CLONING AND SEQUENCE ANALYSIS OF THE GENE CODING FOR A *LEUCONOSTOC* BACTERIOCIN

BY

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To Mommy and in memory of Daddy

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"I can do all things through Christ who strengthens me.....

Now to our God and Father be glory forever and ever. Amen"

(cf. Philippians 4 verse 13 and 20)

I declare that **"Cloning and Sequence Analysis of the Gene coding for a Leuconostoc Bacteriocin"** is my own work and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

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ABSTRACT

Previous studies have shown that *Leuconostoc (Lc.) carnosum* Ta11a produces a bacteriocin that has been designated leucocin B-Ta11a [Papathanasopoulos, 1993, M.Sc thesis, University of the Witwatersrand]. Leucocin B-Ta11a is active against *Listeria monocytogenes* and several lactic acid bacteria. An 8.9 MDa plasmid in *Leuconostoc carnosum* Ta11a hybridised to a 36-mer oligonucleotide probe (JF-1) that is homologous to the amino-terminal sequence of the leucocin A-UAL187 structural gene. A library of *Lc. carnosum* Ta11a plasmid DNA was constructed by partial digestion of DNA with *Sau*3A and ligation into the *Bam*H1 site of pUC118. A plasmid (pJF8.1), containing a 4.9 kb insert was identified by Southern blotting and hybridisation to JF-1. A subclone of this plasmid, with a 2.3 kb insert (pJF5.5), was generated by internal deletion of a 2.6 kb *Xba*1 fragment and religation of the plasmid. Sequence analysis of pJF8.1 and pJF5.5 revealed the presence of two open reading frames (ORF). ORF1 codes for a protein of 61 amino acid residues. This protein product is proposed to be the prepeptide of a 37 amino acid bacteriocin, leucocin B-Ta11a, by virtue of DNA sequence homology to leucocin A-UAL187 [Hastings *et al.*, 1991. J. Bacteriol 173: 7491-7500]. The 24 amino acid residue amino-terminal extension, possibly cleaved during processing of the prepeptide may function as a leader peptide. The amino-terminal extension of leucocin B-Ta11a differed from the similar region in leucocin A-UAL187 by seven residues. The predicted protein of the ORF2 consists of 113 amino acids and is identical to the amino acid sequence of the cognate ORF of the leucocin A-UAL187 operon. Expression of leucocin B-

Ta11a was attempted in *Escherichia coli* JM103 transformed with pJF8.1 and pJF5.5. Results of inhibition studies with various cell fractions of the transformed strains showed that no bacteriocin was produced by these transformants.

LIST OF FIGURES

List of Tables

Contents

CHAPTER 1

THE GENETICS OF BACTERIOCINS OF LACTIC ACID BACTERIA

1.0 Introduction

Lactic acid bacteria (LAB) play an essential role in many food fermentation processes [McKay and Baldwin, 1990]. LAB used in fermentations include *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Propionibacterium* and *Carnobacterium*. LAB are used as starter cultures in the dairy industry and for vegetable fermentations and are commonly associated with meat and meat products [Ahn and Stiles, 1990(a); Daba *et al.*, 1991; Hastings and Stiles, 1991; Papathanasopoulos *et al.*, 1991; Lewus *et al.*, 1991; Hechard *et al.*, 1992; Barefoot and Nettles, 1993; Jiménez-Díaz *et al.*, 1993]. Strains of LAB produce metabolic end products such as hydrogen peroxide, diacetyl and organic acids, some of which impart unique flavour and sensory qualities to food [Daeschel, 1989]. Growth of LAB also appears to inhibit the growth of certain other microorganisms *in vivo*. This could be caused by the lowering of pH as a result of acid production and thereby creating unfavourable growth conditions, or by the inhibitory effect of antimicrobial compounds such as hydrogen peroxide, bacteriocins, diacetyl and organic acids. The recent explosion of research activity in the bacteriocin field has resulted in an increased understanding of LAB, the proteins produced by them and the genetic organisation of the operons that encode these functions.

1.1 Definition of bacteriocins

Bacteriocins are defined as proteinaceous substances that show antimicrobial activity against species usually closely related to the producer organism [Tagg *et al.*, 1976; Klaenhammer, 1988]. Recent studies have shown that some of these bacteriocins may consist of complexes of proteins or

proteins interacting with different types of molecules. The active complexes for lactacin F [Klaenhammer, 1993] and lactococcin G [Nieto-Lozano *et al.*, 1992] consist of two interacting proteins, leuconocin S appears to be a protein associated with carbohydrates [Lewus *et al.*, 1992] and plantaricin S a protein associated with lipid components [Jiménez-Díaz *et al.*, 1993].

It has been suggested recently [Klaenhammer, 1993] that bacteriocins be divided into several classes:

I) the lantibiotics, which are small membrane-active peptides (±5 kDa) containing post-translationally modified lanthionine residues (eg. nisin).

Class II - IV are non-lantibiotics which contain no lanthionine residues. Subdivisions of the latter group have been suggested by Klaenhammer [1993] as follows:

II) small, heat-stable, non-lanthionine containing membrane-active peptides (<10 kDa) characterised by Gly-Gly-1**+1 Xaa processing site in the bacteriocin precursor. Subgroups within class II are:

IIa) *Listeria*-active peptides with a consensus sequence in the N-terminus of -Tyr-Gly-Asn-Gly-Val-Xaa-Cys- (eg. pediocin PA-1, leucocin A-UAL187).

IIb) Poration complexes consisting of two proteinaceous peptides for activity (eg. lactacin F).

IIc) Thiol-activated complexes requiring reduced cysteine residues for activity (eg. lactococcin B).

III) Large, heat labile-proteins (>30 kDa) (eg. helveticin J).

IV) Complex bacteriocins, composed of protein plus one or more chemical moieties (lipid or carbohydrate) required for activity (eg. plantaricin S).

The term bacteriocin, therefore, encompasses a large group of

heterogeneous antibacterial compounds differing in spectrum of activity, biochemical properties and mode of action [Klaenhammer, 1988; Stiles and Hastings, 1991].

1.2 Potential for use

Consumer trends suggest that more natural and less processed foods are preferred. There is therefore a move away from chemical preservatives for use in food. Bacteriocins may be potentially useful as natural preservatives. LAB producing bacteriocins have been isolated from foods and therefore have existed in food systems without known adverse effects. Nisin is the best characterised bacteriocin of LAB and is produced by *Lactococcus lactis* ssp. *lactis*. It is used in the U.S.A. in processed cheese spreads, canned goods, milk and other dairy products to inhibit growth, and production of gas and toxin by clostridial spores. Nisin also inactivates thermophilic spoilage organisms and extends the shelf life of dairy products [Delves-Broughton, 1990]. It has been shown recently that nisin, in combination with EDTA, a chelating agent, inhibited growth of pathogenic organisms including *E. coli* and *Salmonella* [Stevens *et al.*, 1991].

Trials, conducted in order to test the effectiveness of production of pediocin *in situ* during fermentation of dried sausage, showed that *Listeria* growth was effectively inhibited in all samples tested [Foegeding *et al.*, 1992]. A recent report indicated that use of nisin in combination with pediocin AcH had a wider inhibitory spectrum than each of the two bacteriocins individually [Hanlin *et al.*, 1993]. Elucidation of the genetics, regulation of expression and mode of action of these bacteriocins will facilitate the improvement of bacteriocin-producing cultures used in food fermentation and preservation. There is also the possibility of increasing the activity spectrum of these

bacteriocins by protein engineering.

1.3 Genetics of bacteriocins

With a few exceptions such as helveticin J [Joerger and Klaenhammer, 1986; 1990], and possibly plantaricins S and T [Jiménez-Díaz *et al.*, 1993], most bacteriocins of LAB are plasmid encoded [Ray *et al.*, 1989; Mortvedt and Nes, 1990; Muriana and Klaenhammer, 1991(b); Hastings and Stiles, 1991; Marugg *et al.*, 1992]. Elucidation of the genetic structure of bacteriocins is a rapidly developing field, with the cloning and analysis of several bacteriocin genes adding to the existing pool of knowledge about this field of research.

Recent advances in the field of bacteriocin genetics are summarised and discussed in this review, with a specific focus on the bacteriocins produced by the *Leuconostoc* species.

1.3.1 *Lactococcus* ssp.

The numerous bacteriocins produced by the lactococci are listed in Table 1. The genetic determinants of nisin and the lactococcins have been identified and extensively characterised.

Nisin

The discovery of nisin was first reported in 1944 [Mattick and Hirsch, 1944]. Since then, nisin has been widely studied as the model of the lantibiotics. It is a class I bacteriocin of molecular weight 3.352 kDa and contains five thioether cross-bridges and three dehydrated residues formed from serine or threonine [Liu and Hansen, 1990]. The genetic determinants for nisin production have been linked to both the chromosome [Buchman *et al.*, 1988;

Table 1. Characteristics of some bacteriocins produced by *Lactococcus* spp.

Bacteriocin	Producer	Genetic locus	Molecular weight (kDa)	Characteristics	Reference
Nisin	Some *L. lactis* subsp. *lactis* spp.	chromosomal or plasmid 70 kb transposon	3.488	lantibiotic, 34 amino acids	1, 2, 5, 7, 10, 11, 14
Bacteriocin S50	*L. lactis* subsp. *diacetilactis* S50	ND*	ND	ND	8
Lactococcin B	*L. lactis* subsp. *cremoris* 9B4	60 kb plasmid	5.3	47 amino acids, thiol-activated	18, 19
Lactococcin M	*L. lactis* subsp. *cremoris* 9B4	60 kb plasmid	ND	2 peptides	16, 17
Lactococcin A	*L. lactis* subsp. *cremoris* 9B4; LMG2130, *L. lactis* subsp. *lactis* biovar *diacetylactis* WM4	60 kb conjugative plasmid; 55 kb plasmid	5.778	54 amino acids	6, 9, 15, 16, 17
Lactococcin G	*L. lactis* LMG2081	ND	4.346 & 4.110	2 peptides; 39 &35 amino acids	12
Lactacin 481	*L. lactis* subsp. *lactis* CNRZ 481	ND	1.7	lantibiotic	13
Diplococcin	*L. lactis* subsp. *cremoris* 346	83.2 kb conjugative plasmid	5.3	ND	3, 4
Lactostrepcin 5	*L. lactis* subsp. *cremoris* 202	ND	ND	possible lipo-protein	20, 21

*ND = Not determined

References- page 7

References

1) Buchman *et al.*, 1988

2) Cheeseman and Berridge, 1957

3) Davey and Richardson, 1981

4) Davey, 1984

5) Dodd *et al.*, 1990

6) Holo *et al.*, 1992

7) Kaletta and Entian, 1989

8) Kojic *et al.*, 1991

9) Kok *et al.*, 1993

10) Liu and Hansen, 1990

11) Mattick and Hirsch, 1944

12) Nissen-Meyer *et al.*, 1992

13) Piard *et al.*, 1992

14) Steen *et al.*, 1991

15) Stoddard *et al.*, 1992

16) Van Belkum *et al.*, 1989

17) Van Belkum *et al.*, 1991

18) Van Belkum *et al.*, 1992

19) Venema *et al.*, 1993

20) Zajdel *et al.*, 1983

21) Zajdel *et al.*, 1985

Dodd *et al.*, 1990; Steen *et al.*, 1991] and to transconjugal plasmids of *Lactococcus lactis* [Kaletta and Entian, 1989]. The gene coding for the nisin pre-propeptide has been cloned, sequenced and analysed by several research groups [Buchman *et al.*, 1988; Kaletta and Entian, 1989; Dodd *et al.*, 1990; Steen *et al.*, 1991]. The nisin structural gene forms part of a polycistronic operon spanning 8.5 kb [Steen *et al.*, 1991]. This operon contains a putative promoter situated 4 kb upstream from the nisin structural gene. Other genes within the operon include a gene coding for a putative membrane- associated protein of molecular weight 100.5 kDa, a gene coding for a transposase-like protein and an insertion sequence (IS904). The Nip+ (nisin production) phenotype in *Lactococcus lactis* has been consistently linked to sucrose metabolism in these species [Gasson, 1984]. The identification of the IS904 element adjacent to the nisin/sucrose gene block suggests that it may play a role in mediating transfer of this gene block between different strains [Dodd *et al.*, 1990]. Further studies are needed to identify all the genes involved in nisin expression, especially since expression of nisin from cloned fragments has not yet been achieved. This suggests that additional genes, possibly required for processing, modification and transport of the bacteriocin, are needed for expression.

Lactococcin

Lactococcus lactis strains produce three different bacteriocins, designated lactococcin A, M and B. The genetic determinants for these bacteriocins are associated with a 60 kb conjugative plasmid in *Lactococcus lactis* subsp. *cremoris* 9B4 [Neve *et al.*, 1984] and *Lactococcus lactis* subsp. *lactis* Bu2-61 [Van Belkum *et al.*, 1989], with a 55 kb plasmid in *Lactococcus*

lactis subsp. *cremoris* LMG 2130 [Holo *et al.*, 1991] and with a 131 kb plasmid (pNP2) in *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* WM4 [Stoddard *et al.*, 1992]. The strains have different plasmid profiles, but analysis of the protein and DNA sequences of lactococcin A produced by *Lactococcus lactis* subsp. *cremoris* LMG 2130 [Holo *et al.*, 1991] showed that it was identical to the bacteriocin determinants from *Lactococcus lactis* subsp. *lactis* Bu2-61, previously cloned and sequenced by Van Belkum *et al.* [1991]. Although only plasmid p9B4 has been shown experimentally to contain the genetic determinants for all three bacteriocins, comparison of DNA sequences and restriction enzyme digest patterns of cloned fragments from the other plasmids suggests that all three plasmids may carry the genes necessary for lactococcin A, B and M expression [Kok *et al.*, 1993].

The genes necessary for expression of lactococcin A are arranged within an operon consisting of five open reading frames in the same orientation [Stoddard *et al.*, 1992]. The last two open reading frames code for the lactococcin A structural gene (*lcn* A) and the immunity gene (*lci* A), respectively. It was previously reported that *lcn* A and *lci* A are transcribed from a promoter directly upstream of *lcn* A [Van Belkum *et al.*, 1991]. The first open reading frame (ORFX) appeared to have no function. The other two open reading frames in the operon, designated *lcn* C and *lcn* D, are preceded by a putative promoter region that could potentially promote transcription of all four downstream genes, as no termination signals have been observed between *lcn* D and *lcn* A [Stoddard *et al.*, 1992]. Deletion of the promoter element upstream of *lcn* C leads to a Bac$^-$ Imm$^+$ phenotype, which suggests that, although *lcn* A and *lci* A are still expressed, the products of *lcn* C and *lcn* D are required for processing and secretion. The protein products of *lcn* C and *lcn* D are

homologous to other proteins implicated in signal sequence-independent secretion of certain proteins of Gram negative bacteria. It therefore appears that loss of the lcn D, and probably lcn C proteins, results in inhibition of the amino-terminal processing and secretion of lactococcin A into the external media. The result of this inhibition is a Bac- Imm+ phenotype. Holo *et al.* [1991] reported the presence of an inverted repeat sequence which overlaps the promoter region of the *lcn* A gene. It is possible that this palindromic structure represents a binding site for proteins regulating the transcription of *lcn* A. Similar inverted repeats which overlap the Pribnow boxes of colicin genes and act as SOS boxes and binding sites for the lex A repressor protein, have been identified in *E. coli* [Ebina *et al.*, 1982; Van den Elzen *et al.*, 1982; Ebina *et al.*, 1983]. There is no data available to confirm that a similar mechanism of regulation is used for lactococcin A expression.

Purification and sequencing of lactococcin A [Holo *et al.*, 1991] and the subsequent realisation that the nucleotide sequence of this bacteriocin is identical to that obtained by Van Belkum *et al.* [1991] facilitated the elucidation of the mechanism of lactococcin A processing. The protein product of lcn A is a 75 amino acid prepeptide that is cleaved to form the mature bacteriocin of 54 amino acids [Holo *et al.*, 1991]. The release of this 21 amino acid leader peptide appears to be a two step process and cleavage takes place at the carboxyl end of a glycine doublet. Comparisons of the nucleotide sequences of the structural genes for lactococcin A, B and M show similarities with regard to processing signals. The putative amino acid sequences of lactococcin B and M have a high similarity to lactococcin A in the amino-terminal region and all three bacteriocins have an amino-terminal extension of 21 amino acid residues ending with a glycine doublet [Kok *et al.*, 1993].

The operon for expression of lactococcin M, a class IIb type bacteriocin, in *Lactococcus lactis* subsp. *lactis* Bu2-61 contains three open reading frames [Van Belkum *et al.*, 1991; 1992]. Mutation and expression studies implicated the first two open reading frames (*lcn* M and *lcn* N) in bacteriocin production and the third open reading frame in immunity. The protein products of *lcn* M and *lcn* N probably form a heterodimer that is the active antagonistic substance, as disruption of either gene causes a Bac- phenotype.

The genetic determinants for the third bacteriocin of *Lactococcus lactis*, lactococcin B, were cloned and sequenced by Van Belkum *et al.* [1992]. Two genes were identified and these were shown to be transcribed within a single operon. A putative ribosomal binding site and possible rho-independent terminator flanks these two open reading frames. The *lcn* B gene, which codes for a protein of 68 amino acid residues appears to be involved in bacteriocin activity and *lci* B, specifying a protein of 91 amino acid residues, in immunity. Lactococcin B, a small, hydrophobic, positively charged bacteriocin was recently purified [Venema *et al.*, 1993]. The activity of this protein depends on the reduced state of a cysteine residue at position 24. The bacteriocin was shown to have a bactericidal effect on sensitive cells.

In summary, it appears that the genes which code for lactococcin A, B and M expression, respectively are all arranged within an operon and the upstream promoter elements appear to be identical [Kok *et al.*, 1993].

1.3.2 *Lactobacillus (Lb.)* spp.

The first bacteriocin purified from *Lactobacillus* spp. was isolated from *Lb. fermenti* [De Klerk and Smit, 1967]. Purification of this bacteriocin by

Table 2. A description of bacteriocins produced by *Lactobacillus* spp.

Bacteriocin	Producer	Genetic locus	Molecular weight (kDa)	Characteristics	Reference
Lactacin F	*Lb. acidophilus* 11088	chromosomal & episomal	6.3	57 amino acids	14, 15, 16
Helveticin J	*Lb. helveticus* 481	chromosomal	37.0	ND*	7, 8
Lactocin 27	*Lb. helveticus* LP27	chromosomal	12.4	glycolipo-protein	10, 22
Fermenticin	*Lb. fermenti* 466	ND	ND	glycolipo-protein	4
Sakacin A	*Lb. sake* Lb706	60 kb plasmid	4.309	41 amino acids	1,5
Lactocin S	*Lb. sake* L45	50 kb plasmid	<13.7	*ca* 33 amino acids	12, 13
Lactacin A	*Lb. delbrueckii* subsp. *lactis* JCM1106	ND	ND	ND	20
Lactacin B	*Lb. delbrueckii* subsp. *lactis* JCM 1248	ND	ND	ND	20
Brevicin 37	*Lb. brevis*	ND	ND	ND	18
Caseicin B80	*Lb. casei*	ND	40.0-42.0	ND	17
Bavaricin A	*Lb. bavaricus* M1401	ND	3.4-4.0	41 amino acids	11
Plantacin	*Lb. plantarum* NCDO1193	ND	ND	possible glycolipo-protein	24
Plantaricin S	*Lb. plantarum* LPCO10	chromosomal	*ca* 2.5	glycolipo-protein	6
Plantaricin T	*Lb. plantarum* LPCO10	chromosomal	ND	ND	6
Lactacin B	*Lb. acidophilus* N2	chromosomal	6.0-6.5	ND	2, 3
Helveticin V-1829	*Lb. helveticus* 1829	chromosomal	ND	ND	23
Gassericin A	*Lb. gasseri* 33 & 39	ND	ND	ND	21
LA-147	*Lb. acidophilus*	ND	38.5	ND	9

*ND = Not determined

References- page 13

References

1) Axelsson *et al.*, 1993

2) Barefoot and Klaenhammer, 1983

3) Barefoot and Klaenhammer, 1984

4) De Klerk and Smit, 1967

5) Holck *et al.*, 1992

6) Jiménez-Diaz *et al.*, 1993

7) Joerger and Klaenhammer, 1986

8) Joerger and Klaenhammer, 1990

9) Juven *et al.*, 1992

10) Klaenhammer, 1993

11) Larsen *et al.*, 1993

12) Mortvedt *et al.*, 1991

13) Mortvedt and Nes, 1990

14) Muriana and Klaenhammer, 1987

15) Muriana and Klaenhammer, 1991a

16) Muriana and Klaenhammer, 1991b

17) Rammelsberg *et al.*, 1990

18) Rammelsberg and Radler, 1990

19) Schillinger and Lücke, 1989

20) Toba *et al.*, 1991a

21) Toba *et al.*, 1991b

22) Upreti and Hinsdill, 1975

23) Vaughan *et al.*, 1992

24) West and Warner, 1988

dialysis, chromatography and calcium phosphate gel columns showed it to be a lipo-carbohydrate protein complex. The bacteriocin is heat-stable and sensitive to proteinases but not to lysozyme.

Various other species of *Lactobacillus* also produce bacteriocins. A summary of the characteristics of some of these bacteriocins is provided in Table 2. The bacteriocins of lactobacilli that have been well-characterised genetically include helveticin J [Joerger and Klaenhammer, 1986; 1990], lactacin F [Muriana and Klaenhammer, 1989; 1991] and sakacin A [Holck *et al.*, 1992; Axelsson *et al.*, 1993].

Helveticin J

Lb. helveticus 481 produces a 37 kDa protein with inhibitory activity toward closely related LAB [Joerger and Klaenhammer, 1986]. This bacteriocin, helveticin J, is inactivated by proteinases but not lipases, which suggests the absence of a lipid component. The large size of this protein and its heat sensitive nature, is typical of a class III type bacteriocin [Klaenhammer, 1993]. The genes required for helveticin J production are located on the chromosome [Joerger and Klaenhammer, 1986; 1990]. Screening of chromosomal DNA from *Lb. helveticus* bacteriocin producing and non-bacteriocin-producing strains with clones from a library of chromosomal DNA of *Lb. helveticus* 481 resulted in the identification of a cloned 600 bp fragment (HJ4) that hybridised exclusively to total genomic DNA of *Lb. helveticus* bacteriocin- producing strains. HJ4 was used as a probe to identify and isolate a hybrid plasmid (pTRK1135) that restored helveticin J production to strains of *Lb. acidophilus* but not to strains of *E. coli*. Sequencing of the insert of pTRK1135 revealed the presence of five open reading frames (ORF) [Joerger

and Klaenhammer, 1990; Klaenhammer 1993]. ORF3 (*hlvJ*) codes for a protein with a calculated molecular weight of 37.5 kDa, similar to the estimated weight of 37 kDa for helveticin J, as determined by SDS-PAGE. Upstream from ORF3 is a putative ribosomal binding site, as well as ORF2, located 30 bp upstream from the start of ORF3. The protein product of ORF2 is 11.808 kDa and its function is as yet unknown. Its location within the operon suggests an immunity function but the homology of the amino-terminal region to other signal peptides suggests that it may bind to the mature bacteriocin and facilitate its export from the cell. ORF2 and ORF3 are arranged within the operon with a promoter region 218 bp upstream from the start of ORF2 and a putative rho-independent terminator 37 bp downstream of ORF3. An *hlvJ* specific probe was used to identify a RNA transcript of 1600 nucleotides in the helveticin J producer [Klaenhammer, 1993], which is the expected size. The functions of the products of the other ORF's have not yet been reported.

Sakacin A

Sakacin A, a class IIb type bacteriocin, is produced by *Lb. sake* Lb706, and is active against closely related LAB and *Listeria monocytogenes* [Schillinger and Lücke, 1989]. Tests conducted in order to determine the inhibition of *Listeria monocytogenes* in minced meat and raw pork indicated that sakacin A is effective in reducing pathogen numbers by one log cycle [Schillinger and Lücke, 1991]. A protein consisting of a single peptide chain of 41 amino acid residues with a calculated molecular weight of 4.308 kDa is responsible for the observed inhibition [Holck *et al.*, 1992]. Cloning and sequencing of a 1.4 kb DNA fragment from the 60 kb native plasmid of *Lb.*

sake Lb706, enabled the identification of an ORF consisting of 177 bp [Holck *et al.*, 1992], which is the sakacin A structural gene *(sak A)*. This gene codes for a 59 amino acid residue prepeptide that is cleaved at the carboxyl end of residue 18 to yield the mature protein. Other fragments of the 60 kb plasmid from *Lb.sake* Lb706 have subsequently been cloned. These recombinant plasmids restore production of and immunity to sakacin A when transformed into *Lb. sake* Lb706-B, a mutant variant of *Lb. sake* Lb706, deficient in sakacin A production [Axelsson *et al.*, 1993]. Sequence analysis of these cloned fragments identified two ORF's: one coding for a protein of 45 amino acid residues of which the function is still unknown; and a large ORF (*sak B*) that codes for a protein of 430 amino acid residues, implicated in immunity to sakacin A. Mapping of the 60 kb plasmid of *Lb. sake* Lb706 showed that the *sak* A and *sak* B genes are located 1.6 kb apart.

The *sak B* ORF, located downstream of the smaller ORF, is preceded by a putative promoter region and a putative ribosomal binding site. The nucleotide sequence of this gene complements the sequence of a mutated *sak* B gene present in *Lb. sake* Lb706-B. An A-T point mutation in the mutated gene results in a shortened, inactive protein product with a concomitant loss of sakacin expression. Transformation of a plasmid-free variant, *Lb. sake* Lb706X with a recombinant plasmid containing the entire *sak* A-*sak* B region resulted in Sak- Imm- transformants, which implied that additional genes are required for expression of the bacteriocin. Although it appears that the genes required for sakacin A production are clustered in a similar manner to the operons for pediocin [Marugg *et al.*, 1992] and lactococcin [Van Belkum *et al.*, 1991], it appears that the bacteriocin structural gene (*sak* A) and the immunity gene (*sak* B) are transcribed in opposite directions [Axelsson *et al.*, 1993]. The