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

BY

JOSELINE V. FELIX

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Promoter: Prof. J. W. Hastings

Co-promoter: Dr. A. A. Smith

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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J. V. Felix

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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.

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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
Lacto-coccin B	*L. lactis* subsp. *cremoris* 9B4	60 kb plasmid	5.3	47 amino acids, thiol-activated	18, 19
Lacto-coccin M	*L. lactis* subsp. *cremoris* 9B4	60 kb plasmid	ND	2 peptides	16, 17
Lacto-coccin 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
Lacto-coccin 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
Diplo-coccin	*L. lactis* subsp. *cremoris* 346	83.2 kb conjugative plasmid	5.3	ND	3, 4
Lacto-strepcin 5	*L. lactis* subsp. *cremoris* 202	ND	ND	possible lipo-protein	20, 21

*ND = Not determined

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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 l*cn* 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

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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

sak B protein product is homologous to a protein in *Staphylococcus aureus* which regulates expression of several exoproteins. The authors [Axelsson *et al.*, 1993] postulate that a two component signal transducing regulatory system is responsible for sakacin A expression and immunity in *Lb. sake* 706. These components are sakB and another protein preliminarily identified by sequencing downstream of *sak B*.

Lactacin F

Lactacin F, produced by *Lb. acidophilus* 11088 (NCK 88), is a heat-resistant broad spectrum bacteriocin that exhibits antagonistic activity against closely related LAB and *Enterococcus faecalis* [Muriana and Klaenhammer, 1987]. The amino acid sequence of the first 25 residues in the amino-terminal region was identified using purified lactacin F [Muriana and Klaenhammer, 1991]. Although the molecular weight of purified lactacin F was estimated at 2.5 kDa as determined by SDS-PAGE, amino acid compositional analysis indicated that the bacteriocin may contain up to 56 amino acid residues (*ca.* 6.2 kDa). Lactacin F activity was previously associated with a 110 kb conjugal plasmid [Muriana and Klaenhammer, 1987]. Clones containing the putative *laf* structural gene from a lactacin F producing transconjugant, *Lb. acidophilus* T143, were identified using an oligonucleotide probe, the sequence of which was derived from the amino-terminal amino acid sequence [Muriana and Klaenhammer, 1991]. A 2.2 kb *Eco*RI fragment hybridized to the probe and was subcloned into an *E.coli /Lb.acidophilus* shuttle vector. This plasmid (pTRK162) was used to test for expression of lactacin F in two different *Lb. acidophilus* strains, 89 (Laf- Laf^s) and 88C (Laf- Laf^r). Expression of lactacin F occurred in both strains transformed with pTRK162, which implies that the

immunity gene is also present on the 2.2 kb insert. The levels of expression of lactacin F differed between the two recipient strains. The Laf- Laf^r (pTRK162) strains showed larger zones of inhibition and also larger, healthier colony formation than the Laf- Laf^s (pTRK162) strain. Repeated propagation of Laf- Laf^r (pTRK162) and Laf- Laf^s (pTRK162) colonies under conditions optimal for bacteriocin production resulted in the loss of culture viability of the Laf- Laf^s (pTRK162) but not the Laf- Laf^r (pTRK162) strain. The authors [Muriana and Klaenhammer, 1991] postulated that, although the 2.2 kb fragment appears to contain both structural and immunity genes, the reduced levels of expression in these hosts is probably due to a lack of expression signals upstream of the operon that were not present on the cloned fragment. It is possible that the increased level of lactacin F expression in Laf- Laf^r (pTRK162) is due to the inherent resistance of the recipient strain.

Genetic determinants of lactacin F

Sequence analysis of the entire 2.2 kb *Eco*RI fragment that encodes lactacin F production, revealed the presence of four open reading frames, *laf* A, *laf* X, ORFY and ORFZ [Muriana and Klaenhammer, 1991; Klaenhammer, 1993]. This polycistronic operon is flanked by a functional promoter upstream and a functional terminator downstream of the operon. The lactacin F structural gene was initially identified within the *laf* A open reading frame using a derived sequence obtained from the amino acid sequence of the amino-terminal region of mature lactacin F [Muriana and Klaenhammer, 1991]. Lactacin F is produced as a 75 amino acid prepeptide that is cleaved at the carboxyl end of a glycine doublet to release a 57 amino acid mature bacteriocin and an 18 amino acid residue leader peptide. The latter possibly functions as a signal peptide.

Disruption of the lactacin F operon by means of site-directed mutagenesis, deletions and alterations, showed that disruption of either *laf* A or *laf* X but not ORFY results in loss of lactacin F production in Laf- Laf^s (pTRK162) hosts. It has been reported recently that complementation of *laf* A and *laf* X genetic determinants, in trans, results in recovery of lactacin F production [Klaenhammer, 1993]. Furthermore, complementation of the gene products of a *laf* X-containing clone and a *laf* A-containing clone on an agar plate resulted in inhibitory activity against *Lb. delbrueckii* ssp. *lactis* 4797. The gene product of *laf* A alone showed antagonistic activity, but against a narrower spectrum of organisms than demonstrated by the Laf A/Laf X complement.

Laf X codes for a protein of 62 amino acid residues, which exhibits hydropathy characteristics similar to lactacin F [Klaenhammer, 1993]. A putative Gly-Gly processing motif, located 14 residues from the N-terminus suggests that Laf X is processed in a similar manner to other class IIb bacteriocins. Predicted structure and hydropathy profiles of Laf X suggest that it may exert antagonistic activity, but this has not yet been demonstrated.

In summary, lactacin F is a class IIb bacteriocin because its activity is due to the interaction of two peptides, laf A and laf X, and it induces membrane permeability in *Enterococcus faecalis* .

Although the 128 amino acid residue protein product of ORFZ has been implicated in immunity, it appears not to be necessary for lactacin F expression in Laf- Laf^s hosts. The possibility of the expression host having either a functional ORFZ or an alternative gene product that substitutes for the ORFZ gene product, cannot be ruled out. The hydropathy profiles of the predicted protein of ORFZ predict a membrane integrated protein with four trans-membrane segments in its structure. It was suggested that such a membrane-

associated protein may either facilitate export of the mature bacteriocin or provide immunity to a membrane-active bacteriocin [Klaenhammer, 1993].

1.3.3. *Pediococcus* ssp.

Pediococci are widely used for meat and vegetable fermentations [Pederson, 1949; Nettles and Barefoot, 1993]. Various species of bacteriocin-producing pediococci have been reported and are described in Table 3. Characterisation of the genes of pediococci bacteriocins have concentrated mainly on the bacteriocins produced by various strains of *Pediococcus acidilactici*. A comparison of the sequence analysis of the gene coding for pediocin AcH (*pap*) [Motlagh *et al.*, 1992] and the genes responsible for pediocin PA-1 production (*ped* genes) [Marugg *et al.*, 1992] showed that an identical bacteriocin had been characterised in both these studies [Klaenhammer, 1993]. The *Pediococcus acidilactici* strains that produce these bacteriocins were originally isolated from fermented sausage and commercial starter cultures [Bhunia *et al.*, 1987; Gonzalez and Kunka, 1987].

Pediocin PA-1/AcH

Gonzalez and Kunka [1987] associated pediocin PA-1 production by *Pediococcus acidilactici* with a 9.4 kb plasmid (pSRQ11) present within the organism. Pediocin PA-1 exhibits antagonistic activity against closely related LAB. The bacteriocin was purified to homogeneity and the amino acid sequence determined in two separate studies [Henderson *et al.*,1992; Nieto Lozano *et al.*, 1992]. Analysis of the amino acid sequencing data indicated that

Table 3. A description of bacteriocins produced by *Pediococcus* spp.

Bacteriocin	Producer	Genetic locus	Molecular weight (kDa)	Characteristics	Reference
Pediocin PA-1	*P. acidilactici* PA 1.0	9.4 kb plasmid	4.6	44 amino acids	5, 7, 8
Pediocin AcH	*P. acidilactici* H	11.4 kb plasmid	2.7	44 amino acids	1, 2, 9
Pediocin A	*P. cerevisiae* FBB-61	20.94 kb plasmid	ND*	ND	3, 4
Unnamed	*P. pentosaceus* N4p, N5p	ND	ND	ND	11
Unnamed	*P. cerevisiae* FBB-63	16.2 kb plasmid	ND	ND	6
Pediocin SJ-1	*P. acidilactici* SJ-1	7.1 kb plasmid	*ca* 4.0	glyco-protein	10

*ND = Not determined

References

1) Bhunia *et al.*, 1987
2) Bhunia *et al.*, 1988
3) Daeschel and Klaenhammer, 1985
4) Fleming *et al.*, 1975
5) Gonzalez and Kunka, 1987
6) Graham and McKay, 1985
7) Marugg *et al.*, 1992
8) Nieto-Lozano *et al.*, 1992
9) Ray *et al.*, 1989
10) Schved *et al.*, 1993
11) Strasser de Saad *et al.*, 1993

the protein consists of 44 amino acid residues with a calculated molecular weight of 4.629 kDa. The mature bacteriocin contains two disulphide bridges and no lanthionine residues [Henderson *et al.*, 1992; Marugg *et al.*, 1992]. Although pediocin PA-1 and pediocin AcH are reputed to be the same bacteriocin, certain differences between their respective activity spectra and genetic location have been reported. Bhunia *et al.* [1988] reported a wider spectrum of activity for pediocin AcH than that reported for pediocin PA-1 [Henderson *et al.*, 1992]. This includes inhibition of growth of *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens* and *Pseudomonas putida*. Gonzalez and Kunka [1987] reported that loss of plasmid pSQR11 resulted in a Bac- Imm+ phenotype in *Pediococcus acidilactici* PA 1.0, which suggests that immunity is chromosomally determined. This is contrary to the findings of Ray *et al.* [1989], that the genes for both production of and immunity to pediocin AcH are linked to a 7.4 MDa conjugal plasmid (pSMB74).

A recent study by Marugg *et al.* [1992] represents the first successful cloning and expression of pediocin in *E. coli*. Sequence and mutational analysis revealed that the four genes responsible for pediocin production, designated *ped* A, *ped* B, *ped* C, *ped* D, are arranged within an operon [Fig. 1]. A single putative promoter, situated upstream of *ped* A appears to be responsible for transcription of the complete operon. The bacteriocin structural gene (*ped* A) was identified by comparison of the gene derived sequence to the amino acid sequence of the purified protein [Henderson *et al.*, 1992]. These results were confirmed by mutational studies, in which a mutation in *ped* A resulted in loss of pediocin production in *E. coli* [Marugg *et al.*, 1992].

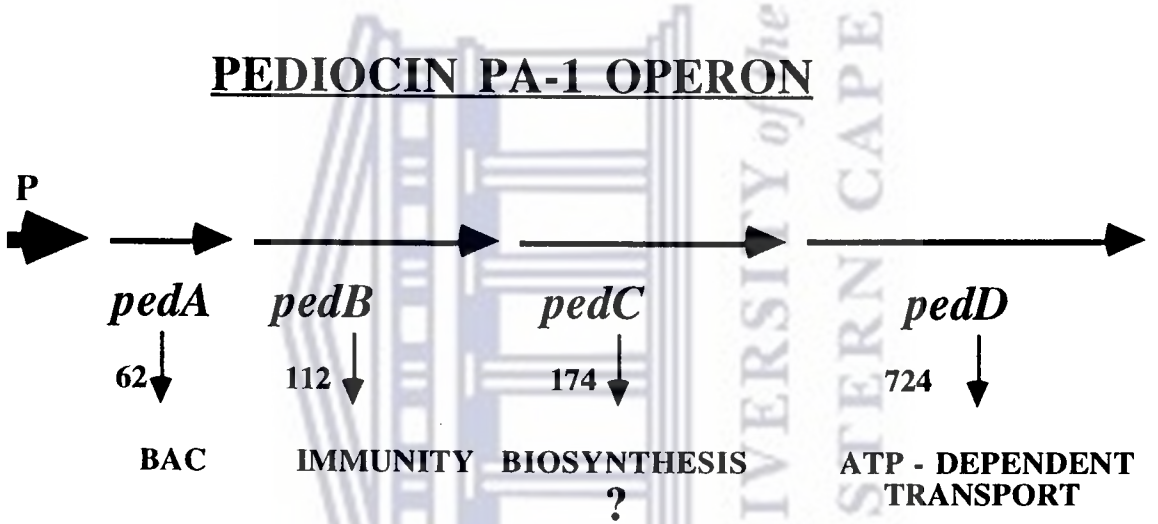

Fig. 1. A schematic overview of the pediocin PA-1 operon indicating the position of the genes within the operon [Marugg*et al.*, 1992]. P indicates a promoter region.

The bacteriocin is produced as a 62 amino acid prepeptide that is cleaved at the carboxyl end of a glycine doublet (residue 18) to yield a 44 amino acid mature bacteriocin. These data are similar to that reported for the cloning and sequencing of the *pap* gene from *Pediococcus acidilactici* H [Motlagh *et al.*, 1992].

The protein product of *ped* B appears to have no function in pediocin expression in *E. coli* [Marugg *et al.*, 1992]. The protein has been implicated in immunity on the basis of a similar arrangement in other well-characterised bacteriocins [Van Belkum *et al.*, 1991]. The exact role of *ped* B in pediocin production needs to be investigated further in pediocin sensitive hosts, as *E. coli* is insensitive to pediocin PA-1. It also appears that immunity to pediocin may be chromosomally determined, as loss of the bacteriocin-producing plasmid does not result in sensitivity to this bacteriocin [Gonzalez and Kunka, 1987]. The function of *ped* C is currently under investigation, but a possible biosynthesis function has been suggested, on the basis of homology between the deduced protein of *ped* C and *spa* B, a protein implicated in subtilin biosynthesis [Klein *et al.*, 1992]. Mutational analysis of *ped* D, a gene coding for a polypeptide of 724 amino acid residues, revealed that this protein is essential for pediocin production [Marugg *et al.*, 1992]. The predicted ped D protein shows strong homology to a family of ATP- dependent transport proteins. The authors [Marugg *et al.*, 1992] suggest that ped D is essential for secretion rather than synthesis of pediocin, because a single mutation in *ped* D results in loss of pediocin production.

1.3.4. *Carnobacterium* spp.

The first report of bacteriocins produced by *Carnobacterium* spp. was published in 1990 [Schillinger and Holzapfel, 1990]. *Carnobacterium* is a new genus proposed by Collins *et al.* [1987] to accommodate strains of *Lactobacillus* that differ with regard to certain growth requirements. Strains of all four species included in this genus produce bacteriocins [Schillinger and Holzapfel, 1990] and some of these are described in Table 4. These proteinaceous compounds are heat-stable and exhibit a bactericidal mode of action primarily against other carnobacteria.

Carnobacteriocins

Carnobacterium piscicola LV17 produces bacteriocins that are active against other LAB as well as *Enterococcus faecalis* and *Listeria monocytogenes* [Ahn and Stiles, 1990(b)]. The antagonistic substance was shown to be heat-stable, unaffected by non-proteolytic enzymes and active between pH 2 and 11. This is unusual as most bacteriocins of LAB are active at low pH only in the purified form [Barefoot and Klaenhammer, 1984; Liu and Hansen, 1990; Hastings *et al.*, 1991]. Bacteriocin activity was related to 40 MDa and 49 MDa plasmids [Ahn and Stiles, 1990(b)]. Mutants containing only one of these plasmids become sensitive to the wild-type strain, but acquisition of either the 40 MDa or the 49 MDa plasmid results in partial bacteriocin activity. A strain with bacteriocin activity equivalent to the wild-type, but with a different plasmid profile was also identified. Complete bacteriocin activity was restored in transconjugants containing either all three plasmids present in *Carnobacterium piscicola* LV17 (pCP9, pCP40 and

Table 4. A description of bacteriocins produced by *Carnobacterium* spp.

Bacteriocin	Producer	Genetic locus	Molecular weight (kDa)	Characteristics	Reference
Carnocin U149	*Carnobacterium* spp.U149	ND*	4.635	possible lantibiotic, *ca* 37 amino acids	5
Unnamed	*Carnobacterium piscicola* LV61	ND	ND	ND	4
Carnobacteriocin B1 and B2	*Carnobacterium piscicola* LV17B	40 MDa plasmid	4.541, 4.969	38 amino acids, 34 amino acids	1, 2, 3
Carnobacteriocin A1, A2, and A3	*Carnobacterium piscicola* LV17A	49 MDa plasmid	*ca* 5.0, 5.123, 5.127	ND	1, 2, 6

* ND = Not determined

References

1) Ahn and Stiles, 1990b
2) Ahn and Stiles, 1992
3) Quadri *et al.*, 1992
4) Schillinger and Holzapfel, 1990
5) Stoffels *et al.*, 1992
6) Worobo *et al.*, 1992

pCP49) or an 88 MDa plasmid derived from the 40 MDa and 49 MDa plasmids [Ahn and Stiles, 1992].

Carnobacterium piscicola LV17A is a mutant which contains only the 49 MDa plasmid [Worobo *et al.*, 1992]. Three bacteriocins were produced, purified and designated carnobacteriocin A1, A2 and A3 respectively. The three proteins have similar N-terminal amino acid sequences. A probe derived from the amino acid sequences was used to identify and clone the genes responsible for carnobacteriocin production in *Carnobacterium piscicola* LV17A. The nucleotide sequence of the genes was determined and the gene structure elucidated.

Carnobacteriocins B1 and B2 are produced by *Carnobacterium piscicola* LV17B, a mutant strain of *Carnobacterium piscicola* LV17, which contains only the 40 MDa plasmid [Quadri *et al.*, 1992]. The bacteriocins produced were purified and the amino acid sequences determined. The structure of these bacteriocins show considerable homology to leucocin A-UAL187 [Hastings *et al.*, 1991], as a probe derived from the leucocin A-UAL187 amino-terminal sequence hybridised to the genes responsible for carnobacteriocin B1 and B2 production. Cloning and sequence analysis of the genes which code for carnobacteriocin B1 and B2 reveal that the two genes are very similar to each other [Quadri *et al.*, 1992], and also to leucocin A [Hastings *et al.*, 1991].

Carnocin U149

Carnocin U149 is produced by a *Carnobacterium* spp. isolated from fish [Stoffels *et al.*, 1992]. The bacteriocin, purified to homogeneity by cation exchange-, hydrophobic interaction- and reverse phase chromatography, is

heat-stable and active over a wide pH range. The bacteriocin is similar to leucocin A-UAL187 [Hastings *et al.*, 1991] in that the purified bacteriocin is very unstable and rapidly loses activity. The molecular weight of carnocin U149 as determined by mass spectroscopy is 4.635 kDa [Stoffels *et al.*, 1992]. The sequence of the first 7 amino acid residues of purified carnocin U149 was determined, but thereafter Edman degradation was blocked. The detection of cysteic acid residues after oxidation with formic acid suggests that this bacteriocin is a class 1 lantibiotic [Klaenhammer, 1993].

1.3.5 *Leuconostoc* spp.

The first description of *Leuconostoc* spp. producing substances inhibiting growth of other leuconostocs and *Lactococcus lactis* was reported by Orberg and Sandine [1984]. These inhibitory substances were not characterised any further and it is uncertain whether or not they were bacteriocins. Harding and Shaw [1990] reported that the newly classified *Leuconostoc gelidum* showed inhibition toward a range of lactobacilli and leuconostocs as well as against the food-borne pathogen *Listeria monocytogenes*. No inhibitory activity was detected against other spoilage organisms or pathogenic bacteria tested. The inhibitor was shown to be of proteinaceous nature and heat stable for up to 1 hour at 100^{o}C. A molecular weight of greater than 10 kDa was estimated after dialysis, and the action of the bacteriocin appears to be bactericidal.

Recent studies have identified more bacteriocin-producing leuconostocs and a description of these are presented in Table 5. These organisms have been isolated from a diverse group of foodstuffs ranging from vacuum-packaged processed meat to goats' milk [Hastings and Stiles, 1991; Daba *et al.*, 1991

Lewus *et al.*, 1992; Héchard *et al.*, 1992; Mathieu et al., 1993]. The best-characterised bacteriocins of leuconostocs with regard to genetic determinants and protein structure are leucocin A-UAL187 from *Lc. gelidum* UAL187 [Hastings and Stiles, 1991; Hastings *et al.*, 1991] and mesentericin Y105 from *Lc. mesenteroides* ssp. *mesenteroides* [Héchard *et al.*, 1992; Cenatiempo, pers. comm.[1]].

Mesentericin Y105

Mesentericin Y105, produced by *Lc. mesenteroides* ssp. *mesenteroides*, is a class IIa type bacteriocin with activity only against various strains of *Listeria monocytogenes* [Héchard *et al.*, 1992; Klaenhammer, 1993]. The protein is stable at low pH and very high temperature - up to 100°C for 120 min at pH 4.5, and activity is retained after long-term storage at -20°C. The molecular weight of the protein was estimated to be between 2.5 and 3.0 kDa, after SDS-PAGE, whereas the deduced molecular weight based on the amino acid sequence of the purified protein, is 3.666 kDa. The primary structure of mesentericin Y105, as determined by microsequencing of the purified protein, is very similar to that of leucocin A-UAL187 [Hastings *et al.*, 1991]. The only difference is that Ala22 and Ile26 are replaced by phenylalanine and valine respectively, in the leucocin A -UAL187 protein. Although protein sequencing showed that the bacteriocin consists of 36 amino acid residues, sequencing of the cloned gene revealed the presence of a tryptophan residue at position 37 [Cenatiempo, pers. comm.[1]]. The amino-terminal extension of mesentericin Y105 differs from that of leucocin A-UAL187 at seven positions.

[1]Footnote: Yves Cenatiempo, Institut de Biologie Moleculaire Genetique,URA CNRS 1172, Universite de Poitiers, 40 Avenue du Recteur, 86022 Poitiers Cedex, France.

Table 5. A description of bacteriocins produced by *Leuconostoc* spp.

Bacteriocin	Producer	Genetic locus	Molecular weight (kDa)	Characteristics	Reference
Leucocin A	*Lc. gelidum* 187-22	7.6 MDa plasmid	3.930	37 amino acids	4, 5
Leuconocin S	*Lc. paramesen-teroides* OX	ND*	*ca*10.0 & *ca* 2.0	glycoprotein	7
Mesenterocin 5[a]	*Lc. mesenteroides* UL5	ND	4.5	ND	2
Mesentericin Y105	*Lc mesenteroides* Y105	ND	3.666	36 amino acids	1, 6
Unnamed	*Lc. gelidum* IN139	ND	>10.0	ND	3
Carnosin	*Lc. carnosum* La44a	ND	2.5-6.0	ND	9
Mesenterocin 52	*Lc. mesenteroides* ssp. *mesenteroides* FR52	ND	< 10.0	ND	8

*ND = Not determined

[a] Recently reclassified as pediocin

References

1) Cenatiempo, pers. comm.[1]
2) Daba *et al.*, 1991
3) Harding and Shaw, 1990
4) Hastings *et al.*, 1991
5) Hastings and Stiles, 1991
6) Hechard *et al.*, 1992
7) Lewus *et al.*, 1992
8) Mathieu *et al.*, 1993
9) Van Laack *et al.*, 1992

[1]Footnote: Yves Cenatiempo, Institut de Biologie Moleculaire Genetique,URA CNRS 1172, Universite de Poitiers, 40 Avenue du Recteur, 86022 Poitiers Cedex, France.

Leucocin A-UAL187

Leucocin A-UAL187, produced by *Lc. gelidum* UAL187, is a class IIa type bacteriocin. A protein consisting of 37 amino acid residues with a molecular weight of 3.930 kDa, as determined by mass spectroscopy, is responsible for antagonistic activity [Hastings *et al.*, 1991]. The crude bacteriocin is active after boiling for 20 min and also at very low pH [Hastings and Stiles, 1991]. The purified bacteriocin was shown to be unstable at all pH values and temperatures tested and was only stabilised after addition of bovine serum albumin (BSA) [Hastings *et al.*, 1991].

The genes required for leucocin A-UAL187 production are present on a 7.6 MDa (11.7 kb) plasmid (pLG7.6), one of three native plasmids present in *Lc. gelidum* UAL187 [Hastings and Stiles, 1991]. Loss of pLG7.6 results in loss of bacteriocin production and immunity. The protein was purified to homogeneity by ammonium sulphate precipitation and chromatographic procedures (hydrophobic interaction, gel permeation and RP-HPLC) and the sequence of the first 13 amino acid residues determined by Edman degradation [Hastings *et al.*, 1991]. A mixed oligonucleotide probe, designed from the amino-terminal region of the protein, was used to identify the structural gene, which was present on a 2.9 kb *Hpa*II fragment of pLG7.6 cloned into pUC118. Sequence analysis of the recombinant plasmid (pJH6.1) showed two contiguous open reading frames (ORF) flanked by a promoter region and ribosomal binding site upstream of the first ORF and a putative rho- independent terminator situated downstream of the last ORF. This finding is similar to the conserved pattern of gene arrangement found in other bacteriocins of LAB such as lactococcin M and A [Van Belkum *et al.*, 1989, 1991] and pediocin PA-1 [Marugg *et al.*, 1992], where the genes required for production are all confined to a single operon. This operon consists of a bacteriocin structural gene, a possible immunity gene, a possible processing/biosynthesis gene and a gene coding for an ATP-dependent transport protein. Only two of these components

were identified within the leucocin A-UAL187 operon. The first ORF codes for the prepeptide of mature leucocin, a protein of 61 amino acid residues. The purified protein lacks the first 24 amino acids present in the amino-terminal region of the prepeptide. Although the cleaved 24 amino acid peptide is unlike conventional signal peptides, as it lacks the hydrophobic core, the authors proposed a similar function for this peptide. Cleavage of the prepeptide occurs at the carboxyl end of the glycine doublet, at the typical val-val-gly-gly processing site. The second ORF codes for a protein of 113 amino acid residues. The function of this gene has not been determined experimentally, but it is suggested to be an immunity protein, on the basis of a similar gene arrangement in other bacteriocin producers [Van Belkum *et al.*, 1991; Marugg *et al.*, 1992; Axelsson *et al.*, 1993]. Expression studies, conducted in order to test for production of leucocin A-UAL187 in several hosts such as *Escherichia coli*, *Lactococcus* and *Carnobacterium* transformed with pJH6.1 were unsuccessful. This suggests that additional information not present on the cloned fragment may be needed for expression of this protein.

Mesenterocin 5

This bacteriocin, produced by *Lc. mesenteroides* UL5, is active against *Listeria monocytogenes* but not active against several lactic acid bacteria used in food fermentations [Daba *et al.*, 1991]. The molecular weight of this bacteriocin was estimated to be 4.5 kDa, after direct detection in SDS-PAGE gels. Recent characterisation of the genetic determinants of mesenterocin 5 showed that the bacteriocin is identical to pediocin PA-1 and that the producer strain is a *Pediococcus* [Klaenhammer, 1993].

Leuconocin S

Leuconocin S, produced by *Lc. paramesenteroides*, is only heat-stable up to 60°C for 30 min [Lewus *et al.*, 1992]. Bacteriocin activity is lost after treatment with proteinases or α-amylase, which implies that both a protein and a carbohydrate moiety are essential for inhibitory activity. This bacteriocin exhibits a broad inhibition spectrum, which includes inhibitory activity against *Clostridium botulinum* and *Staphylococcus aureus*. Leuconocin S is hence a class IV type bacteriocin and is the first bacteriocin in this class to be isolated from *Leuconostoc* species [Klaenhammer, 1993].

Although various bacteriocins produced by the *Leuconostoc* spp. have been identified and the genetic determinants of certain of these bacteriocins cloned, expression of a bacteriocin from *Leuconostoc* in a heterologous host has not been reported thus far. It, therefore, seems likely that additional, as yet unidentified, genes are required for expression of these bacteriocins. Further analysis of bacteriocin-producing species within this genus will possibly reveal characteristics of the genetic determinants required for expression of a *Leuconostoc* bacteriocin in a heterologous host.

Recently, a strain of *Lc. carnosum* was isolated which produces a bacteriocin [Papathanasopoulos, 1993]. *Lc. carnosum* Ta11a was isolated from spoiled South-African vacuum-packaged vienna sausages. The bacteriocin is produced during the exponential growth phase and exhibits a bacteriolytic mode of action when tested against *Lc.mesenteroides* TA10c. The crude bacteriocin is stable at 100°C for up to 30 minutes in the pH range between 2 and 9.

The genetic determinants required for production of leucocin B Ta11a, the bacteriocin produced by *Leuconostoc carnosum* Ta11a were identified and analysed in this study. The approach adopted included cloning of plasmid DNA from *Leuconostoc carnosum* Ta11a followed by sequencing and analysis of the isolated clones.

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture maintenance

A description of all strains and plasmids, except the indicator organisms used in this study, is given in Table 6. For storage purposes, all cultures of lactic acid bacteria were grown for 24 hours in a modified tryptone soy broth (20g casein protein, 3.0g soy meal peptone, 2.5g glucose, 2.5g di-potassium hydrogen phosphate, 2.8g beef extract, 60g glycerol, 5.0g sodium chloride per 1000ml) at 25°C and thereafter stored in aliquots at -70°C. All other cultures were grown for 24 hours in Nutrient broth (Biolab, Midrand) and thereafter stored in aliquots containing 15% glycerol at -20°C. Working cultures of LAB, *E. coli* and all other organisms were maintained on MRS (Biolab, Midrand) plates, Luria-Bertani (LB) [Sambrook *et al.*, 1989] agar plates and Nutrient agar (Biolab, Midrand) plates, respectively, with ampicillin (Boehringer Mannheim, GmbH, Mannheim, Germany) added to a final concentration of 50 µg/ ml when selecting for the presence of the pUC plasmids. Working cultures were stored at 4°C and subcultured fortnightly. All chemicals used were of an analytical grade. Glassware was autoclaved before use and distilled water used for all solutions.

2.2 Growth conditions

Cultures of LAB were grown in MRS broth (Biolab, Midrand), at 25°C. All other non-lactic acid bacteria indicator organisms were cultured in Nutrient broth (Biolab, Midrand) at 37°C, with the exception of *Micrococcus* M2 that was grown at 25°C. *E. coli* was cultured in Luria-Bertani broth [Sambrook *et al.*, 1989], pH 7.0, at 37°C with shaking and ampicillin was added to a final concen-

Table 6. Bacterial strains and plasmids used in this study

	Characteristics	Reference
Bacterial strains		
Escherichia coli JM103	*thr, rps* L, *end A, sbc15, hsdR4,* Δ(*lac pro* A, B), F´ *tra* D36 *pro* AB, *lac*IZ ΔM15	3
Leuconostoc carnosum Ta11a	LcnB$^+$Imm$^+$ containing native 8.9 MDa plasmid	2
Plasmids		
pUC 118	lac_z´, *Amp*^{r}, 3.2 kb	4
pJH6.1	*Amp*^{r}, *lcn*A, 6.1 kb	1
pJF8.1	*Amp*^{r}, *lcn*B, 8.1 kb	This study
pJF5.5	*Amp*^{r}, *lcn*B, 5.5 kb	This study

References

1) Hastings *et al.*, 1991

2) Papathanasopoulos, 1993

3) Sambrook *et al.*, 1989

4) Viera and Messing, 1987

tration of 50 µg/ml when selecting for the presence of the pUC plasmids. Media were solidified by the addition of Agar (Biolab, Midrand) to a final concentration of 1.5% unless otherwise stated. All cultures were subcultured at least twice before use.

2.3 Spectrum of activity

Tests to determine the spectrum of activity were performed using the deferred method of detection [Barefoot and Klaenhammer, 1983]. A 2 µl aliquot of an overnight culture of the producer organism was spotted onto dry MRS plates and incubated for 24 hours. These plates were then overlaid with 8ml MRS soft agar (MRS, containing 0.8% agar) containing a 1% inoculum from a 24 hour culture of the appropriate indicator organism. Plates were incubated for 24 hours at the optimum growth temperature of the indicator organism and examined for clear zones of growth inhibition.

2.4 DNA isolation

Plasmid DNA was isolated from *Lc. carnosum* Ta11a using a modification of the method of Anderson and McKay [1983]. After the addition of lysozyme, the mixture was incubated for one hour. DNA was treated with RNAse (Boehringer Mannheim, GmbH, Mannheim, Germany) and Pronase E each added to a final concentration of 1mg/ml, before purification with the Geneclean II Bio101 kit (La Jolla, CA) as per manufacturer's instructions. Isolation of plasmid DNA from *E. coli* was done by the alkaline lysis method using the Qiagen plasmid purification kits (Diagen, GmbH, FRG) for large-scale preparations and the method of Sambrook *et al.* [1989] for mini preparations of plasmid DNA. The DNA samples were electrophoresed on horizontal agarose gels (0.8% agarose) in 0.5 x

TAE (pH 8.0), stained with ethidium bromide at a final concentration of 0.5 μg/ml and the DNA viewed under UV light.

2.5 Isolation of DNA fragments from agarose gels

Fragments of DNA were separated on a 1% low melting temperature horizontal agarose gel (Seaplaque FMC Bioproducts, Rockland, USA) according to standard procedures [Sambrook *et al.*, 1989]. The gel was stained with ethidium bromide as described previously and the DNA viewed under UV light. The DNA fragment of interest was excised and the weight determined. An appropriate volume of 25 x agarase buffer was added and the mixture heated at 65°C until the agarose had melted. After cooling to 45°C, Agarase (Boehringer Mannheim, GmbH, Mannheim, Germany) was added at a concentration of 1U per 100mg of agarose and the mixture incubated at 45°C for 1 hour. The DNA was used for further manipulation without precipitation.

2.6 Southern blotting

The procedures for Southern blotting of DNA from agarose gels were as described by Sambrook *et al.* [1989]. The DNA was denatured and neutralised within the gel and thereafter transferred to Nytran filters (Schleicher & Schuell, GmbH, FRG). Filters were air-dried and baked at 80°C for 2 hours. Hybridisation of filters was performed as described in section 2.8.

2.7 Colony hybridisations

Cultures of *E. coli* were spotted onto Nytran filters with a sterile toothpick and incubated at 37°C for approximately six hours or until colonies were visible on

the filter. Filters were treated as described by Sambrook *et al.* [1989] to lyse the colonies and baked for 2 hours at 80^{o}C to fix the DNA. Hybridisation of filters was performed as described in section 2.8.

2.8 Hybridisation of filters

A 36-mer oligonucleotide probe (JF-1) (5´-CGTACTTGAGGCAATGGTATTATGAAGCAGCTGTAT -3´), designed to be homologous to the the nucleotide sequence coding for the amino-terminal region of leucocin A-UAL187, was used in all filter hybridisation experiments. The probe (JF-1) was synthesized at the Synthetic DNA Laboratory, Department of Biochemistry, University of Cape Town, Cape Town, South Africa, using an AutoGen 6500 DNA synthesizer. The probe was labelled with digoxigenin-11-dUTP using a digoxigenin (DIG) nucleic acid labelling kit (Boehringer Mannheim, GmbH, Mannheim, Germany). Filters were prehybridised in prehybridisation solution (6 X SSC; 10mM EDTA; 5 x Denhardts solution; 0.5% SDS) for at least 1 hour at 37^{o}C before labelled probe was added. All hybridisations were performed at 37^{o}C for 18 hours or more in a Techne Hybridiser (Techne Limited, Cambridge, UK). The filters were washed twice with solution A (2x SSC, 0.5% SDS) for 30 minutes at 37^{o}C with shaking, followed by two washes in solution B (0.1x SSC, 0.1% SDS) for thirty minutes at 37^{o}C with shaking. Positive signals were visualised using a DIG nucleic acid detection kit (Boehringer Mannheim, GmbH, Mannheim, Germany), according to the supplier's recommendations.

2.9 Cloning and restriction mapping.

Restriction endonucleases, calf intestinal phosphatase and T4 DNA ligase (Boehringer Mannheim, GmbH, Mannheim, Germany) were used according to the manufacturer's instructions. A molar ratio of 1:5 of vector : insert was routinely used in all ligation experiments.

Transformation of plasmids into *E. coli* JM103 were performed by electroporation using a Biorad Gene Pulser (Biorad, California). Cells for electroporation were prepared from a log-phase culture of *E. coli* JM103 (OD_{260} of 0.5- 0.8). The cells were centrifuged for 10 minutes at 4000xg at 4°C. The supernatant was discarded and cells were washed in sterile ice-cold water and concentrated by centrifugation at 4000xg for 10 minutes at 4°C. Electroporation-competent cells were resuspended in 1/50 of the original volume in sterile ice-cold 10% glycerol. Aliquots of cells (40µl) were incubated with approximately 1 ug of DNA for 60 seconds on ice. Electroporation was performed in a 0.2 cm cuvette at 25 µF, 2.5 kV and 200Ω. Pulsed cells were immediately diluted with 1ml LB-broth and incubated at 37°C for 60 minutes. Cultures were spread onto Luria-Bertani agar plates containing 50µg/ml ampicillin. 50µl of a 2% solution of 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) and 10µl of a 100mM solution of isopropyl-ß-D-thiogalactopyranoside (IPTG) were added for every 200µl of culture plated and positive clones were identified by α-complementation.

Positive transformants (white colonies) were further screened by either colony hybridisation or Southern transfer and hybridisations as described previously.

2.10 Nucleotide sequencing of the leucocin B-Ta11a gene.

Double-stranded DNA was sequenced in both directions by the Sanger dideoxy chain termination method using the Sequenase 2.0 kit (United States Biochemical, Ohio, USA) and ^{32}P-dCTP. DNA (4µg) was denatured by the addition of NaOH to a final concentration of 0.4 M. The denatured DNA was precipitated with 3 M sodium acetate (pH 5.3) and 96% ethanol. After the pellet was air-dried an appropriate volume of annealing buffer, water and primer was added and the mixture incubated at 65^{o}C for 10 minutes and thereafter at 37^{o}C for 30 minutes. Primers used included the pUC118/M13 multiple cloning site (MCS) universal forward and reverse primers as well as JHA1, JHA2, JHA3, JHA4 and JHA7 [Hastings *et al.*, 1991], respectively. Sequencing gels containing 8% polyacrylamide and 7M urea were electrophoresed in 1x TBE (pH 8.0) at 50 Watts for 3 hours. A second loading of the sample was performed and the gel run for a further 3 hours at 50 Watts. The gel was dried at 75^{o}C for 1 hour under vacuum, and exposed overnight to Agfa Curix X-ray film, at -70^{o}C. The hydrophilicity profiles of the leucocin A-UAL187 and leucocin B-Ta11a genes were generated using the GCG Sequence Analysis Software package [Devereux *et al.*, 1984].

2.11 Expression of bacteriocin

Cultures of *E. coli* JM103 transformed with pUC118, pJF5.5 or pJF8.1 respectively, were grown for 24 hours at 37^{o}C with shaking. A 1ml aliquot of an overnight culture was centrifuged at 12000xg and 4^{o}C for one minute. The supernatant (Fraction 1) was transferred to a new tube and the cell pellet retained. The pellet was suspended in 100µl of freshly prepared lysozyme solution (1mg/ml

in 20 w/v sucrose, 30mM Tris-Cl, 1mM EDTA, pH 8.0), left on ice for 10 minutes and centrifuged as before. The supernatant, containing the periplasmic fraction (Fraction 2), was transferred to a clean tube and stored at 4°C until required. The cell pellet was resuspended in 0.1 M Tris-Cl, pH 8.0, and the cells lysed by freeze-thawing in dry-ice and at 37°C, alternately until the suspension appeared clear. The mixture was centrifuged as before and the supernatant containing cytoplasmic proteins (Fraction 3), stored at 4°C until required. Membrane proteins (Fraction 4) were solubilised by treating the pellet with 1% Triton X-100. Samples of all fractions were analysed for inhibitory activity by the spot-on-lawn test (section 2.15), direct detection in sodium-dodecyl-sulphate-polyacrylamide (SDS-polyacrylamide) gels (section 2.13) and detection after elution from SDS-polyacrylamide gels (section 2.14).

2.12 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the method described by Sambrook *et al.* [1989]. A stacking gel of 6% and a 20% separating gel was routinely used for all separations. Samples of 20µl of fractions 1 to 4 (section 2.11) respectively, were loaded on the gel, along with a molecular weight marker (Sigma, Cat. No. MW-SDS-175). The gel was run at 10mA until the samples entered the separating gel, after which the current was changed to 15mA, for approximately 1 hour and 30 minutes. Duplicate gels were run and either stained or used for detection of bacteriocin activity after electrophoresis. Before staining, gels were fixed for 30 minutes in fixing solution (10% acetic acid, 25% isopropanol) and thereafter stained with Coomassie blue G-250 according to Sambrook *et al.* [1989].

2.13 Direct detection of bacteriocin activity in SDS-polyacrylamide gels

After electrophoresis, the gel was fixed in a solution containing 10% acetic acid and 20% isopropanol for 2 hours and washed with distilled water for 1 hour, after which each lane was cut out vertically. Each segment was placed on a dry MRS plate and overlaid with MRS soft agar containing a 1% inoculum of a 24 hour culture of *Listeria monocytogenes* NCTC 7973. The plates were incubated at 37^{O}C for 24 hours and examined for zones of growth inhibition.

2.14 Elution of bacteriocin from SDS-polyacrylamide gels

After electrophoresis the lanes were cut out vertically and each lane was cut into fragments of approximately 3mm. Each fragment was crushed in a tube containing 100µl of elution buffer (0.2 M sodium actetate, pH 4.5) and incubated at room temperature overnight. Samples were centrifuged for 1 minute at 12000xg and 20µl of supernatant spotted onto a lawn of *Listeria monocytogenes* NCTC 7973 in MRS soft agar (MRS, containing 0.8% agar). The plates were incubated at 37^{O}C for 24 hours and examined for zones of growth inhibition .

2.15 Spot-on-lawn assay

Samples of 20µl of fraction 1 to 4 (section 2.11) were spotted onto a lawn of *Listeria monocytogenes* NCTC 7973 in MRS soft agar (MRS, with 0.8% agar). The plates were incubated at 37^{O}C for 24 hours and examined for zones of growth inhibition.

CHAPTER 3

RESULTS

3.1 Spectrum of activity.

The antimicrobial activity of *Lc. carnosum* Ta11a was tested against a range of closely related Gram positive bacteria (see section 2.3), as some bacteriocins of LAB are very specific and have a very narrow inhibition spectrum. *Lc. carnosum* Ta11a showed antimicrobial activity against several closely related Gram positive indicator organisms including *Listeria monocytogenes* (Table 7). *Lc. carnosum* Ta11a did not inhibit growth of *Lc. gelidum* UAL 187-22 or the Gram negative indicator organisms that were tested (Table 7).

3.2 Genetic characterisation.

3.2.1 Cloning of the leucocin B-Ta11a operon

Plasmid DNA from *Lc. carnosum* Ta11a was isolated and fractionated as described in section 2.4. The DNA was transferred to a filter (see section 2.6), and hybridised to the probe, JF-1, at 37°C (see section 2.8.) An 8.9 MDa plasmid from *Lc. carnosum* Ta11a hybridised to the probe. The results obtained are shown in lane B of Fig. 2 (1) and 2 (2). The three positive signals visible on the filter in lane B of Fig. 2 (1) are the various forms of the 8.9 MDa plasmid which have different electrophoretic mobilities (covalently closed circular, open circular and linear).

A library containing *Sau*3A fragments of the plasmids of *Leuconostoc carnosum* Ta11a in pUC118 was constructed as represented in Fig. 3. The plasmid DNA was partially digested with *Sau*3A and DNA fragments were ligated with pUC118, previously linearised with *Bam*H1 and treated with calf intestinal phosphatase (see section 2.9). The ligation mixture was used to trans-

Table 7. Antimicrobial activity spectrum of *Leuconostoc carnosum* Ta11a, as determined by the spot-on-lawn assay

Indicator strain	Inhibition
Moraxella phenylpyruvica[a]	-
Pseudomonas fluorescens[a]	-
Flavobacterium odoratum[a]	-
Serratia liquefaciens[a]	-
Salmonella typhimurium	-
Lactobacillus confusus[a]	+
Lactococcus E9	-
Micrococcus M2[a]	-
Proteus mirabilis[a]	-
Lactobacillus fermentum	+
Leuconostoc mesenteroides LA44a	-
Leuconostoc paramesenteroides lA75a	+
Lactobacillus curvatus lA45c	+
Leuconostoc gelidum UAL187-22	-
Listeria monocytogenes	+
Leuconostoc mesenteroides TA10c	+

[a] Results used with kind permission from M. Papathanasopoulos [Papathanasopoulos, 1993]

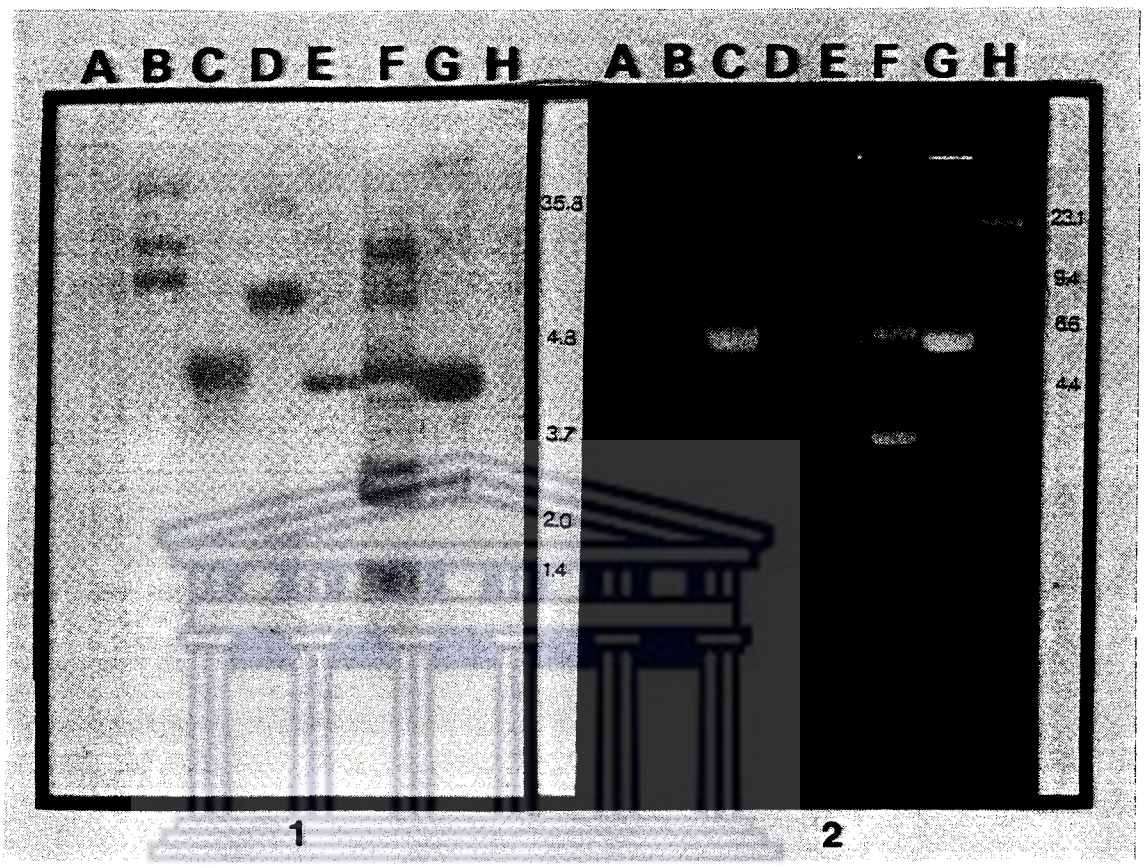

Fig. 2. Characterisation of leucocin B-Ta11a clones. (2) DNA was digested with restriction endonucleases and fractionated on a 0.8% agarose gel. The DNA was visualised by ethidium bromide staining of the gel. **(1)** Southern blot of the gel (2), hybridised to the probe, JF-1. Lane A, *E. coli* V517 (plasmid size marker (MDa)); Lane B, *Leuconostoc carnosum* Ta11a plasmids; Lane C, pJH6.1; Lane D, pJF8.1; Lane E, pJF8.1*Xba*1; Lane F, pJF5.5; Lane G, pJF5.5 *Xba*1; Lane H, λ*Hin*dIII (size marker (kb)).

form *E. coli* JM103 by electroporation, and transformants were then mixed with X-gal and IPTG and plated onto LB plates containing ampicillin (see section 2.9). Clones containing inserts were selected on the basis of α-complementation. Positive clones were further screened to determine whether the insert contained the leucocin B-Ta11a structural gene. Colonies were streaked onto the filter, treated to lyse the colonies amd fix the DNA, and hybridised to JF-1 at 37°C (see sections 2.7, 2.8.)

Four clones showed a positive signal after hybridisation to the probe (results not shown). The plasmids from these clones were isolated (see section 2.4) and the inserts characterised by restriction endonuclease digestion, Southern blotting and hybridisation using JF-1 as a probe. Two of the four clones contained inserts of 4.9 kb, but the inserts of the remaining clones were considered too small to contain all the genes required for expression of the bacteriocin as the pediocin PA-1 operon which mediates expression of the bacteriocin in *E. coli* spans a distance of 5.6 kb [Marugg *et al.*, 1992] Only one of the recombinant plasmids containing the 4.9 kb insert was characterised further. This plasmid was designated pJF8.1 and used for all subsequent experiments. The results obtained after Southern blotting and hybridisation of pJF8.1 to JF-1 are shown in lane D of Fig. 2 (1) and 2 (2). The results presented in lanes C to G will be discussed later in the text.

3.2.2 Characterisation of pJF8.1

Restriction endonuclease digestion analysis of pJF8.1 showed that only one *Bam*H1 site was present on the plasmid [Fig. 5 (1), lane D] as the insert was not released after digestion with *Bam*H1. Cloning of the *Sau*3A digested

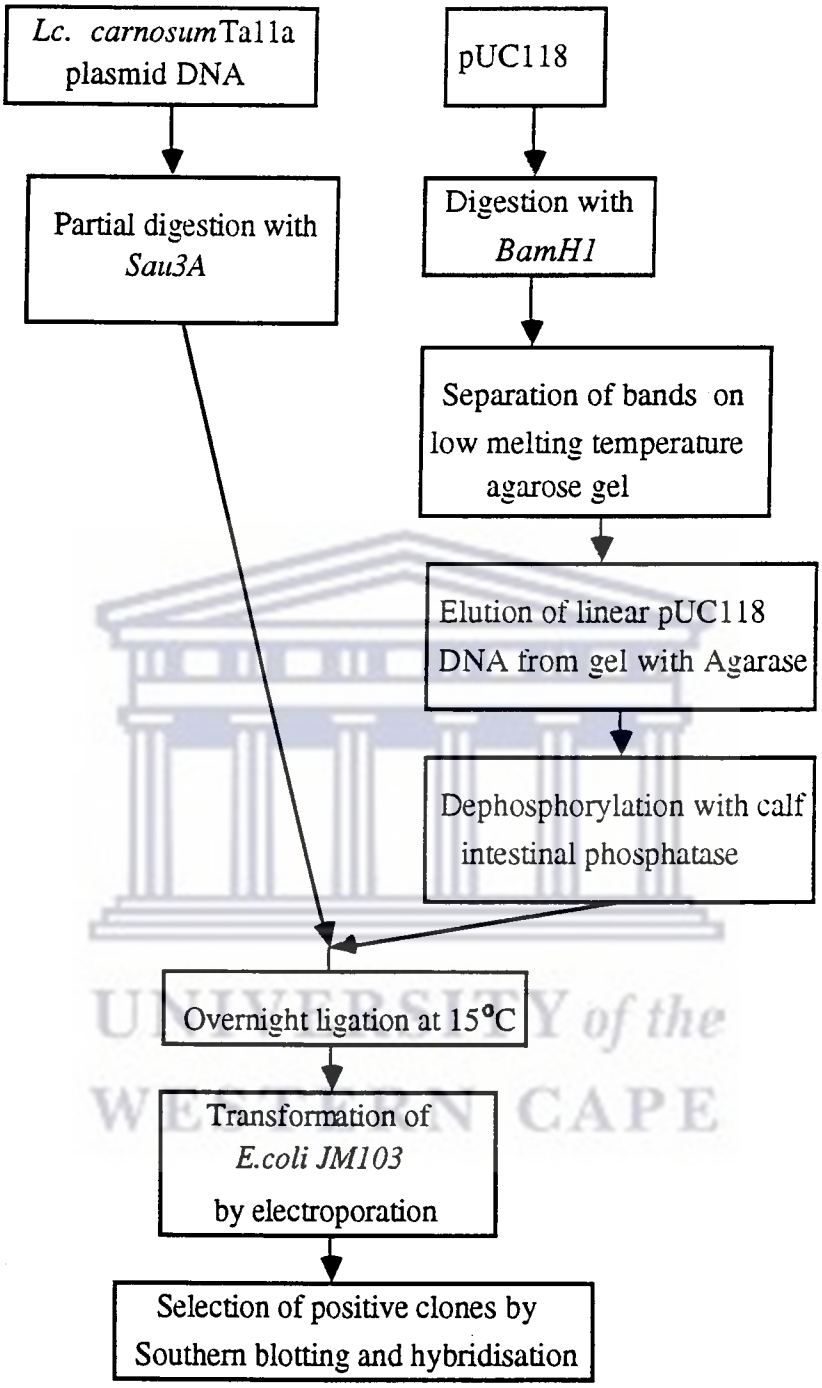

Fig. 3. A schematic representation of the strategy used in cloning the leucocin B-Ta11a structural gene.

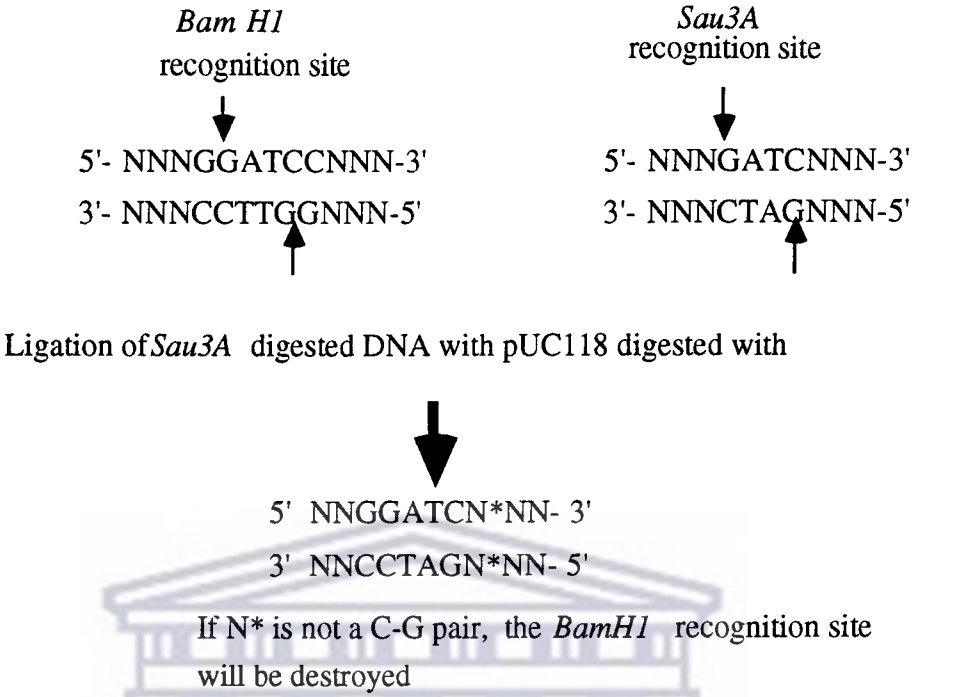

Fig. 4.1. Sequence requirement for restoration of a *Bam*H1 recognition site when cloning with *Sau*3a fragments.

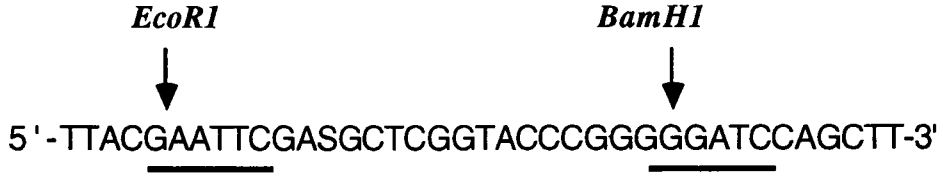

Fig. 4.2. Nucleotide sequence of part of pJF8.1 indicating the position of the restored *Bam*H1 site. The *Eco*R1 site is part of the pUC118 multiple cloning site.

DNA into the *Bam*H1 site of pUC118, therefore, resulted in the restoration of one *Bam*H1 site and the destruction of the other site. It is possible to restore the *Bam*H1 site only if the nucleotide marked with an asterisk in Fig. 4.1 is a C-G pair. The results of the restriction mapping of pJF8.1 indicated that the *Bam*H1 site in the 5´ position on pJF8.1, as indicated in Fig. 6, was restored. The 2.3 kb DNA fragment seen in lane F of Fig. 5 (1) shows that the restored *Bam*H1 site is in the 5´ position in pJF8.1, as this 2.3 kb fragment is obtained when the 5.5 kb *Xba*1 fragment, shown in lane E of Fig. 5 (1), is digested with *Bam*H1. If the restored *Bam*H1 site were in the 3´position, the *Xba*1 DNA fragment would not be cleaved as the *Bam*H1 and *Xba1* sites are adjacent to each other in the pUC118 MCS. The position of this site was confirmed by sequencing pJF8.1 with the pUC118 reverse primer. The nucleotide sequence flanking the restored *Bam*H1 site is given in Fig.4.2. Details of sequencing experiments are described in section 2.10.

Further restriction endonuclease digestion of pJF8.1 was performed to identify the region of the insert that contains the structural gene. A 2.3 kb *Xba*1 fragment of the *Xba*1/*Bam*H1 digest hybridised to JF-1 as shown in lanes D, E and F of Fig.5 (1) and 5 (2). Two other fragments obtained, did not hybridise to JF-1 as shown in lane F of Fig. 5 (1) and 5 (2). The very faint bands observed in lane B of Fig5 (1) and 5 (2) are due to the very low concentration of DNA loaded onto the gel. A subclone of the 2.3 kb fragment in pUC118 was generated [Fig.2 (1), lane E] by internal deletion of the 2.6 kb *Xba*1 fragment. A summary of the cloning procedure is given in Fig. 7. The insert of this subclone, designated pJF5.5, was characterised by restriction enzyme digests and Southern blotting and hybridisation with JF-1. Fig. 2 (1) an 2 (2), lane F, shows the undigested plasmid pJF5.5, and lane G the plasmid digested with *Xba*1 at its single *Xba*1 site.

3.2.3 Sequence analysis of the leucocin B-Ta11a operon

Sequence analysis was performed in order to identify and characterise the genes responsible for production of leucocin B-Ta11a. Plasmid DNA from both pJF8.1 and pJF5.5 was used for sequencing with the pUC118 universal forward and reverse primers, and primers JHA1, JHA2, JHA3, JHA4 and JHA7 [Hastings *et al.*, 1991], respectively (see section 2.10).

Analysis of the sequencing data revealed the presence of at least two open reading frames (ORF's), each preceded by a putative ribosomal binding site (RBS) (Fig.8). ORF1 was proposed to be the gene coding for leucocin B-Ta11a by virtue of homology with leucocin A-UAL187 [Hastings *et al.*, 1991]. Consensus sequences, which indicated a putative promoter, were identified at positions -35 and -10 upstream of the ORF1 as shown in Fig.8. ORF1 codes for a protein of 61 amino acids comprising the proposed leucocin B-Ta11a structural gene and an amino-terminal extension of 24 amino acid residues. The predicted hydrophilicity profiles of the amino-terminal extensions of leucocin A-UAL187 and leucocin B-Ta11a shown in Fig.10, were generated and compared to determine whether a change in amino acid sequence may cause a change in hydrophilicity of the protein, which could possibly affect the function of the protein. These results will be discussed in chapter 4. A comparison of the amino acid sequence of the proposed leucocin B-Ta11a bacteriocin, with the amino acid sequence of leucocin A-UAL187 and mesentericin Y105 is given in Fig. 9 and will be discussed in chapter 4.

The predicted protein product of ORF2 (Fig.8) has the same amino acid sequence as the leucocin A-UAL187 proposed immunity protein [Hastings *et al.*, 1991] .

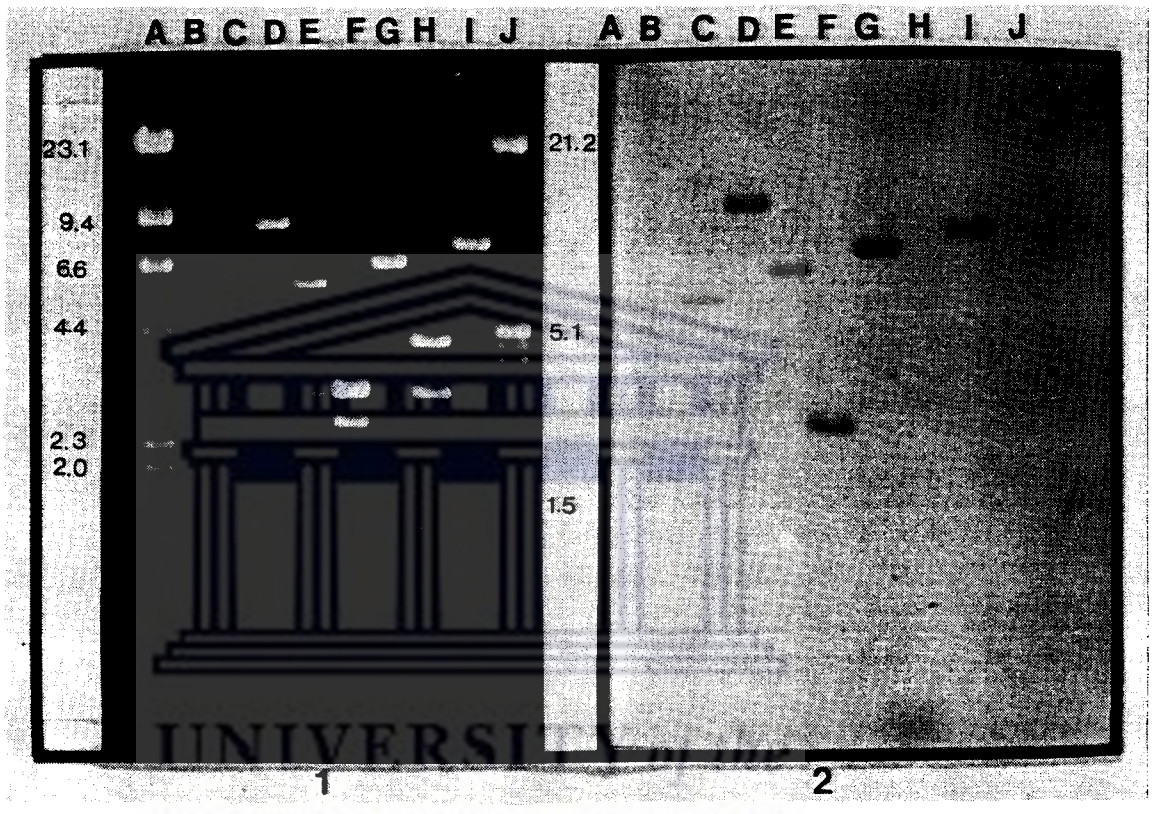

Fig. 5. Restriction endonuclease digestion analysis of pJF8.1, which is a cloned 4.9 kb DNA fragment containing part of the leucocin B-Ta11a operon, in pUC118. **(1).** The plasmid, pJF8.1, was digested with restriction endonucleases and fractionated on a 0.8% agarose gel. DNA was visualised by staining with ethidium bromide. **(2).** The gel was transferred to a filter by Southern blotting and hybridised with JF-1. Lane A, λ*Hin*dIII (size marker); Lane B, *Lc. carnosum Ta11a* plasmids; Lane C, pJF8.1; Lane D, pJF8.1*Bam*H1; Lane E, pJF8.1*Xba*1; Lane F, pJF8.1*Xba*1/*Bam*H1; Lane G, pJF8.1*Bam*H1/*Cla*1; Lane H, pJF8.1*Xba*1/*Cla*1; Lane I, pJF8.1*Cla*1; Lane J, λ*Eco*R1/*Hin*dIII (size marker).

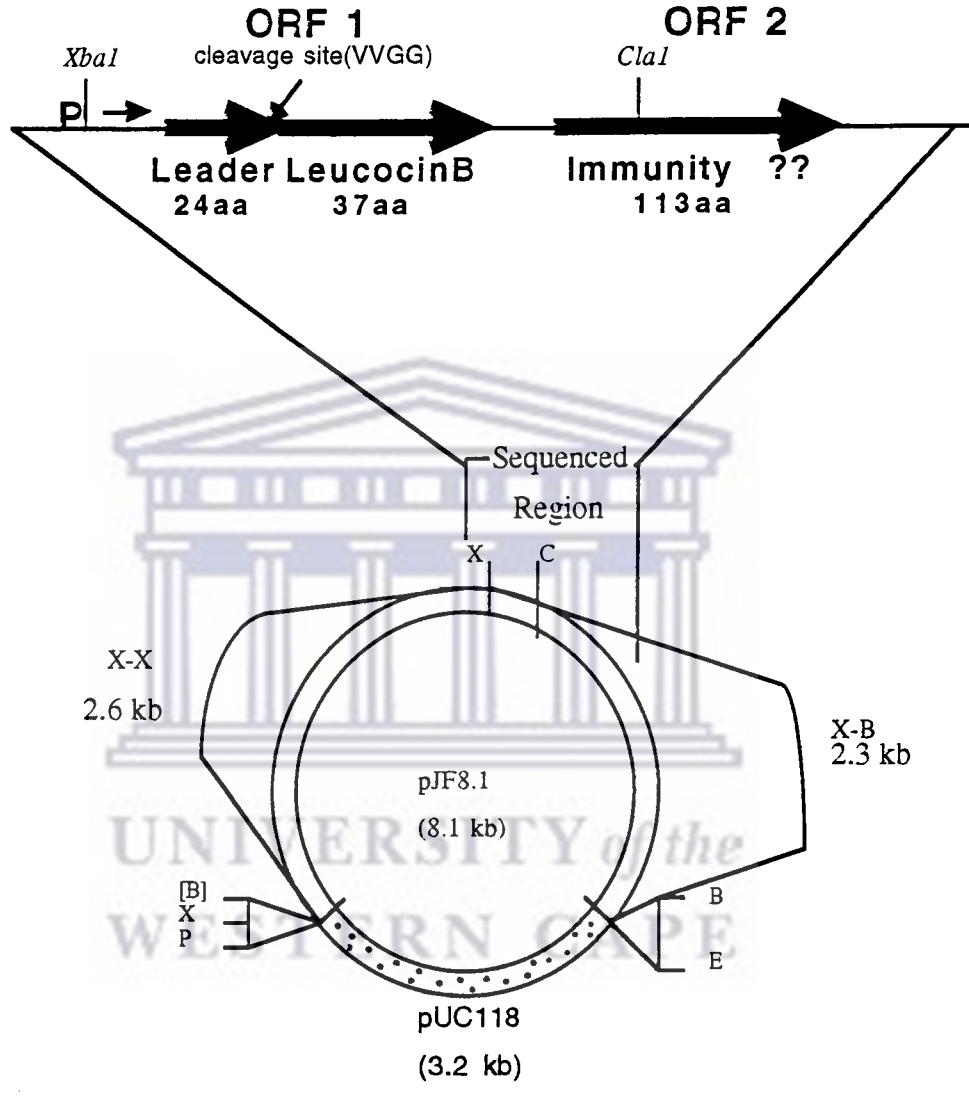

Fig. 6. Schematic overview of the restriction endonuclease analysis of a 4.9 kb fragment from *Leuconostoc carnosum* Ta11a cloned into the MCS of pUC118. The sequenced region has been enlarged to show the exact location of the leucocinB structural gene as well as the putative immunity gene. The dotted (.) region indicates the vector and the clear region the insert. P, putative promoter. X-*Xba*1, Ps-*Pst*1, B-*Bam*H1, C-*Cla*1, E-*Eco*R1, [B]-destroyed *Bam*H1 site.

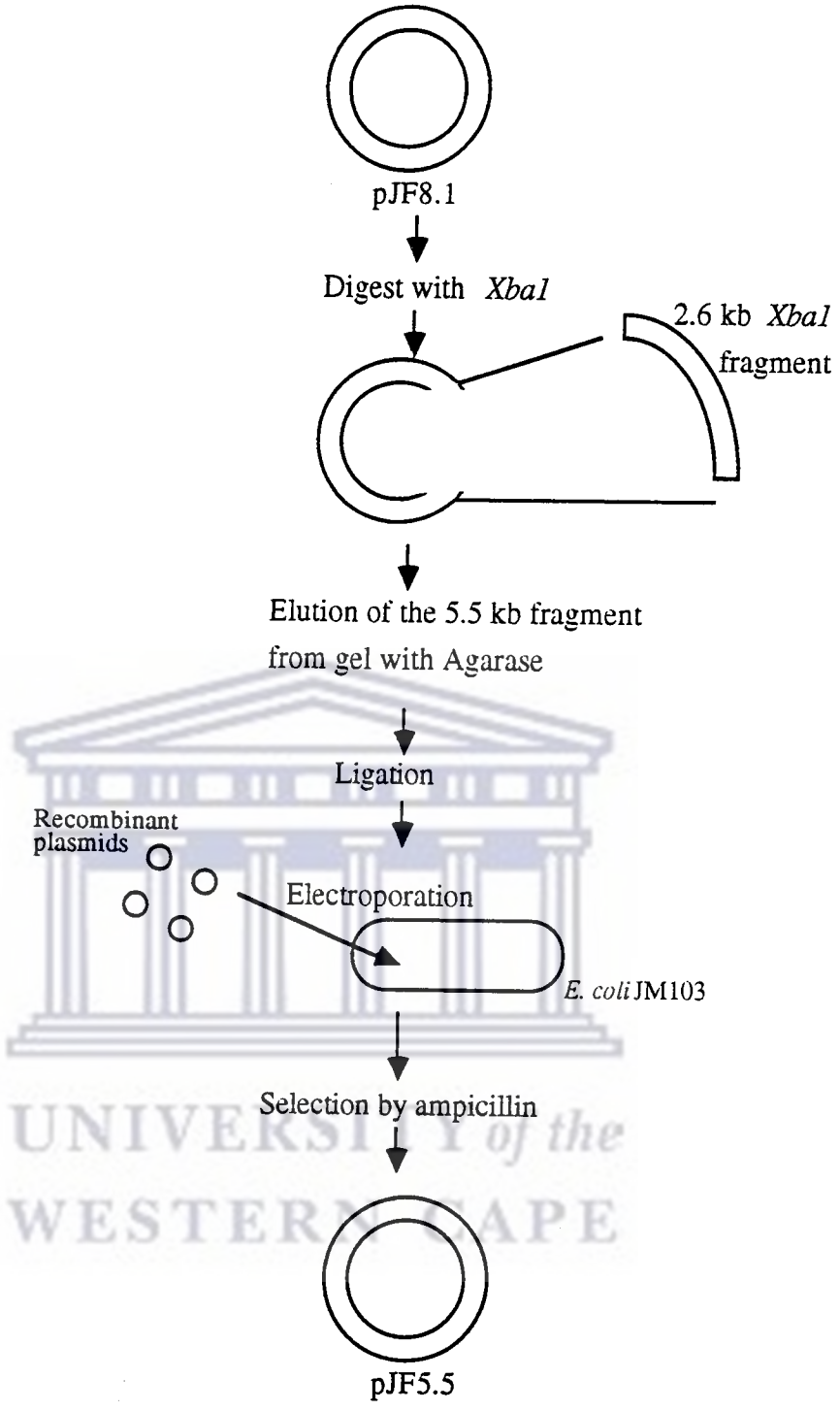

Fig. 7. A schematic representation of the cloning strategy used to create a subclone of pJF8.1, containing the proposed leucocin B-Ta11a structural and immunity genes.

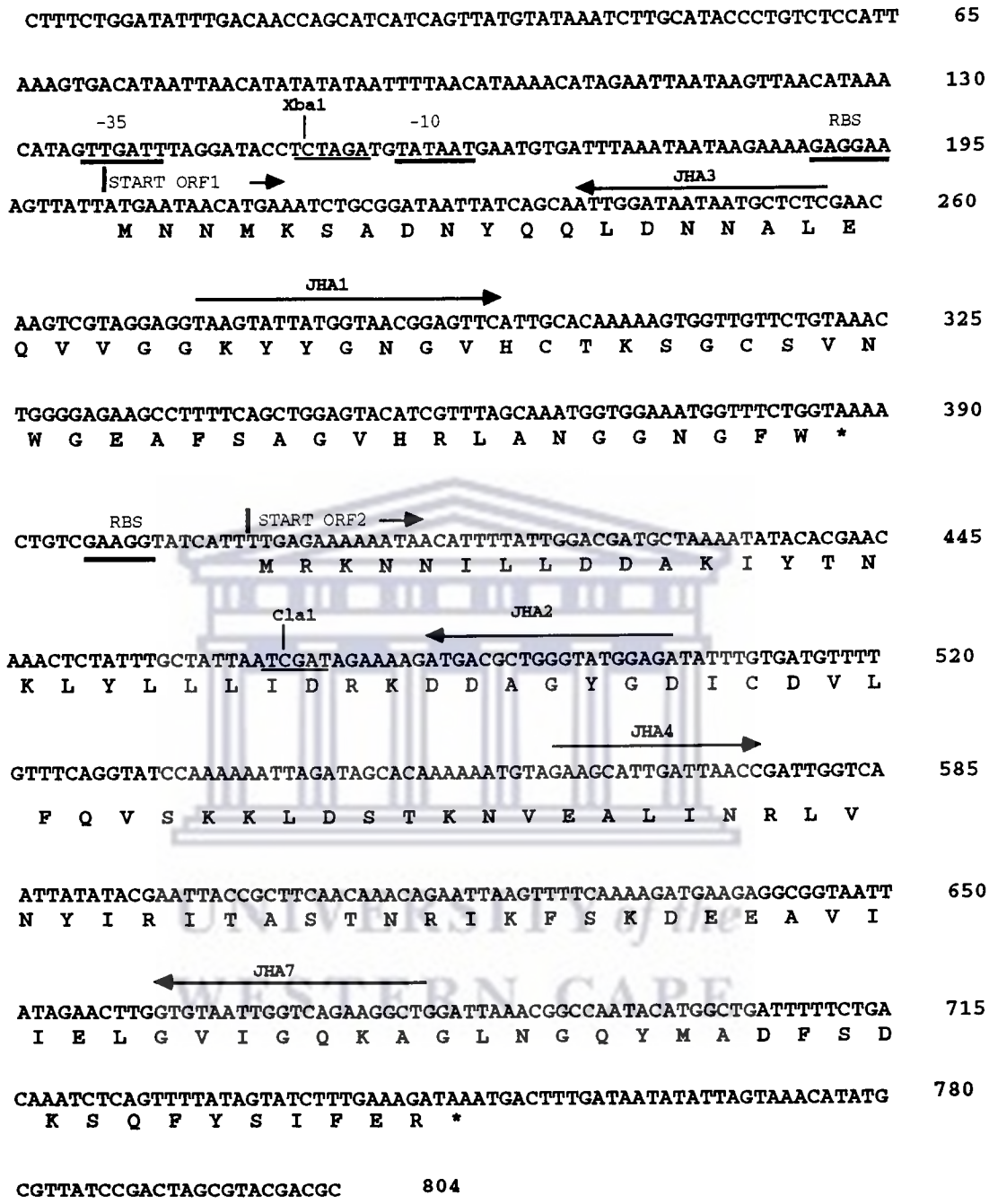

Fig. 8. Nucleotide sequence analysis of the region of pJF8.1 containing the leucocin B-Ta11a genetic determinants. ORF1 (proposed leucocin B-Ta11a precursor) and ORF2 (putative immunity gene) are indicated, with the translation products given below the nucleotide sequence. (*) indicates a stop codon. RBS, ribosomal binding site

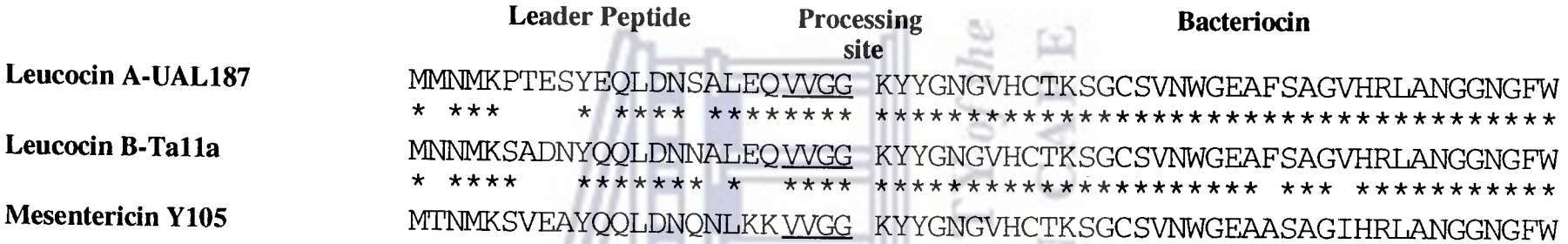

Fig. 9. Amino acid sequence comparison of leuconostoc bacteriocins: leucocin A-UAL187 [Hastings *et al.*,1991], leucocin B-Ta11a and mesentericin Y105 [Héchard *et al.*, 1992; Cenatiempo, pers. comm.[1]]. Similarities between amino acids are indicated with *.

[1]Footnote: Yves Cenatiempo, Institut de Biologie Moleculaire Genetique,URA CNRS 1172, Universite de Poitiers, 40 Avenue du Recteur, 86022 Poitiers Cedex, France.

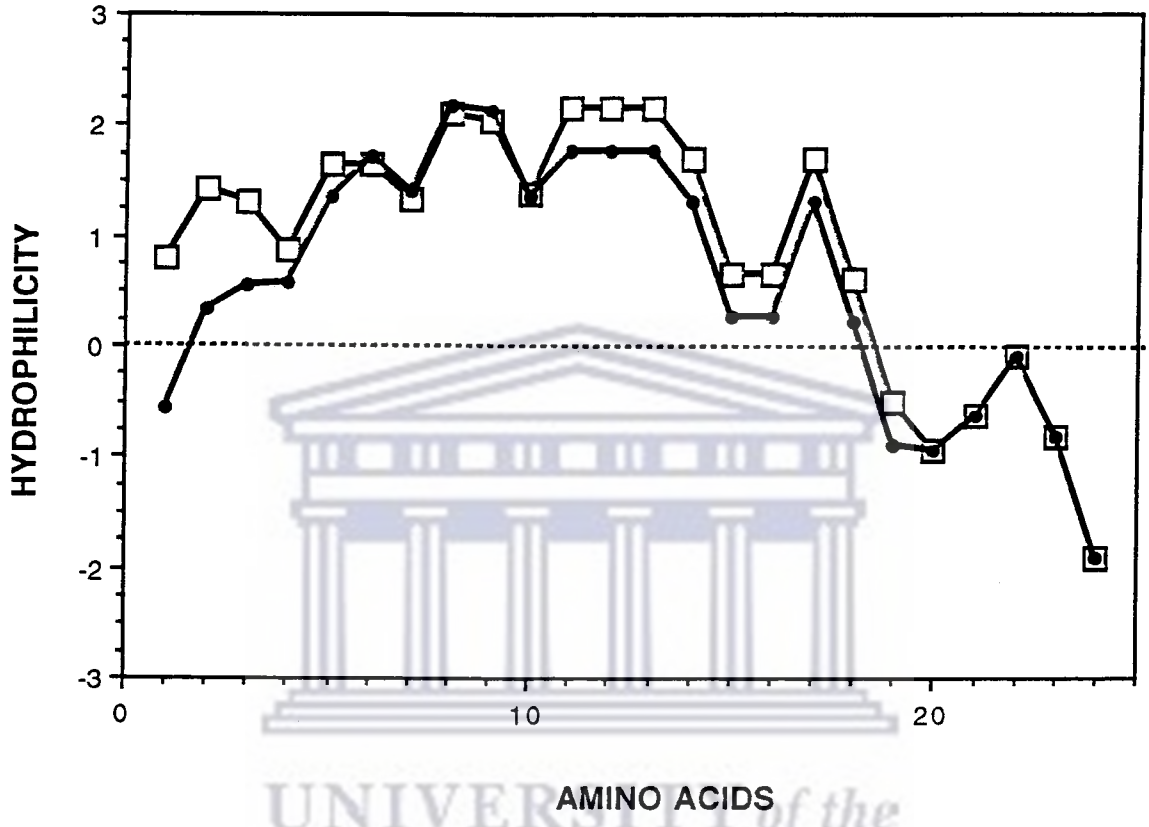

Fig. 10. Comparison of the hydrophilicity profiles (Kyte-Doolittle) of the leucocin A-UAL187 and leucocin B-Ta11a leader peptides. Symbols: —□—, LeucocinB leader; —●—, LeucocinA leader.

3.3 Expression of bacteriocin

E. coli JM103 containing the recombinant plasmids pJF8.1 or pJF5.5 were tested for expression of leucocin B-Ta11a as described in section 2.11. The various fractions obtained were analysed by direct detection in SDS-polyacrylamide gels (see section 2.13), elution of the bacteriocin from SDS-polyacrylamide gels (see section 2.14) and the spot-on-lawn assay (see section 2.15). No zone of growth inhibition of *Listeria monocytogenes* by leucocin B-Ta11a was noted for any fraction tested with any of the detection procedures.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Discussion

The isolation and characterisation of *Lc carnosum* Ta11a has been reported previously [Papathanosopoulos, 1993]. *Lc. carnosum* Ta11a produces a bacteriocin that is inactivated by proteinases and is very stable at high temperature and low pH. The bacteriocin is not inactivated by amylase or chloroform which suggests that no carbohydrates or lipids are required for activity unlike leuconocin S [Lewus *et al.*, 1992]. The bacteriocin, designated leucocin B-Ta11a, is similar to other *Leuconostoc* bacteriocins, in that it is a small, heat-stable protein of the class IIa type [Klaenhammer, 1993].

The relatively narrow spectrum of activity of leucocin B-Ta11a, with antagonistic activity against closely related LAB and *Listeria monocytogenes*, is similar to other *Leuconostoc* bacteriocins already described [Harding and Shaw, 1990; Daba *et al.*, 1991; Hastings and Stiles, 1991; Lewus *et al.*, 1992; Héchard *et al.*, 1992; Van Laack *et al.*, 1992; Klaenhammer, 1993]. Mesentericin Y105 is only active against various *Listeria* species [Héchard *et al.*, 1992] and LA-147 only against *Lb. leichmanii* [Juven *et al.*, 1992]. Leucocin B-Ta11a exhibits a bacteriolytic mode of action when tested against *Lc. mesenteroides* TA10c [Papathanosopoulos,1993].

Bacteriocin production and immunity are often plasmid encoded [Gonzalez and Kunka, 1987; Hastings and Stiles, 1991; Hastings *et al.*, 1991; Van Belkum *et al.*, 1991; Van Belkum *et al.*, 1992; Klaenhammer, 1993;]. Hybridisation of plasmid DNA of *Lc. carnosum* Ta11a to a 36-mer oligonucleotide probe, homologous to the nucleotide sequence of the amino-terminal region of leucocin A-UAL187 [Hastings *et al.*, 1991], linked bacteriocin production by *Lc. carnosum*

Ta11a to an 8.9 MDa plasmid. This result implies sequence homology between leucocin B-Ta11a and leucocin A-UAL187. This is not unexpected as pediocin PA-1 [Marugg *et al.*, 1992], sakacin A [Holck *et al.*, 1992], sakacin P [Tichaczek *et al.*, 1992], leucocin A-UAL187 [Hastings *et al.*, 1991] and curvacin A [Tichaczek *et al.*, 1992] have recently been shown to contain a consensus sequence of -Tyr-Gly-Asn-Gly-Val-Xaa-Cys- in their amino-termini. The amino acid sequence of mesentericin Y105 [Héchard *et al.*, 1992; Cenatiempo, pers. comm.[1]] showed a primary structure closely resembling that of leucocin A-UAL187 [Hastings *et al.*, 1991]. A similar homology has now been demonstrated for the proposed leucocin B-Ta11a bacteriocin [Fig. 9].

The primary structure of the proposed leucocin B-Ta11a structural protein is similar to that of leucocin A-UAL187 [Hastings *et al.*, 1991] and mesentericin Y105 [Héchard *et al.*, 1992; Cenatiempo, pers. comm.[1]] in that it may be synthesised as a 61 amino acid precursor which is cleaved at the val-val-gly-gly processing site. This 37 amino acid structural protein of leucocin B-Ta11a is identical to that of leucocin A-UAL187, but differs from mesentericin Y105 at two residues [Fig. 9]. The amino-terminal extension of leucocin B-Ta11a differs by seven residues from both leucocin A-UAL187 and mesentericin Y105 [Fig. 9]. A comparison of the predicted hydrophilicity of the amino-terminal extension of leucocin B-Ta11a with that of leucocin A-UAL187 [Fig. 10] suggests that the differences in amino acid sequence do not greatly affect the hydrophilicity of these peptides except at the amino-terminal end. The replacement of the Met(-2) in the leucocin A-UAL187 amino-terminal extension with an asparagine residue at the same position in the leucocin B-Ta11a molecule causes a notable increase in hydrophilicity of this region

[1]Footnote: Yves Cenatiempo, Institut de Biologie Moleculaire Genetique,URA CNRS 1172, Universite de Poitiers, 40 Avenue du Recteur, 86022 Poitiers Cedex, France.

of the protein. The function of these peptides has not yet been determined, but they have been implicated in expression of the bacteriocin across the cytoplasmic membrane [Klaenhammer, 1993]. The suggested prepeptide of leucocin B-Ta11a contains a val-val-gly-gly sequence at position -24. This sequence motif is similar to that of leucocin A-UAL187 and mesentericin Y105 and common to many other bacteriocin genes [Klaenhammer, 1993]. The structure of the proposed amino-terminal extension of leucocin B-Ta11a does not show the typically charged amino-terminus and hydrophobic core common to most secretory peptides [Von Heijne,1983; Gierasch, 1989]. This suggests that, either the functioning of this region may be different to that of normal secretory peptides, or the possibility that the amino-terminal extension could have additional functions that accounts for the difference in structure.

It is interesting to note that while the leucocin A-UAL187 producing strain inhibits growth of *Lc. carnosum* Ta11a, the latter does not inhibit growth of *Lc. gelidum* 187-22 (results not shown). If the protein product of ORF2 functioned solely as an immunity protein, it is expected that *Lc. carnosum* Ta11a would be immune to leucocin A-UAL187. It is possible that the proposed prepeptide or amino-terminal extension may have an effect on immunity, as the only differences detected between the two bacteriocins are between the leader region of leucocin A-UAL187 and the proposed leader region of leucocin B-Ta11. This phenomenon has been described previously in *Staphylococcus epidermidis,* where the products of both *pepA*, the bacteriocin structural gene, and ORF1, the presumed immunity protein, are required for immunity [Reis and Sahl, 1992]. Another possibility that needs further investigation is that *Leuconostoc gelidum* 187-22 produces two bacteriocins, one the same as leucocin B-Ta11a and another one that is different and active against *Lc. carnosum* Ta11a.

An interesting similarity in the amino-terminal leader regions of the bacteriocins produced by *Leuconostoc* spp. is that they consist of 24 amino acids, whereas in most other genera of LAB they are 18 to 21 residues, beginning with a methionine (M) and followed by lysine (K). The leader regions of leucocin A-UAL187, mesentericin Y105 and the proposed leader region of leucocin B-Ta11a also show the presence of the M (-21), K (-20) motif but this is preceded by three amino acids with methionines consistently placed at residues -24 and -21. This arrangement is similar to the dual start motif of bacteriophage lysis proteins [Blasï *et al.*, 1989; Nam *et al.*, 1990], in which a dual start motif produces two identical peptides with the exception of 3 residues at the amino-terminus. One protein functions as a lysis effector and the other as a non-lethal lysis inhibitor (immunity function). As bacteriocins are lysis effectors, it may be that the production of a second protein is required for host immunity. Further work is required to investigate the possibility of the existence of a dual start motif and the functions of the two proteins, which differ in length by three residues, in immunity.

The lack of expression of leucocin B-Ta11a in *E. coli* could possibly be a result of the size of the cloned fragment. The size of the fragment downstream of the promoter, effectively involved in bacteriocin production, is only 2.3 kb. If this operon is organised in a similar manner as the pediocin operon [Marugg *et al.*, 1992] all the genes required for expression will not be present on a fragment of this size. Other research groups have expressed bacteriocin activity from cloned fragments of this size [Van Belkum *et al.*, 1991; Van Belkum *et al.*, 1992], but expression was in lactic acid bacteria, where the recipient may possess copies of the genes required for biosynthesis and transport, thereby complementing the function of those genes lost during the cloning procedure. Further experiments, cloning larger fragments of the 8.9 MDa plasmid of *Lc. carnosum* Ta11a may possibly lead

to the identification of the other genes required for expression of bacteriocins in *Leuconostoc* spp.

4.2 Conclusion

The recent explosion of research activity on the bacteriocins produced by LAB has made a great contribution to our understanding of the genetic determinants involved in protein processing and expression in these organisms. These studies have however mainly concentrated on the bacteriocins produced by *Lactococcus*, *Lactobacillus* and more recently *Carnobacterium* spp.

This study involved the identification, cloning and characterisation of the genes responsible for production of leucocin B-Ta11a, a bacteriocin produced by *Leuconostoc carnosum* Ta11a. This represents only the third study reported of the genetics of a bacteriocin produced by a *Leuconostoc* spp.

An operon consisting of two open reading frames with a putative promoter and ribosomal binding site situated upstream of the open reading frames was identified on a 4.9 kb *Sau*3A fragment of an 8.9 MDa plasmid of *Leuconostoc carnosum* Ta11a. Comparison of the nucleotide sequence of a 61 amino acid protein, proposed to be the prepeptide precursor of leucocin B-Ta11a, with leucocin A-UAL187 and mesentericin Y105 showed that the leuconostoc bacteriocins exhibit a high degree of similarity in the structural gene with diversity mainly in the amino-terminal extension. This 61 amino acid protein may be cleaved at the carboxyl end of a glycine doublet releasing the proposed active bacteriocin of 37 amino acids and a 24 amino acid amino-terminal extension. This is similar to other bacteriocins of *Leucocnostoc* spp. that have been identified, leucocin A-UAL187 [Hastings and Stiles, 1991; Hastings *et al.*, 1991] and mesentericin Y105 [Héchard *et al.*, 1992; Cenatiempo, pers. comm.[1]]. The amino-terminal extensions of these bacteriocins

[1]Footnote: Yves Cenatiempo, Institut de Biologie Moleculaire Genetique,URA CNRS 1172, Universitede Poitiers, 40 Avenue du Recteur, 86022 Poitiers Cedex, France.

differ in length from the amino-terminal extensions of other bacteriocins of LAB which are usually 18 to 21 amino acids. These amino-terminal extensions also have a characteristic Met (-24), Met (-21) motif. The function of this characteristic arrangement of amino acids is not yet known and needs to be investigated as it may play a role in self-immunity. Attempts to express leucocin B-Ta11a in *E. coli*, from cloned fragments containing the proposed bacteriocin structural and immunity genes, have not been successful. A similar finding was reported by Hastings *et al.* [1991], who attempted to express leucocin A-UAL187 from cloned fragments containing the structural and immunity genes in several hosts, including *E. coli*. Larger fragments of the plasmids of bacteriocin-producing *Leuconostoc* spp. should be cloned to include the sequenced region as well as a section of DNA greater than 5 kb, situated downstream or upstream of the genes already identified. This may lead to the identification of the other genes required for expression of the bacteriocin, particularly since the genes mediating expression of pediocin PA-1 in *E. coli* span a region of approximately 5.6 kb [Marugg *et al.*,1992] and the genes required for expression of parental levels of lactococcin A in *L. lactis* subsp. *lactis* LM0230 span a region of approximately 5.2 kb [Stoddard *et al.*, 1992]. These operons both contain the structural and immunity genes as well as two other genes that were shown to be involved in processing and expression of the bacteriocins. The genes involved in processing and synthesis are situated downstream of the pediocin structural and immunity genes [Marugg *et al.*,1992], but upstream of the same genes in the case of lactococcin A [Stoddard *et al.*, 1992].

There is a lack of knowledge and understanding of the cell machinery involved in protein expression in *Leuconostoc* spp. Once expression of these bacteriocins has been obtained, mutation analysis studies could be undertaken to elucidate the exact role of each gene in bacteriocin expression and immunity, as well

as the amino acids that are important for activity of the bacteriocin. Identification of the latter will assist in genetic engineering of bacteriocins to enhance the activity spectrum. An increased understanding of the mechanism involved in protein expression in *Leuconostoc* spp. could facilitate the development of cloning vectors for increased expression of either bacteriocins, which would serve as a natural preservative, or other useful proteins in food systems.

Appendix I

Media

Luria-Bertani medium

10g Tryptone

5g Yeast extract

10g Sodium chloride

2g glucose

Add 900ml H_2O. Adjust pH to 7.0 and make up to 1 liter with H_2O. Sterilize by autoclaving for 20 mins at 121^{o}C on a liquid cycle.

Electrophoresis Buffers

50x Tris-acetate buffer (TAE)

242g Tris base

57.1ml glacial acetic acid

100ml 0.5 M EDTA (pH 8.0)

Make up to 1 liter with distilled H_2O.

10x Tris-borate

108g Tris base

55g boric acid

40ml 0.5 M EDTA (pH 8.0)

Make up to 1 liter with distilled H_2O.

Solutions for Hybridisation

20x SSC

175.3g Sodium Chloride

88.2g Sodium citrate

Add distilled $H_2O$to 800ml. Adjust the pH to 7.0 with 10 N NaoH. Make up to 1 liter with distilled H_2O.

10%SDS

Dissolve 100g of sodium dodecyl sulphate (SDS) in 900ml distilled H_2O. Heat solution to 68°C to assist dissolution. Make up to 1 liter with distilled H_2O.

50x Denhardts solution

5g Ficoll

5g polyvinylpyrrolidone

5g bovine serum albumin (Fraction IV)

Add distilled H_2O to 500ml. Filter and store at -20°C.

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