production of bacteriocins for specific practical purposes through the development of food-grade inducible systems. Furthermore, the systems could be used as a model to study signal transduction. The specific interaction between the inducer peptides and the histidine protein kinase could also serve as a model to study protein-protein interaction.

5.4. Carnobacteriocins B2 and BM1: Immunity functions

The molecular basis of the mechanism(s) whereby the producer organisms protect themselves against the antimicrobial effect of their bacteriocins is poorly understood. We investigated the immunity function that protects the producer organism against carnobacteriocin B2. Immunity to carnobacteriocin B2 requires the protein CbiB2. The genetic determinant of CbiB2 is located downstream of the genetic determinant of carnobacteriocin B2 and these two genes form part of the same transcriptional unit (Quadri et al., 1995). This protein increases the level of resistance of sensitive homologous and heterologous hosts to externally added carnobacteriocin B2. However, CbiB2 does not increase the level of resistance to the closely related carnobacteriocin BM1. The immunity to carnobacteriocin BM1 is probably encoded by *cbiBM1*, located downstream of the carnobacteriocin BM1 gene.

Carnobacteriocin B2, like other bacteriocins, targets the cytoplasmic membrane of sensitive cells and it causes dissipation of the proton motive force with leakage of intracellular components through the probable formation of pores (Van Belkum, 1995). Expression of CbiB2 within the bacterial cell protects against the antimicrobial action of CbnB2 applied externally, but addition of purified CbiB2 to the medium does not protect sensitive strains. Preincubation of CbiB2 with carnobacteriocin B2 does not inactivate the bacteriocin. These observations agree with the cellular localization of CbiB2. Immunolocalization experiments indicate that the majority of the intracellular pool of CbiB2 is located in the cytoplasm. The cellular localization of CbiB2 differs from that reported for the immunity protein of the class II bacteriocin lactococcin A. In this case, between one third and one half of the cellular pool of the immunity protein is associated with the membrane (Nissen-Meyer et al., 1993; Venema et al., 1994). The presence of a transmembrane spanning segment in the immunity protein has been demonstrated

(Venema et al., 1994). The model for the mode of action of the immunity protein predicts that the presence of the immunity protein associated with the receptor in the membrane, prevents the successful insertion of lactococcin A into the cytoplasmic membrane to form pores (Venema et al., 1995). A different mode of action has been proposed for the immunity protein of the lantibiotic nisin A. The immunity protein has a consensus lipoprotein signal sequence, suggesting that it is a extracellular membrane-anchored lipoprotein. It could protect the producer organism by specifically binding nisin A molecules and preventing their insertion into the membrane (Kuipers et al., 1993). In the case of the carnobacteriocin B2 immunity protein, it seems unlikely that the immunity protein directly inhibits the interaction of the extracellular bacteriocin with the membrane. Rather, CbiB2 may interfere with the formation of a functional pore complex in the membrane, or it may block the functional pore to prevent the efflux of intracellular components by interacting with the portion of the pore structure facing the cytoplasm.

Further study is required to formulate an appropriate model for the mode of action of the immunity protein. The introduction of changes in the amino acid sequence of CbiB2 by site directed mutagenesis and the analysis of the phenotype of sensitive strains expressing the altered immunity proteins, would allow the identification of key residues required for the mode of action of the immunity protein. Furthermore, because CbiB2 can be expressed in *E. coli*, large quantities of isotopically labeled CbiB2 could be readily available for use in NMR studies to determine the solution structure of the protein. NMR information on CbiB2 solution structure, combined with information on the biological activity derived from protein engineering of CbiB2, may facilitate the formulation of a model and the understanding of the immunity mechanism.

5.5. Carnobacteriocins B2 and BM1: Their Application

When considering the potential for application of a bacteriocin as biopreservative agent in meat systems several points should be taken into account in order to assess the economic feasibility. Some of these points are:

- Production, stability and effectiveness of the bacteriocin in meat: is the bacteriocin being produced in meat? Is the bacteriocin stable in meat? Is the bacteriocin in meat free to exert its inhibitory effect?

- Target organism(s) to be inhibited: Gram-positive, Gram-negative, sporeforming bacteria, etc.; does the bacteriocin inhibit the target organism in meat?

- Strategy to deliver the bacteriocin in the product: addition of pure or partially purified bacteriocin, fermented sterile culture supernatant containing the bacteriocin or inoculation of the product with a defined culture(s) that produces the bacteriocin while growing in the product.

- Effect of the specific strategy used on the organoleptic properties of the product: production of flavor and odor defects, discoloration. This is especially important when inoculation of the product is required to deliver the bacteriocin.

- Guidelines and regulations for food additives and preservatives and food processing: is the specific strategy used in agreement with the existing regulations or does it require a modification of existing regulations?

- Consumer perception and acceptability of the modified product: is the consumer willing to accept small changes in the organoleptic properties of the product or an increased cost in exchange for a safer product? Is the consumer willing to

accept the use of nonconventional preservatives such as bacteriocins or the addition of defined cultures to products that are not commonly perceived as fermented products with high numbers of organisms?

Because of the narrow spectrum of antimicrobial activity of the carnobacteriocins the cost of their addition to meat products would not be justifiable. The potential inoculation of meat products in modified atmosphere packaging (MAP) with elevated levels of carbon dioxide, including vacuum packaging, with a carnobacteriocin-producing *Carnobacterium* spp. could be used to achieve a predictable and defined LAB population in the product. This hypothesis was tested by adding *C. piscicola* LV17 to MAP beef. Although *C. piscicola* LV17 grew well at 7 °C, its growth was not predictable at 2 °C (Leisner et al., 1995). Moreover, no bacteriocin activity was detected in meat samples inoculated with *C. piscicola* LV17 and the presence of *C. piscicola* LV17 reduced the aerobic storage life after the vacuum storage period (Leisner et al., 1995; McMullen, 1995).

These results suggest that *C. piscicola* LV17 and the bacteriocins that it produces are nether suitable for enhancing safety nor for extending shelf life of MAP beef; however, they might be applicable to other meat products such as processed meats (McMullen and Stiles, 1995). Although, the carnobacteriocins alone will probably not be of use, the biochemical and genetic information obtained for these and other antimicrobial peptides could be useful to engineer new and more suitable bacteriocins and bacteriocin-producing LAB.

It is possible to engineer LAB strains genetically to produce several bacteriocins. By using the *sec*-dependent signal sequence of the class II bacteriocin divergicin A (Worobo et al., 1995) several heterologous bacteriocins, including carnobacteriocin B2, have been produced in *C. divergens* LV13 (McCormick, 1995). This strategy has expanded the spectrum of antimicrobial activity of the producer strain. Alternatively,

information about function-structure relationship of the carnobacteriocins and other antimicrobial peptides could provide rational tools to design new bacteriocins more suitable for commercial applications. Properties such as specific activity, spectrum of inhibitory activity, solubility and stability could be changed through protein engineering to obtain bacteriocins with desired characteristics.

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