Characterization of bacteriocin 423

produced by

Lactobacillus pentosus

by

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DECLARATION

I the undersigned hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

Worldwide, bacteriocins, particularly those produced by food-related lactic acid bacteria, are receiving attention due to the possible use of these peptides as natural preservatives in food, replacing potentially harmful chemical preservatives.

Bacteriocins are ribosomally synthesized proteins or peptides that inhibit closely related microorganisms. Most bacteriocins produced by lactic acid bacteria are small, heat resistant peptides that inhibit other Gram-positive bacteria, including food-borne pathogens such as *Listeria monocytogenes*, *Bacillus cereus, Clostridium perfringens* and *Staphylococcus aureus*, but do not inhibit Gram-negative bacteria, molds or fungi. Bacteriocins are produced as inactive prepeptides that become active after the N-terminal leader peptide is cleaved off. Small heat resistant bacteriocins are either lantibiotics (Class I), containing unusual post-translationally modified amino acids, or peptides that are non-lanthionines (Class II). The Class II bacteriocins are further divided into four different groups: Class IIa, the anti-listerial bacteriocins containing the YGNGV consensus sequence in the N-terminal of the protein, Class IIb, bacteriocins consisting of two peptides, Class IIc, bacteriocins that are secreted via the *sec* pathway, and Class IId, bacteriocins that do not belong in the previous three subgroups.

A bacteriocin producing lactic acid bacterium was isolated in our laboratory from traditionally home fermented South African sorghum beer. The producing bacterium was found to be a facultative heterofermentative *Lactobacillus* sp. and was identified as *Lactobacillus plantarum* or *Lactobacillus pentosus* by using the API 50 CHL carbohydrate fermentation system and numerical analysis of total soluble cell protein patterns. RAPD-PCR analysis identified the strain as *L. plantarum*, but 16S rRNA sequencing confirmed its identification as *L. pentosus*.

The bacteriocin, first designated plantaricin 423 and later bacteriocin 423, was identified as a Class IIa small heat resistant anti-listerial bacteriocin containing the YGNGV consensus motif. Bacteriocin 423 inhibited a variety of Gram-positive bacteria, including *Lactobacillus* spp., *Leuconostoc* spp., *Oenococcus oeni*, *Pediococcus* spp., *Enterococcus* spp., *Propionibacterium* spp., *Staphylococcus* spp., *Bacillus* spp., *Clostridium* spp. and *Listeria* spp. The bacteriocin was inactivated by proteolytic enzymes and active over a wide pH range (pH 1-10). Bacteriocin 423 lost 50 % of its activity after autoclaving for 15 min at 121°C, but was not affected by lesser heat treatments.

Bacteriocin production was increased by optimizing the growth medium, which consisted of glucose, tryptone, yeast extract, potassium phosphate, sodium acetate, ammonium citrate,

manganese sulphate, Tween 80 and casamino acids.

The bacteriocin was found to be plasmid-encoded. Genetic analysis of the bacteriocin operon indicated a high percentage of homology to the operon of another Class IIa bacteriocin, pediocin PA-1, although the structural genes of the two bacteriocins were markedly different. The structural gene of bacteriocin 423 was amplified by PCR and cloned into a yeast/*E. coli* vector between the *ADH1* promoter and terminator sequences and fused in-frame to the *MFα1* secretion signal sequence. *Saccharomyces cerevisiae* transformed with this plasmid expressed the bacteriocin.

The sequence of prebacteriocin 423 (MMKKIEKLTEKEMANIIGGKYYGNGVTCGKHSCSVN WGQAFSCSVSHLANFGHGKC) is similar, but not identical to any other reported Class IIa anti-listerial peptide.

OPSOMMING

Bakteriosiene, veral dié wat deur melksuurbakterieë geproduseer word, wek belangstelling as gevolg van die moontlike gebruik van hierdie natuurlike antimikrobiese proteïene as preserveermiddels in voedselprodukte, in plaas van potensieël gevaarlike chemiese preserveermiddels.

Bakteriosiene is ribosomaal-vervaardigde proteïene wat naverwante bakterieë inhibeer. Die meeste bakteriosiene wat deur melksuurbakterieë geproduseer word, is klein en hittebestand. Hierdie bakteriosiene inhibeer ander Gram-positiewe bakterieë, insluitend patogene soos *Listeria monocytogenes, Bacillus cereus, Clostridium perfringens* en *Staphylococcus aureus*, maar inhibeer nie Gram-negatiewe bakterieë, giste of swamme nie. Bakteriosiene word as onaktiewe prepeptiede geproduseer, wat ge-aktiveer word wanneer die N-terminale leierpeptied afgesplits word. Klein hittebestande bakteriosiene is óf lantibiotika (Klas I), met ongewone aminosure, óf normale peptiede (Klas II). Laasgenoemde klas kan verder in vier groepe verdeel word. Klas IIa is anti-listeriese bakteriosiene met 'n YGNGV-aminosuurvolgorde in die N-terminale kant van die peptied. Klas IIb sluit in bakteriosiene wat uit twee peptiede bestaan. Klas IIc is *sec*-afhanklike bakteriosiene, en Klas IId sluit in al die bakteriosiene wat nie in die eerste drie groepe geklassifiseer kan word nie.

'n Bakteriosien-produserende melksuurbakterie is uit tradisionele tuisgefermenteerde Suid-Afrikaanse sorghumbier geïsoleer. Die bakterie is as 'n fakultatief heterofermentatiewe *Lactobacillus* sp. geïdentifiseer. Die bakterie is verder as 'n *Lactobacillus plantarum* of *Lactobacillus pentosus* geïdentifiseer deur middel van die API 50 CHL-koolhidraat fermentasiesisteem en numeriese analiese van totale oplosbare selproteïenprofiele. Met RAPD-PCR analiese is die organisme as *L. plantarum* geïdentifiseer, maar 16S rRNA nukleotiedopeenvolging het die identiteit van die organisme as *L. pentosus* bevestig.

Bakteriosien 423, aanvanklik geklassifiseer as plantaricin 423, is 'n klein Klas IIa, hittebestande en anti-listeriese bakteriosien met die YGNGV motief, wat verskeie Gram-positiewe bakterieë inhibeer. Bakteriosien 423 het verskeie Gram-positiewe organismes geïnhibeer, onder andere *Lactobacillus* spp., *Leuconostoc* spp., *Oenococcus oeni*, *Pediococcus* spp., *Enterococcus* spp., *Propionibacterium* spp., *Staphylococcus* spp., *Bacillus* spp., *Clostridium* spp., en *Listeria* spp. Proteolitiese ensieme inaktiveer die bakteriosien. Die peptied was oor 'n pH reeks van 1-10 aktief. Outoklavering vir 15 min by 121°C het die aktiwiteit van die peptied halveer, maar die bakteriosien is nie geïnaktiveer met ander hittebehandelings nie.

Produksie van die bakteriosien is verhoog deur die groeimedium te optimiseer. Die

groeimedium het bestaan uit glukose, triptoon, gisekstrak, kaliumfosfaat, natriumasetaat, ammoniumsitraat, mangaansulfaat, Tween 80 en casaminosure.

Die bakteriosien se genetiese determinante is op 'n plasmied gesetel. Genetiese analiese van die bakteriosien operon het 'n hoë homologie met 'n ander Klas IIa bakteriosien, pediocin PA-1, getoon, maar die strukturele gene van die twee bakteriosiene verskil merkbaar. Die strukturele geen van bakteriosien 423 is met PKR ge-amplifiseer en in 'n gis/*E. coli*-vektor tussen die ADH1 promotor- en termineerderopeenvolgings, in leesraam met die MFα1 sekresiesein, gekloneer. *Saccharomyces cerevisiae* wat met hierdie plasmied getransformeer is, het bakteriosien 423 uitgedruk. Die aminosuurvolgorde van prebakteriosien 423 (MMKKIEKLTEKEMANIIGGKYYGNGVTCGKHSCSVNWGQAFSCSVSHLANFGHGKC) is verwant aan, maar nie identies aan, ander Klas IIa anti-listeriese peptiede.

BIOGRAPHICAL SKETCH

Carol Ann van Reenen (née M^{c}Callum) was born in Cape Town on 19 August 1958. She matriculated at Napier High School, Napier, in 1976. In 1979 she obtained a B.Sc. degree at the University of Stellenbosch, majoring in Microbiology and Genetics. In 1980 she was employed by the Cape Provincial Administration and in 1981 by the University of Stellenbosch in the Department of Medical Microbiology at Tygerberg Hospital. During this period (1980-1981) she also enrolled as a part-time student and obtained a B.Sc. (Hons.) in Medical Sciences in December 1981. From February 1982 to January 1986 she was employed by the Department of Agricultural Economics and Marketing at the Regional Veterinary Laboratory in Stellenbosch as a virologist. From February 1986 to December 1988 she was employed as a full-time technical officer, and between January 1989 and April 1997 as a part-time technical officer in the Department of Microbiology, University of Stellenbosch. She obtained her M.Sc. (*cum laude*) in Microbiology in March 1992. The title of her M.Sc. thesis was "Microbial respiration, ATP and biomass in soils of Swartboskloof, Jonkershoek, under mountain fynbos subjected to a prescribed burn", with Prof. M.A. Loos as study-leader.

Mrs. van Reenen is married to Dr. A.J. van Reenen of the Institute of Polymer Science, Department of Chemistry, University of Stellenbosch. She has two sons and resides in Stellenbosch.

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PREFACE

This dissertation is presented as a compilation of manuscripts. Each chapter is introduced separately and is written according to the style of the respective journal. Three papers stemmed from this research. The Appendix is added for additional information pertaining to this research.

Chapter 4 **Van Reenen, C.A. and Dicks, L.M.T**. 1996. Evaluation of numerical analysis of Random Amplified Polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus plantarum* from *Lactobacillus pentosus*. *Current Microbiology* **32**, 183-187.

Chapter 5 **Van Reenen, C.A., Dicks, L.M.T. and Chickindas, M.L**. 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology* **84**, 1131-1137.

Chapter 6 **Van Reenen, C.A., Chikindas, M.L., van Zyl, W.H. and Dicks, L.M.T.** Characterization and heterologous expression of bacteriocin 423. Prepared for publication in *Journal of Bacteriology*.

Appendix **Verellen, T.L.J., Bruggeman, G., van Reenen, C.A., Dicks, L.M.T. and Vandamme, E.J**. 1998. Fermentation optimization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423. *Journal of Fermentation and Bioengineering* **86**, 174-179.

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INTRODUCTION

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INTRODUCTION

Consumers worldwide have become increasingly aware that chemical preservatives such as sulfur dioxide, benzoic acid, sorbic acid, nitrate and nitrite may have adverse effects on human health. This issue, as well as a demand for minimally processed food, have resulted in research focussing on naturally occurring metabolites produced by lactic acid bacteria to inhibit the growth of undesirable contaminants and food-borne pathogens (De Vuyst and Vandamme, 1994; Harlander, 1993).

Bacteria are capable of producing a wide range of molecules that may be inhibitory towards themselves or other organisms. These molecules include toxins, bacteriolytic enzymes, bacteriophages, by-products of primary metabolic pathways, antibiotic substances, and bacteriocins (Jack *et al.*, 1995). Bacteriocins are defined as antimicrobial peptides and proteins that are ribosomally synthesized by bacteria, and which inhibit the growth of closely related organisms. These peptides are produced as inactive prepeptides, which are subsequently separated from a leader peptide to form biologically active peptides (Jack *et al.*, 1995).

Lactic acid bacteria are used in the food industry as starter cultures for the fermentation of raw milk, meat and vegetable products. These organisms alter the flavour, texture and appearance of raw food products in a desirable way, and since these organisms have been consumed by humans and animals for centuries without any adverse effects, lactic acid bacteria have GRAS (generally recognized as safe) status. In addition, food is preserved due to the production of antimicrobial substances, primarily acid from sugar fermentation, resulting in a lower pH environment that is inhibitory to most microorganisms (Davidson and Hoover, 1993). Other antimicrobial substances such as bacteriocins are normal by-products, which are recognized as a potential source of food biopreservatives, particularly for minimally processed food and to control the emergence of psychrotrophic food-borne pathogens (Davidson and Hoover, 1993). Bacteriocins are often heat resistant, and could therefore be applied to food in combination with heat treatments. Bacteriocins appear to be stable in food, biodegradable, digestible, safe to health, and active at low concentrations. In addition, the antimicrobial spectrum of bacteriocins produced by lactic acid bacteria is restricted to Gram-positive bacteria, and often includes food spoilage bacteria such as *Bacillus cereus, Clostridium perfringens, Listeria monocytogenes* and *Staphylococcus aureus* (De Vuyst and Vandamme, 1994).

Although Antonie van Leeuwenhoek and Pasteur recorded observations of the antagonistic

interaction between bacteria in 1676 and 1877, respectively, the first clear indication of the inhibition of organisms by a bacteriocin, colicin V, produced by *Escherichia coli*, was documented in 1925 (Jack *et al.*, 1995). Nisin, a bacteriocin produced by *Lactococcus lactis* was discovered in 1927, but only structurally characterized in 1971.

Numerous bacteriocins produced by lactic acid bacteria have been described in the last decade. Continual research of bacteriocins produced by the different genera of lactic acid bacteria is motivated by the potential use of these bacteriocins as preservatives. Several factors may influence the successful application of bacteriocins as antimicrobial agents in food, including the presence of proteolytic enzymes, lipid content and concentrations of certain salts such as sodium chloride (Ray, 1994). Bacteriocins may be applied by using the producers as starter cultures, using the bacteriocin as an additive to food, or genetically engineering a starter culture to produce the bacteriocin (Harlander, 1993). Increased knowledge of the structure and mode of action of bacteriocins may lead to the production of gene cassettes that may enhance the activity of peptides which may not be appropriate for use as preservatives in their native state (Stiles, 1993). The use of bacteriocins is not limited to food products, but can also be applied to health care products such as toothpaste, skin care products and possibly as an alternative therapeutic agent for the treatment of infections caused by antibiotic-resistant Gram-positive bacteria (Harlander, 1993, Ross *et al.*, 1999). Nisin, a lanthionine-containing antimicrobial peptide produced by *L. lactis* subsp. *lactis*, is the only approved food-grade bacteriocin used in the United States. Nisin is bactericidal against Gram-positive bacteria, including food pathogens such as *L. monocytogenes*, and is used to prevent mastitis in cows, control oral infections, dental caries and acne (Harlander, 1993, Ross *et al.*, 1999).

During 1993, 400 lactic acid bacteria were isolated in our laboratory from various typically home-fermented South African food products. These organisms were tested for the production of antimicrobial substances against various Gram-positive organisms, including *Pediococcus acidilactici*, *Lactobacillus casei*, *L. monocytogenes* and *Streptococcus mutans,* and *E. coli*. Several of the 400 isolates produced antimicrobial compounds. Strain 423, isolated from sorghum beer, produced a broad-spectrum antibacterial protein that inhibited a variety of Gram-positive bacteria, including *Listeria* spp., *Bacillus* spp., *Clostridium* spp., *Staphylococcus* spp., *Propionibacterium* spp., *Leuconostoc* spp. and *Oenococcus oeni*. The aim of this study was to identify strain 423, to characterize the antibacterial protein (called plantaricin 423, or bacteriocin 423) and to identify and characterize the genes encoding the protein. This involved cloning of the structural gene of the bacteriocin and expression in a different host organism. Since bacteriocin 423 inhibited *Oenococcus oeni*, the most common

organism involved in malolactic fermentation in wine, *Saccharomyces cerevisiae* was chosen to express bacteriocin 423. Malolactic fermentation is undesirable in wine produced in countries with warmer climates, such as South Africa.

Strain 423 was subsequently identified as either a *Lactobacillus plantarum* or a *Lactobacillus pentosus*, and therefore more attention is given to the taxonomy and bacteriocins produced by these species in the literature survey. Similarly, since bacteriocin 423 was identified as a Class IIa bacteriocin, attention is focussed on the properties of Class IIa bacteriocins.

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CHAPTER 2

THE LACTIC ACID BACTERIA

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2.1 TAXONOMY OF FOOD-RELATED LACTIC ACID BACTERIA

2.1.1 Introduction

The taxonomy of lactic acid bacteria is currently primarily based on phenotypic and genetic characteristics, rather than on the more traditional morphological and physiological differences (Klein *et al.*, 1998). Other characteristics that are used to classify lactic acid bacteria into different genera include mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, growth at high salt concentrations, acid or alkaline tolerance, fatty acid composition and mobility of lactate dehydrogenase (Axelsson, 1993). Methods used for phenotypic and phylogenetic classification include comparative analysis of SDS-PAGE of whole cell protein patterns, 16S and 23S rRNA sequence analysis, DNA base composition, DNA homologies and RAPD-PCR (random amplified polymorphic DNA - polymerase chain reaction) band patterns (Pot *et al.*, 1994; Stiles and Holzapfel, 1997).

Phylogenetically, the Gram-positive bacteria form two lines of descent, the *Clostridium* branch, with a G+C content of less than 50 mol%, and the *Actinomyces* branch, with a G+C content higher than 50 mol% (Schleifer and Ludwig, 1995). Lactic acid bacteria are Gram-positive, catalase negative, non-sporeforming rods, cocci or coccobacilli with a G+C content of less than 53% (Stiles and Holzapfel, 1997). Lactic acid bacteria have complex nutrient requirements. Homofermentative lactic acid bacteria convert hexoses to lactic acid, and do not ferment pentoses or gluconate. Facultative heterofermentative lactic acid bacteria ferment hexoses to lactic acid and pentoses to lactic acid and acetic acid. Some heterofermentative lactic acid bacteria also ferment hexoses to acetic acid, ethanol, and formic acid under glucose limitation (Pot *et al.*, 1994). Obligately heterofermentative lactic acid bacteria convert glucose to lactic acid, carbon dioxide, ethanol and/or acetic acid, and pentoses to lactic acid and acetic acid (Pot *et al.*, 1994). The genera of the lactic acid bacteria associated with foods include *Carnobacterium, Enterococcus*, *Lactobacillus, Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus, Vagococcus* and *Weissella*. *Bifidobacterium* spp. were originally considered as members of the lactic acid bacteria, since they produce lactate and acetate as major fermentation end-products. Although the genus *Bifidobacterium* has a G + C content of more than 50 mol%, and is therefore closer related to *Actinomyces*

than to lactic acid bacteria (Schleifer and Ludwig, 1995), it is included in this review due to its importance in the food industry.

Gram-positive, non-sporing lactic acid bacteria not involved with food production or spoilage include the genera *Alloiococcus* (Aguirre and Collins, 1992) *Aerococcus* (Collins *et al.*, 1990; Stiles and Holzapfel, 1997) and *Melissococcus* (Bailey and Collins, 1982).

New species and strains with novel phenotypic characteristics are continually being isolated, and therefore the classification of lactic acid bacteria remains dynamic and intensively studied (Pot *et al.*, 1994).

2.1.2 The genus *Lactobacillus*

Based on their fermentative characteristics, the genus *Lactobacillus* is divided into three groups, *viz.* obligately homofermentative, facultative heterofermentative, and obligately heterofermentative (Stiles and Holzapfel, 1997). Lactobacilli from all three groups are used in the food industry, and are associated with numerous different habitats, such as the normal microflora in human cavities, plants, soil, water, sewage, food fermentations, cereal products, silage and food spoilage (Hammes and Vogel, 1995).

Group I, the obligately homofermentative lactobacilli, ferments hexoses such as glucose almost exclusively to lactic acid. These organisms lack the enzymes glucose 6-phosphate dehydrogenase (G-6-PDH) and 6-phosphogluconate dehydrogenase (6-P-GDH) and cannot ferment pentoses or gluconate (Pot *et al.*, 1994). The species in this group include *Lactobacillus acidophilus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus aviarius* subspp. *araffinosus* and *aviarius*, *Lactobacillus crispatus*, *Lactobacillus delbrueckii* subspp. *bulgaricus, delbrueckii* and *lactis*, *Lactobacillus farciminis*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus helveticus, Lactobacillus jensenii, Lactobacillus johnsonii, Lactobacillus kefiranofaciens, Lactobacillus kefirgranum, Lactobacillus mali, Lactobacillus ruminis*, *Lactobacillus salivarius* subspp. *salicinus* and *salivarius*, and *Lactobacillus sharpeae.* A new species, *Lactobacillus fornicalis*, was recently added to this group (Dicks *et al.*, 2000). Within this group, *L. acidophilus, L. delbrueckii, L. helveticus, L. farciminis* and *L. kefiranofaciens* are important in the food industry (Klein *et al.*, 1998; Stiles and Holzapfel, 1997).

On the basis of DNA-DNA hybridization studies, two subgroups are identified within the obligate homofermentative group. Subgroup I includes *L. delbrueckii* and *L. jensenii. Lactobacillus delbrueckii* is associated with plant and dairy products that are fermented at high temperatures (45-50°C). *Lactobacillus delbrueckii* subsp. *bulgaricus* is a yogurt starter

organism, together with *Streptococcus thermophilus*, while it is also used with *L. delbrueckii* subsp. *lactis*, *L. helveticus* and *S. thermophilus* in cheese starter cultures. *Lactobacillus delbrueckii* and *L. jensenii* are indistinguishable by physiological tests, but have different mol% G + C values (Pot *et al.*, 1994). Subgroup 2 includes *L. acidophilus*, a heterogeneous species composed of several subgroups, viz. *L. amylovorus, L. crispatus, L. gallinarum, L. gasseri and L. johnsonii.* These organisms cannot be differentiated phenotypically, and comparative methods such as mol % G + C, electrophoretic analysis of total soluble cellular proteins or lactate dehydrogenase and cell wall studies are necessary (Dicks and Van Vuuren, 1990; Pot *et al.*, 1994). Certain strains of *L. acidophilus* are used in acidophilus milk production and as a probiotic, while *L. delbrueckii* subsp. *delbrueckii*, *L. acidophilus* and *L. farciminis* are involved with sourdough fermentation and *L. johnsonii* is also used in yogurts. *Lactobacillus acidophilus* can be distinguished from *L. gasseri* with 23S rRNA sequencing (Stiles and Holzapfel, 1997).

The facultative heterofermentative group (Group II) has both dehydrogenase enzymes (G-6-PDH and 6-P-GDH). These organisms ferment hexoses to lactic acid and may produce gas from gluconate, but not from glucose. Pentoses are fermented to lactic and acetic acid via an inducible pentose phosphoketolase pathway (Pot *et al.*, 1994). Group II is represented by *Lactobacillus acetotolerans*, *Lactobacillus agilis, Lactobacillus alimentarius, Lactobacillus bifermentans, Lactobacilllus casei, Lactobacillus coryniformis* subsp. *coryniformis* and *torquens, Lactobacillus curvatus, Lactobacillus graminis, Lactobacillus hamsteri, Lactobacillus homohiochii, Lactobacillus intestinalis, Lactobacillus murinus*, *Lactobacillus paracasei* subspp. *paracasei* and *tolerans*, *Lactobacillus paraplantarum, Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus rhamnosus* and *Lactobacillus sakei* (Stiles and Holzapfel, 1997). Three subgroups occur within this group (Collins *et al.*, 1991). Subgroup 1 is represented by *L. casei*, an organism associated with sourdough and cheese fermentations, cheese spoilage and is also used as a probiotic (Klein *et al.*, 1998). The *L. casei* group comprises the species *L. casei*, *L. paracasei* and *L. rhamnosus* (Klein *et al.*, 1998). A revision of this group was proposed by Dicks *et al.* (1996) after an initial unsuccessful "Request for an opinion" (Dellaglio *et al.*, 1991). It has been suggested that the name *L. paracasei* be rejected and the species designated *L. casei*, and the species *Lactobacillus zeae* be revived (Dicks *et al.*, 1996). Subgroup 2 contains the species *L. plantarum* that is used as a starter organism in fermented sausages, cereal products and may occur as a spoilage organism in citrus juice, wine and cheese. Other species included in this subgroup are *L. paraplantarum* (Curk *et al.*, 1996) and *L. pentosus* (Zanoni *et al.*, 1987). *Lactobacillus pentosus* is very closely related to *L. plantarum* as determined by 16S rRNA sequence analysis (Collins *et al.*, 1991). *Lactobacillus*

sakei, L. curvatus and *L. bavaricus* represent the third subgroup (Pot *et al.*, 1994). Although they are phenotypically different, *L. sakei* and *L. bavaricus* cannot be distinguished by DNA-DNA hybridization studies and it has been proposed to reject the name *L. bavaricus* (Torriani *et al.*, 1996). *Lactobacillus sakei* and *L. curvatus* share 50% DNA similarity (Pot *et al.*, 1994). Both of the latter two species are important starter cultures in fermented meat products and are implicated in the spoilage of cold stored vacuum packed meat (Stiles and Holzapfel, 1997). It has been suggested that *L. sakei* be divided into two subgroups on the basis of SDS-PAGE protein patterns. These subgroups are represented by the organisms *L. sakei* subsp. *sakei* and *L. sakei* subsp. *carnosus* (Torriani *et al.*, 1996). Similarly, *L. curvatus* should be divided into two subgroups, represented by *L. curvatus* subsp. *curvatus* and *L. curvatus* subsp. *melibiosus* (Klein *et al.*, 1996; Torriani *et al.*, 1996). Methods for the rapid detection and identification of these organisms, using 23S rRNA probes (Hertel *et al.*, 1991) or RAPD-PCR (Berthier and Ehrlich, 1999), have been developed.

Group III, the obligate heterofermentative lactobacilli, ferment hexoses to lactic acid, acetic acid and/or ethanol and carbon dioxide. Gas is produced from glucose. Pentose is fermented to lactic and acetic acids via the pentose phosphoketolase pathway (Pot *et al.*, 1994). The species *Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus collinoides, Lactobacillus fermentum, Lactobacillus fructivorans, Lactobacillus fructosus, Lactobacillus hilgardii, Lactobacillus kefir, Lactobacillus malefermentans, Lactobacillus oris, Lactobacillus panis, Lactobacillus parabuchneri, Lactobacillus parakefir, Lactobacillus pontis, Lactobacillus reuteri, Lactobacillus sanfranciscensis, Lactobacillus suebicus, Lactobacillus vaccinostercus* and *Lactobacillus vaginalis* are obligate heterofermentative. *Lactobacillus sanfranciscensis, L. brevis* and *L. fermentum* are used in sourdough fermentations (Stiles and Holzapfel, 1997). *Lactobacillus panis* and *L. pontis* are associated with rye sourdough fermentations (Stiles and Holzapfel, 1997). *Lactobacillus reuteri* produces a broad-spectrum antimicrobial substance reuterin (Earnshaw, 1992). *Lactobacillus reuteri* and *L. fermentum* can only be distinguished from one another genetically (Stiles and Holzapfel, 1997). Some species within this group such as *L. brevis* and *L. buchneri* cause spoilage of food products (Stiles and Holzapfel, 1997).

2.1.3 The genus *Carnobacterium*

Collins *et al.* (1987) suggested the genus *Carnobacterium* to accommodate atypical lactobacilli, based on biochemical, chemical and physiological criteria. *Carnobacterium* spp. have been isolated from fresh and vacuum-packaged meat and meat products, poultry, fish

and seawater (Schillinger and Holzapfel, 1995). 16S rRNA sequences of these species show a high degree of homology, forming a phylogenetically closely related group, distinct from other lactic acid bacteria (Collins *et al.*, 1987). *Carnobacterium* spp. can be distinguished from *Lactobacillus* spp. by its inability to grow at pH 4.5 or on acetate agar. Four species have been identified: *Carnobacterium divergens*, *Carnobacterium gallinarum*, *Carnobacterium mobile* and *Carnobacterium piscicola*. *Carnobacterium divergens* and *Carnobacterium piscicola* occur mainly on vacuum-packaged meat products stored at low temperatures. *Carnobacterium gallinarum* and *C. mobile* have only been isolated from chicken meat (Schillinger and Holzapfel, 1995). *Carnobacterium* spp. are distinguished from each other based on acid production and fatty acid content. Phylogenetically, the genus *Carnobacterium* is closer related to the genera *Enterococcus* and *Vagococcus* than to *Lactobacillus* (Collins *et al.*, 1987).

2.1.4 The genus *Enterococcus*

Originally part of the streptococci, the genus *Enterococcus* became a separate genus in 1984, with *Streptococcus faecalis* and *Streptococcus faecium* renamed as *Enterococcus faecalis* and *Enterococcus faecium*, respectively (Schleifer and Kilpper-Bälz, 1984). Subsequently, several *Streptococcus* spp. have been renamed *Enterococcus*. The genus *Enterococcus* is closely related to the genera *Vagococcus*, *Tetragenococcus* and *Carnobacterium* (Collins *et al.*, 1989).

Enterococcus faecalis and *E. faecium* are important in the food and health industry where they are used as probiotics and starter cultures. Their success as probiotic organisms lies in their ability to survive in the gastrointestinal tract (GIT) of humans and animals (Klein *et al.*, 1998). *Enterococcus faecium* is associated with the fermentation of various southern European cheeses. Enterococci may also indicate faecal contamination of food, are frequently involved in human infections, and are possibly involved in food-borne illnesses (Stiles and Holzapfel, 1997). *Enterococcus faecium* and *E. faecalis* can easily be distinguished from other homofermentative lactic acid bacteria based on physiological characteristics (Devriese and Pot, 1995).

Within the genus *Enterococcus*, 16S rRNA sequence analyses have revealed the existence of several phylogenetically related species groups (Devriese and Pot, 1995). *Enterococcus faecalis* does not fit in any currently identified group. This organism is often the dominating enterococcal species in the human GIT. In animals, the occurrence of this organism is age dependent (Devriese and Pot, 1995).

The *E. faecium* species group includes the species *E. faecium, Enterococcus durans, Enterococcus hirae* and *Enterococcus mundtii*. These species are distinguished from one another by pigment production and biochemical characteristics. *Enterococcus faecium* occurs mainly in the GIT of humans and animals, but has also been isolated from plants, frozen-, dried- and processed food and raw milk and milk products (Devriese and Pot, 1995). *Enterococcus faecium* is less pathogenic than *E. faecalis*, but its multiple drug resistance properties, which have implications for possible hospital infections and epidemics, have received much attention. *Enterococcus durans* usually occurs in milk and dairy products, *E. hirae* in the gut of dogs and other domestic animals, and *E. mundtii* in plants (Devriese and Pot, 1995).

The *Enterococcus avium* group consists of the species *E. avium, Enterococcus malodoratus, Enterococcus pseudoavium* and *Enterococcus raffinosus*. These species are differentiated by carbohydrate fermentations. They are mostly associated with animals and are rarely isolated from humans (Devriese and Pot, 1995).

The *Enterococcus gallinarum* species group consists of the species *E. gallinarum, Enterococcus casseliflavus* and probably *Enterococcus flavescens*. These organisms are motile and have a natural low resistance to the antibiotic vancomycin. They differ from each other by pigment formation, carbohydrate fermentations and haemolysis. The actual habitat of *E. gallinarum* and *E. flavescens* has not been confirmed. *Enterococcus casseliflavus* occurs mainly in plants (Devriese and Pot, 1995).

The *Enterococcus cecorum* species group currently consists of two species, *E. cecorum* and *Enterococcus columbae*. *Enterococcus cecorum* is the enterococcal species most frequently isolated from adult chicken intestines, and has also been isolated from the intestines of other animals. *Enterococcus columbae* is isolated mostly from pigeons. The two species are differentiated by carbohydrate fermentation reactions (Devriese and Pot, 1995).

Other enterococci that are unrelated to food and health include *Enterococcus sulfureus, Enterococcus saccharolyticus* and *Enterococcus dispar* (Devriese and Pot, 1995).

2.1.5 The genus *Lactococcus*

Lactococci are non-motile spheres or ovoid cells that may occur singly, in pairs or in chains (Teuber, 1995). Although the history of the lactic streptococci started more than a century ago, the genus *Lactococcus* was only described 15 years ago (Schleifer *et al.*, 1985). The nutritionally fastidious species of this genus can easily be distinguished from *Pediococcus* and *Leuconostoc* spp. by the products produced from glucose fermentation. *Lactococcus*

comprises the species *Lactococcus lactis* subspp. *lactis*, *diacetylactis*, *cremoris* and *hordniae*, *Lactococcus garvieae*, *Lactococcus plantarum*, *Lactococcus raffinolactis* and *Lactococcus piscium* (Klijn *et al.*, 1991; Pot *et al.*, 1994; Stiles and Holzapfel, 1997). *Lactococcus lactis* have been isolated from raw milk and milk products. The subspecies *lactis* and *cremoris* are the main starter cultures used in the production of cheese (Stiles and Holzapfel, 1997). *Lactococcus lactis* subsp. *cremoris* can be distinguished from the closely related *L. lactis* subsp. *lactis* by differential medium and SDS-PAGE of whole-cell proteins (Descheemaeker *et al.*, 1994). The use of oligonucleotide probes proved to be a highly sensitive and rapid technique for the specific identification of *L. lactis* subsp. *cremoris* (Salama *et al.*, 1991).

The habitats of the other *Lactococcus* spp. have not been extensively researched. *Lactococcus garvieae* has been isolated from cows with mastitis, *L. plantarum* from frozen peas, *L. raffinolactis* from raw milk, *L. lactis* subsp. *hordniae* from a leafhopper, and *L. piscium* from a diseased rainbow trout (Schleifer *et al.*, 1985; Williams *et al.*, 1990). Lactococci are not found in faecal material or soil. Acid production from carbohydrates as well as rRNA gene restriction analysis can be used to identify and differentiate the *Lactococcus* spp. (Köhler *et al.*, 1991; Rodrigues *et al.*, 1991; Teuber, 1995).

2.1.6 The genera *Leuconostoc*, *Oenococcus* and *Weissella*

Leuconostocs are classified as facultative anaerobic cocci, but in media other than milk these organisms appear elongated and may be classified as rods (Stiles, 1994). Species from this genus produces D (-) lactate from glucose, as opposed to L (+) lactate that is produced by lactococci and DL-lactate that is produced by heterofermentative lactobacilli (Stiles and Holzapfel, 1997). *Leuconostoc*, *Lactobacillus* and *Pediococcus* spp. are phylogenetically closely related (Collins *et al.*, 1991). *Leuconostoc* is the predominant genus among lactic acid bacteria on plants, but are also found in milk and dairy products (Garvie, 1986; Stiles and Holzapfel, 1997) and vacuum-packed meats (Shaw and Harding, 1984). The genus *Leuconostoc* is divided into the species *Leuconostoc mesenteroides* subspp. *mesenteroides*, *dextranicum* and *cremoris*, *Leuconostoc paramesenteroides*, *Leuconostoc pseudomesenteroides*, *Leuconostoc lactis*, *Leuconostoc carnosum*, *Leuconostoc gelidum*, *Leuconostoc amelibiosum*, *Leuconostoc citreum*, *Leuconostoc fallax* and *Leuconostoc argentinum* (Dellaglio *et al.*, 1995).

The species *Leuconostoc oenos* (Garvie, 1986), an important organism in wine and related habitats, was recently reclassified as *Oenococcus oeni* (Dicks *et al.*, 1995).

Following taxonomic studies on atypical lactobacilli isolated from dry fermented Greek sausage, Collins *et al.* (1993) proposed the reclassification of *Leuconostoc paramesenteroides* and related organisms to the genus *Weissella*. Several species have been proposed: *Weissella paramesenteroides*, *Weissella hellenica*, *Weissella confusa, Weissella halotolerans, Weissella kandleri, Weissella minor* and *Weissella viridescens* (Collins *et al.*, 1993). The differentiation of these organisms from *Leuconostoc* requires the use of a combination of taxonomic methods such as acid production from sugar fermentations, dextran formation, lactic acid configuration and murein type.

2.1.7 The genera *Pediococcus* and *Tetragenococcus*

Pediococcus is a phylogenetically heterogeneous genus comprising eight species (Collins *et al.*, 1990). Pediococci are non-motile cocci of uniform size. The mode of division of pediococci to form tetrads is characteristic of this genus. *Pediococcus damnosus* is associated with beer, wine and cider, *Pediococcus dextrinicus* with beer and silage, *Pediococcus parvulus* with sauerkraut and silage, and *Pediococcus inopinatus* with sauerkraut and beer. *Pediococcus pentosaceus* and *Pediococcus acidilactici* are associated with vegetable material, milk and dairy products, while *P. pentosaceus* is also used as a starter culture in fermented sausages (Simpson and Taguchi, 1995).

Collins *et al.* (1990) proposed the genus *Tetragenococcus* to accommodate the organism *Pediococcus halophilus.* This species plays an important role in the production of soy sauce (Stiles and Holzapfel, 1997). 16S rRNA sequence analysis revealed that the species is more related to the genera *Enterococcus* and *Carnobacterium* than to *Pediococcus* and other lactic acid bacteria (Collins *et al.*, 1990). The species *Pediococcus urinae-equi* is more closely related to the genus *Aerococcus* (Collins *et al.*, 1990).

2.1.8 The genus *Streptococcus*

The streptococci are spherical or ovoid cells arranged in chains or pairs (Teuber, 1995). This genus originally comprised four groups, the enterococci, lactic streptococci, viridans streptococci and pyogenic streptococci. Before the advent of molecular taxonomy, the streptococci were identified using properties such as haemolytic changes on blood agar plates and serological methods, such as the Lancefield groupings (Hardie and Whiley, 1995). Lactic streptococci mostly belonged to serological group N, pathogenic streptococci to groups A, B,

and C, and the enterococci to group D (Teuber, 1995). The four groups comprising *Streptococcus* have in recent years been subdivided into three genera, viz. *Enterococcus*, *Streptococcus* and *Lactococcus* (Pot *et al.*, 1994). The organisms still classified as *Streptococcus* are of the oral (viridans) and the pyogenic groups. These organisms are of medical importance since they occur in the alimentary, respiratory and urogenital tracts of humans as normal flora, and some organisms, particularly of the pyogenic group, are pathogens (Pot *et al.*, 1994). The only food-related species is *S. thermophilus* (*Streptococcus salivarius* subsp. *thermophilus*), an important starter culture for cheese and yogurt, together with the organisms *L. delbrueckii* subsp. *bulgaricus*, *L. lactis* and (or) *L. helveticus* (Stiles and Holzapfel, 1997).

2.1.9 The genus *Vagococcus*

Vagococcus fluvialis, a motile organism isolated from chicken faeces and river water, was originally identified as *Streptococcus fluvialis,* reacting with Lancefield group N antiserum. 16S rRNA sequence analysis revealed a closer relationship to the genus *Enterococcus* than to the lactic streptococci (*Lactococcus*) and a new genus, *Vagococcus*, was proposed (Collins *et al.*, 1989). Based on the high level of 16S rRNA sequence similarities, two new species, *Vagococcus salmoninarum*, isolated from rainbow trout (Wallbanks *et al.* 1990), and *Vagococcus lutrae*, isolated from a common otter, (Lawson *et al.*, 1999), have subsequently been described.

2.1.10 The genus *Bifidobacterium*

Members of the genus *Bifidobacterium* are non-motile, anaerobic rods mainly found in the intestinal tract of humans and animals (Stiles and Holzapfel, 1997). In addition, *Bifidobacterium* spp. form part of the normal microflora of the vagina, cervix, oral cavity, insect intestines and sewage (Sgorbati *et al.*, 1995). *Bifidobacterium* spp. appear to contribute to the maintenance of a balanced microflora in the intestine. This beneficial probiotic effect has resulted in the inclusion of *Bifidobacterium* spp. in milk and milk by-products, and pharmaceutical products (Sgorbati *et al.*, 1995; Stiles and Holzapfel, 1997). Sgorbati *et al.* (1995) listed 29 different species of *Bifidobacterium*, of which the most important in the food industry are *Bifidobacterium bifidum*, *Bifidobacterium longum* and *Bifidobacterium breve*.

2.2 INDUSTRIAL IMPORTANCE OF LACTIC ACID BACTERIA

Historically, the fermentation of food was based on the activity of natural microflora that contaminated raw material (De Vuyst and Vandamme, 1994). These fermentations were gradually improved, and nowadays, worldwide, huge industries exist that produces fermented food products. Milk alone can be fermented to more than 1000 products, each different in flavour and texture. Therefore, great demands exist on the metabolic activity, stability and bacteriophage resistance of starter cultures (Mäyrä-Mäkinen and Bigret, 1993). *Lactobacillus* spp. and *Lactococcus* spp. are usually the major components of starter cultures used in the production of fermented dairy products (Teuber, 1995).

Starter cultures can be mesophilic, growing at temperatures of 10-40°C, or thermophilic, with optimum growth temperatures being 40-50°C (Mäyrä-Mäkinen and Bigret, 1993). These cultures may be a single strain of a specific species, different known strains of a species, different known strains of different species, or species and strains that are partly or totally unknown (Mäyrä-Mäkinen and Bigret, 1993). Most mesophilic starters include *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *diacetylactis*, *L. lactis* or *L. mesenteroides* subsp. *cremoris*. Thermophilic starter cultures include *S. thermophilus*, *L. delbrueckii* subspp. *lactis* and *bulgaricus* and *L. helveticus* (Mäyrä-Mäkinen and Bigret, 1993). The physiological functions of starter cultures are important, since they influence the organoleptic quality of the end-product. The function of starter cultures is to ferment sugars with a subsequent decrease in pH and reduction of growth of adventitious microflora, to hydrolyze proteins to create the correct texture and taste, to synthesize flavour compounds and texturing agents, and to produce inhibitory components (Mäyrä-Mäkinen and Bigret, 1993). Technological improvement of lactococci in particular, has become a major area of research. Important functions such as lactose utilization, casein degradation, citrate uptake, bacteriocin production, bacteriophage resistance and polysaccharide formation are often associated with unstable and naturally transferable plasmids. Since lactococci have the ability to transfer genes by conjugation and transduction, genetically improved starter cultures are being developed which, through their acquired properties, will improve the overall quality of the food products (Teuber, 1995; Von Wright and Sibakov, 1993).

Lactic acid bacteria are also utilized in the production of cereal based foods such as sourdough breads, made from rye or wheat, and products made from maize, sorghum, millet and other cereal grains (Salovaara, 1993). Sourdough fermentation renders dough with rye flour more suitable for baking. Dough acidification and salt inhibit amylases that would otherwise degrade starch after gelatinization. In addition, compounds that improve the flavour

and aroma of bread are produced by the microorganisms (Lücke, 1996). Organisms associated with sourdough fermentations include *L. sanfranciscensis, L. pontis, P. pentosaceus, L. plantarum, L. brevis, L. fructivorans, L. fermentum* and *L. reuteri* (Lücke, 1996). In the production of fermented meat products, the organisms *P. acidilactici*, or other *Pediococcus* spp., *L. plantarum, Micrococcus varians*, *Staphylococcus xylosus* and *Staphylococcus carnosus* are used as starter cultures in combination with various concentrations of sugar, salt and spices (Lücke, 1996; Mäyrä-Mäkinen and Bigret, 1993). Lactic acid bacteria used in meat fermentations are all homofermentative. Production of lactic acid decreases the pH, leading to inhibition of spoilage bacteria as well as accelerating the formation of curing colour. In various types of spontaneously fermented sausages *L. sakei* and *L. curvatus* were the most competitive at temperatures below 25°C (Lücke, 1996). To ferment vegetables for the production of products such as sauerkraut, pickled cucumbers and green olives, *L. plantarum*, various *Pediococcus* spp., *L. mesenteroides* and *L. brevis* are most often used in combination with NaCl (Mäyrä-Mäkinen and Bigret, 1993). The production of soy sauce involves the initial fermentation of heat-treated raw materials by fungi (various *Aspergillus* spp.), with subsequent fermentation by *Tetragenococcus halophilus* in the presence of 13-19 % (w/v) salt (Lücke, 1996).

Lactic acid bacteria are claimed to be beneficial to human and animal health by improving the integrity of the intestinal microflora (Salminen *et al.*, 1993). Health benefits attributed to lactic acid bacteria include the control of infections in the intestine and urogenital tract, control of lactose intolerance, reduction of carcinogenic enzymes leading to the formation of tumors in the colon, reduction of serum cholesterol levels and stimulation of the immune system and bowel movement (Ray, 1996). Probiotics of lactic acid bacteria are used increasingly to promote health. The term probiotic refers to viable bacteria, cultured dairy products, or food supplements that contain viable lactic acid bacteria (Salminen *et al.*, 1993). Probiotic strains for both humans and animals need to be antagonistic against pathogenic and carcinogenic bacteria, but also stable in acidic environments and in the presence of bile salts. The adherence to intestinal cells and colonization in the intestinal tract is an added advantage. *In vitro* growth and safety in humans are also important considerations (Salminen *et al.*, 1993). Various strains of *L. acidophilus, L. casei, L. delbrueckii* subsp. *bulgaricus, E. faecium* and *Bifidobacterium* spp. are used as probiotics (Ballongue, 1993; Nousiainen and Setälä, 1993; Salminen *et al.*, 1993).

2.3 ANTIMICROBIAL ACTION OF LACTIC ACID BACTERIA

The antimicrobial action of lactic acid bacteria is often a complex interaction of several phenomena to produce a combined effect (Earnshaw, 1992). The antimicrobial effect is attributed to various factors, such as fermentation end-products, and production of substances by individual strains of bacteria, including bacteriocins and other antagonistic systems (Lindgren and Dobrogosz, 1990; De Vuyst and Vandamme, 1994).

2.3.1 Fermentation end-products

The species of the organism(s) involved, the chemical composition of the culture environment and the prevailing physical conditions during the fermentation process, affect the levels and proportions of fermentation end-products that accumulate (Lindgren and Dobrogosz, 1990).

2.3.1.1 Organic acids

The rapid production of organic acids, primarily lactic acid, during the fermentation of carbohydrate-rich food and feed, results in an acidic culture environment which inhibits the growth and metabolic activities of other organisms that may be present (Lindgren and Dobrogosz, 1990). Very few non-lactic bacteria are able to grow at pH levels lower than the threshold pH (3.8-4.4) of lactic acid bacteria (Piard and Desmazeaud, 1991). In addition to the decrease in pH, the undissociated form of the acid molecule causes the collapse of the electrochemical proton gradient, causing eventual cell death of susceptible organisms (Earnshaw, 1992). Other organic acids that are produced include acetic acid, formic acid, malic acid and citric acid. Acetic acid (pKa = 4.75) is a more effective inhibitor than lactic acid (pKa = 3.08) (Davidson and Hoover, 1993). Preservation is usually affected by the simultaneous presence of various acids and/or other antimicrobial compounds (Davidson and Hoover, 1993). An example is the antimould activity observed in sourdough fermentations. The activity was identified as a mixture of acetic, caproic, formic, propionic, butyric and *n*-valeric acids produced by *L. sanfranciscensis* CB1 (Corsetti *et al.*, 1998).

2.3.1.2 Carbon dioxide

Carbon dioxide per se has antimicrobial activity. Carbon dioxide also creates an anaerobic

environment by replacing existent molecular oxygen in the fermentation product, thereby inhibiting the growth of aerobic organisms. The sensitivity of organisms to carbon dioxide can vary greatly. Low concentrations of carbon dioxide may have no effect on the growth of some organisms or may stimulate growth, while higher concentrations may inhibit growth of other organisms (Lindgren and Dobrogosz, 1990).

2.3.1.3 Oxygen metabolites

The production of the oxygen metabolites hydrogen peroxide (H_2O_2), superoxide anions (O_2^-) and hydroxyl radicals ($OH^\bullet$) is dependent on the availability of oxygen and on the particular strains present (Lindgren and Dobrogosz, 1990; Piard and Desmazeaud, 1991). Hydrogen peroxide accumulates because lactic acid bacteria do not produce catalase (Davidson and Hoover, 1993). The bactericidal effect of hydrogen peroxide may be attributed to its strong oxidizing effect on the bacterial cell and to the destruction of basic molecular structures of cell proteins (Lindgren and Dobrogosz, 1990). Free radicals and hydrogen peroxide can damage bacterial nucleic acids (Piard and Desmazeaud, 1991). In milk, the antimicrobial lactoperoxidase system is activated by hydrogen peroxide, to which *Escherichia coli*, *Salmonella* and *Pseudomonas* spp. are sensitive, but which minimally affects Gram-positive bacteria (Earnshaw, 1992; Lindgren and Dobrogosz, 1990). The production of hydrogen peroxide has many complicated controlling factors, and since the oxidizing nature of this compound can have adverse effects on the sensory quality of foods, the use of this compound as a preservative is limited (Earnshaw, 1992). In addition, oxygen metabolites may affect both lactic acid bacteria and other unwanted organisms (Piard and Desmazeaud, 1991).

2.3.1.4 Fatty acids

Under optimal conditions some lactic acid bacteria produce significant amounts of fatty acids, which are known to show antimicrobial activity (Earnshaw, 1992). Short chain fatty acids such as acetic-, propionic- and sorbic acids are used as preservatives in food, while medium-chain fatty acids are used as surface active or emulsifying agents (Jay, 1992). Fatty acids with a carbon chain length of 12 to 16 (C_{12} – C_{16}) are primarily active against Gram-positive bacteria and C_{10} – C_{12} fatty acids against yeasts, while Gram-negative bacteria are not inhibited by fatty acids (Jay, 1992). The most active antimicrobial lipid is monolaurin (Branen *et al.*, 1980; Jay, 1992; Kabara *et al.*, 1977).

Ouattara *et al.* (1997) examined the antibacterial effect of the fatty acids lauric-, myristic-,

palmitic-, palmitoleic-, stearic-, oleic-, linoleic-, and linolenic acid against the meat spoilage organisms *Pseudomonas fluorescens*, *Serratia liquifaciens*, *Brochothrix thermosphacta*, *C. piscicola*, *L. curvatus* and *L. sakei*. Lauric- and palmitoleic acids exhibited the greatest inhibitory effect, while myristic-, palmitic-, stearic-, and oleic acid were ineffective. All the fatty acids failed to inhibit *B. thermosphacta*, *P. fluorescens* and *S. liquifaciens*.

Rao and Reddy (1984) studied the effect of fermentation of whole milk by *L. acidophilus*, *L. bulgaricus* (now *L. delbrueckii* subsp. *bulgaricus*) and *S. thermophilus* on fatty acid composition. Only moderate changes occurred in the amount of glyceride fatty acids and total cholesterol level, while significant increases in the level of the free fatty acids stearic acid and oleic acid were observed. No unusual fatty acids present in lactic acid bacteria were detected.

2.3.1.5 Diacetyl

Diacetyl is produced by organisms able to ferment citrate, such as *L. mesenteroides* subsp. *cremoris* and *L. lactis* subsp. *diacetylactis*. This compound produces a buttery flavour in fermented dairy products and is used as a flavour additive in food (Davidson and Hoover, 1993). Diacetyl is also found in wine, brandy, roasted coffee and silage (De Vuyst and Vandamme, 1994). This compound plays an important role as a biopreservative in combination with other compounds, but the effective concentration is too high for it to be used on its own as a biopreservative (Davidson and Hoover, 1993; Earnshaw, 1992).

2.3.2 Other compounds

Heterofermentative lactic acid bacteria can produce acetaldehyde during carbohydrate fermentation to form ethanol. The absence of alcohol dehydrogenase may result in the excretion of acetaldehyde. Although acetaldehyde appears to possess antagonistic properties (Piard and Desmazeaud, 1991), its antagonistic effect has been relatively undocumented (De Vuyst and Vandamme, 1994). Acetaldehyde produced by *L. bulgaricus* in yoghurt contributes to the aroma of the product (Piard and Desmazeaud, 1991). During fermentation of peanut milk with various lactic acid bacteria, hexanal, one of the substances responsible for undesirable green/beany flavour in peanut milk, disappeared. Simultaneously, the acetaldehyde content of the peanut milk increased during fermentation, with a significant increase in creamy flavour (Lee and Beuchat, 1991).

Lactobacillus reuteri produces a wide spectrum antimicrobial compound, reuterin, during anaerobic growth in the presence of glycerol (Earnshaw, 1992). Reuterin is active against a

variety of Gram-negative and Gram-positive bacteria, yeasts, fungi and protozoa (Daeschel, 1989).

Various unidentified low-molecular mass non-protein antimicrobial substances produced by lactic acid bacteria have been reported (De Vuyst and Vandamme, 1994). *L. plantarum* VTT-E-78076 was found to produce antimicrobial compounds such as methylhydantoin, mevalonolactone and benzoic acid. These low-molecular mass compounds appeared to act co-operatively (Niku-Paavola *et al.*, 1999). Skyttä *et al.* (1993) reported the broad-spectrum antimicrobial activity of three *Pediococcus* spp. (*P. damnosus* VTT-E-76065, *P. pentosaceus* VTT-E-76067 and *P. pentosaceus* VTT-E-76068) isolated from beer. Gram-positive and Gram-negative organisms were inhibited, and the compounds were not sensitive to proteolytic enzymes.

Other factors that play a role in the antimicrobial potential of lactic acid bacteria include competition for nutrients, phage-induced antibacterial proteins (lysins), and coaggregation (De Vuyst and Vandamme, 1994).

2.3.3 Bacteriocins

Bacteriocins can be defined as biologically active proteins or protein complexes that display a bactericidal mode of action towards usually closely related species (De Vuyst and Vandamme, 1994). The potential use of bacteriocins as natural preservatives have resulted in the report of numerous bacteriocins produced by all the genera of the lactic acid bacteria. Bacteriocins are discussed in detail in Chapter 3.

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CHAPTER 3

BACTERIOCINS OF LACTIC ACID BACTERIA

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3.1 INTRODUCTION

Bacteriocins are biologically active proteins or protein complexes that display a bactericidal mode of action towards usually closely related species (De Vuyst and Vandamme, 1994). Numerous bacteriocins produced by most of the genera of lactic acid bacteria have been reported during the last decade, some of which are discussed or mentioned in Sections 3.3.1-3.3.8. More than thirty of these bacteriocins have been fully characterized (Carolissen-Mackay *et al.*, 1997, Nettles and Barefoot, 1993, Piard and Desmazeaud, 1992), but the extent of bacteriocin diversity is still unknown, since several bacteriocins that have been characterized do not belong to any of the currently recognized classes of bacteriocins as described in Section 3.2.1.

Biochemical characterization of several bacteriocins have indicated that they are often identical, even if produced by the same species, but isolated from different sources, for example pediocin PA-1 and pediocin AcH (Gonzalez and Kunka, 1987; Bhunia *et al.*, 1988). Identical bacteriocins such as sakacin A and curvacin A have also been isolated from different organisms (Tichaczek *et al.*, 1993; Holck *et al.*, 1992).

Several bacteriocin-producing organisms have initially been identified incorrectly, for instance the producer of pediocin 5 (Daba *et al.*, 1991; 1994; Huang *et al.*, 1996) and bavaricin MN (Kaiser and Montville, 1996). Some bacteriocin-producing organisms produce only one antimicrobial peptide for instance pediocin PA-1, produced by *Pediococcus acidilactici* PAC1.0 (Marugg *et al.*, 1992), while other producers, for instance *Leuconostoc mesenteroides* TA33a, secrete more than one bacteriocin (Papathanasopoulos *et al.*, 1997).

3.2 CHARACTERISTICS OF BACTERIOCINS

Bacteriocins differ widely in molecular weight, pI, and presence and number of particular groups of amino acids, although differences in antimicrobial activity can not be attributed to particular amino acids or sequence of amino acids (Jack *et al.*, 1995). Most of the low molecular weight bacteriocins are cationic at pH 7, and many of these bacteriocins have greater antimicrobial activity at low pH. Adsorption of bacteriocins to Gram-positive cell surfaces is also pH dependent, with maximum adsorption at or above pH 6 (Jack *et al.*, 1995).

3.2.1 Classification of bacteriocins

Klaenhammer (1993) originally defined four distinct classes of lactic acid bacteria bacteriocins: Class I: lantibiotics, Class II: small (< 10 kDa) heat-stable membrane-active peptides, Class III: large (> 30 kDa), heat-labile proteins, and Class IV: complex bacteriocins. Class II bacteriocins were further divided into *Listeria*-active peptides with an N-terminal consensus sequence (Class IIa), poration complexes requiring two different peptides for activity (Class IIb) and thiol-activated peptides that require reduced cysteine residues for activity (Class IIc), such as lactococcin B (Venema *et al.*, 1993). Subsequent studies have, however, suggested that the cysteine is not essential for activity, and that the thiol-activated peptide group should be excluded from the classification (Nes *et al.*, 1996; Venema *et al.*, 1996). The Class IV complex bacteriocins consisted of bacteriocins that contained a protein and one or more chemical moieties, such as lipid or carbohydrate. The existence of this class was supported by the inactivation of some bacteriocins by glycolytic and lipolytic enzymes. Several bacteriocins produced by *Leuconostoc* spp., such as leuconocin S and carnocin LA54A, are inactivated by α-amylase (Keppler *et al.*, 1994; Lewus *et al.*, 1992). Subsequent studies suggested that these complexes may be artifacts caused by interaction between cell constituents or growth medium and regular peptide bacteriocins (Nes *et al.*, 1996).

Cintas *et al.* (1998) proposed a new class of bacteriocins that are unrelated to that defined for Class II. Enterocin L50, produced by *Enterococcus faecium* L50, are secreted without an N-terminal leader sequence or signal peptide, and are more related to a group of cytolytic peptides secreted by staphylococci.

Currently, bacteriocins are classified as follows (Moll *et al.*, 1999; Nes *et al.*, 1996). Class I, lantibiotics, is further divided into type A lantibiotics and type B lantibiotics. Type A lantibiotics are elongated, cationic, pore forming peptides. Type B lantibiotics are compact, with globular structures, are enzyme inhibitors and are immunologically active (De Vuyst and Vandamme, 1994). Class II, the small heat-stable non-lanthionine peptides, is divided into four groups: Class IIa consists of *Listeria*-active peptides with an N-terminal consensus sequence. Class IIb are two-peptide bacteriocins. Class IIc contains *sec*-dependent bacteriocins, and Class IId contains the small heat-stable non-lanthionine bacteriocins that do not belong to any of the three groups within Class II. Class III consists of large heat labile bacteriocins (Moll *et al.*, 1999). Most of the bacteriocins belong to Class I or Class II. Research has focussed on these two classes, since they are the most abundant and have the best potential for industrial application (Nes *et al.*, 1996). Bacteriocins of food-related lactic acid bacteria that have been charcterized are summarized in Table 3.1.

Table 3.1. Some characterized bacteriocins representing the genera of the lactic acid bacteria and classified according to Nes *et al.* (1996).

Genera	Organism	Bacteriocin[1]	Classification[2]	Reference
***Bifidobacterium* sp.**	*B. bifidum*	bifidocin B	Class IIa	Yildirim *et al.*, 1998
***Carnobacterium* sp.**	*C. piscicola*	carnocin U149	Class I	Stoffels *et al.*, 1992
		carnobacteriocin BM1	Class IIa	Quadri *et al.*, 1994
		carnobacteriocin B2	Class IIa	Quadri *et al.*, 1994
		carnobacteriocin A	Class IId	Worobo *et al.*, 1994
		piscicocin V1a	Class IIa	Bhugaloo-Vial *et al.*, 1996
		piscicolin 61	Class IId	Holck et al., 1994
	C. divergens	divergicin 750	Unknown	Holck *et al.*, 1996
		divergicin A	Class IIc	Worobo *et al.*, 1995
		divercin V41	Class IIa	Métivier *et al.*, 1998
***Enterococcus* sp.**	*E. faecium*	enterocin A	Class IIa	Aymerich *et al.*, 1996
		enterocin B	Class IId	Casaus *et al.*, 1997
		enterocin CRL 35	Class IIa	Farias *et al.*, 1996
		enterocin P	Class IIc	Cintas *et al.*, 1997
		enterocin I	Unknown	Floriano et al., 1998
	E. faecalis	cytolysin	Class I	Gilmore et al., 1994
		enterocin AS-48	Unknown	Gálvez *et al.*, 1989
		bacteriocin 31	Class IIc	Tomita *et al.*, 1996
	E. mundtii	mundticin	Class IIa	Bennik *et al.*, 1998
***Lactobacillus* sp.**				
Homofermentative *Lactobacillus* sp.				
	L. johnsonii	lactacin F	Class IIb	Fremaux *et al.*, 1993
	L. acidophilus	acidocin J1132	Class IIb	Tahara *et al.*, 1996
		acidocin A	Class IIa	Kanatani *et al.*, 1995a
		acidocin B	Class IIc	Leer *et al.*, 1995
		acidocin 8912	Class IId	Kanatani *et al.*, 1995
	L. helveticus	helveticin J	Class III	Joerger and Klaenhammer,1986
		helveticin V-1829,	Unknown	Vaughan *et al.*, 1992
	L. amylovorus	lactobin A	Class IIb	Contreras *et al.*, 1997
	L. salivarius	salivaricin A	Class I	Ross *et al.*, 1993
Facultative heterofermentative *Lactobacillus* sp.				
	L. plantarum	plantaricin C19	Class IIa	Atrih *et al.*, 1993
		plantaricin C	Class I	González, *et al.*, 1994
		plantaricin EF	Class IIb	Andersson *et al.*, 1998
		plantaricin JK	Class IIb	Andersson *et al.*, 1998
		plantaricin S	Class IIb	Jiménez-Diaz *et al.*, 1993
		plantaricin-149	Unknown	Kato *et al.*, 1994

Table 3.1 (continued)

	L. casei	caseicin 80	Class III	Rammelsberg *et al.*, 1990
	L. curvatus	curvaticin FS47	Class IId	Garver and Muriana, 1994
	L. sakei	lactocin S	Class I	Mørtvedt and Nes, 1990
		sakacin P	Class IIa	Tichaczek *et al.*, 1992
		sakacin 674	Class IIa	Holck *et al.*, 1994
		sakacin A	Class IIa	Holck *et al.*, 1992
		bavaricin MN	Class IIa	Lewus and Montville, 1992
	L. bavaricus	bavaricin A	Class IIa	Larsen *et al.*, 1993
***Lactococcus* sp.**	*L. lactis* subsp. *lactis*	nisin A	Class I	Teuber, 1995
		nisin Z	Class I	Mulders *et al.*, 1991
		lactococcin 972	Class IIc	Martínez *et al.*, 1996
	L. lactis subsp. *cremoris*	lactococcin A	Class IId	Van Belkum *et al.*, 1992
		lactococcin B	Class IId	Van Belkum *et al.*, 1992
		lactococcin M	Unknown	Van Belkum *et al.*, 1992
	L. lactis	lacticin 481	Class I	Piard *et al.*, 1990
		lactococcin G	Class IIc	Nissen-Meyer *et al.*, 1992
		lacticin 3147	Class I	Ryan *et al.*, 1996
***Leuconostoc* sp.**	*Leuconostoc* MF215B	leucocin H	Class IIb	Blom *et al.*, 1999
	L carnosum	leucocin B-TA11a	Class IIa	Felix *et al.*, 1994
	L. mesenteroides	mesentericin Y105	Class IIa	Héchard *et al.*, 1992a, b
		mesentericin 52B	Unknown	Revol-Junelles *et al.*, 1996
		leucocin B-TA33b	Unknown	Papathanasopoulos *et al.*, 1998
		leucocin C-TA33c	Class IIa	Papathanasopoulos *et al.*, 1998
	L. gelidum	leucocin A-UAL 187	Class IIa	Hastings and Stiles, 1991
***Pediococcus* sp.**	*P. acidilactici*	pediocin PA-1	Class IIa	Gonzalez and Kunka, 1987
***Streptococcus* sp.**	*S. thermophilus* Sfi13	thermophilin 13	Class IIb	Marciset *et al.*, 1997

[1]Identical bacteriocins were not repeated

[2]Classification:

Class 1, lantibiotics

Class II, small heat-stable non-lanthionine bacteriocins

Class IIa, *Listeria*-active peptides with an N-terminal consensus sequence (YGNGV)

Class IIb, two peptide bacteriocins

Class IIc, *sec*-dependent bacteriocins

Class IId, Class II bacteriocins that do not belong to Class IIa, b or c

Class III, large heat labile bacteriocins

Unknown, sequence data is available, but the classification of the bacteriocin has not been published

3.2.1.1 Class I: Lantibiotics

Lantibiotics (*lan*thionine-containing an*tibiotic* peptides) are small (less than 5 kDa, with 19 to 38 amino acids) membrane-active peptides that contain unusual, posttranslationally modified amino acids such as lanthionine (Lan), β-methyl lanthionine (MeLan) and dehydrated residues (Klaenhammer, 1993, Sahl and Bierbaum, 1998). Posttranslational peptide modification usually involves only the amino acids serine, threonine and cysteine, although lysine, aspertate and isoleucine residues may also be found in modified form (Sahl and Bierbaum, 1998). All lantibiotics currently documented are produced by Gram-positive bacteria (Nes and Tagg, 1996). The gene cluster encoding lantibiotic peptides usually also contain a gene or genes that encode specific enzymes able to facilitate the dehydration of certain residues in the propeptide region, followed by the addition of cysteine residues to form characteristic Lan and MeLan sulfur ring structures (Nes and Tagg, 1996). Considerable differences in the leader peptide sequence of type A lantibiotics have been observed. Nisin A and nisin Z, produced by *Lactococcus lactis*, have a proline residue at the –2 cleavage site, together with several lantibiotics produced by other Gram-positive bacteria such as subtilin, produced by *Bacillus subtilis*, and pep5, epilancin K7, epidermin and gallidermin, produced by *Staphylococcus epidermides* (Nes and Tagg, 1996). The leader peptides of the lantibiotics salivaricin A, produced by *Lactobacillus salivarius*, lacticin 481 (= lactococcin DR) produced by *L. lactis*, streptococcin A-FF22, produced by *Streptococcus pyogenes*, cytolysin LL and cytolysin LS, produced by *Enterococcus faecalis* are all of the double glycine type (Nes and Tagg, 1996). Other lantibiotics produced by lactic acid bacteria include lactocin S, produced by *Lactobacillus sakei,* carnocin U149, produced by *C. piscicola* and mutacin, produced by *Streptococcus mutans* (Nes and Tagg, 1996). A two-peptide lanthionine, cytolysin, has also been reported (Gilmore *et al.,* 1994).

3.2.1.2 Class II: Small heat-stable bacteriocins

These bacteriocins can be defined as small (less than 10 kDa), do not contain any unusual amino acids, are membrane-active and heat resistant up to temperatures of 100°C, or autoclavable. Most of these bacteriocins are characterized by the so-called double glycine (G-G) processing site in the bacteriocin precurser (Klaenhammer, 1993). The bacteriocins of Class II share various features, such as the occurrence of a high content of small amino acids such as glycine, being strongly cationic with pI's between 8 and 11, and the possession of hydrophobic and amphiphilic domains (Abee, 1995).

3.2.1.2.1 Class IIa: *Listeria*-active bacteriocins

Members of this group, also referred to as pediocin-like bacteriocins, are produced by a wide variety of lactic acid bacteria, and several have been biochemically characterized. Although the antimicrobial spectrum of these bacteriocins is different, they are all active against *Listeria* spp. and share a conserved amino acid sequence, YGNGV, in their structure (Table 3.2). The function of the YGNGV consensus motif is not clear, since the mechanism for initial binding of the bacteriocins to the target membranes involves electrostatic interactions between positive amino acid residue groups and negatively charged membrane phospholipid groups, without involvement of the YGNGV motif (Chen *et al.*, 1997a).

Pediocin PA-1 is the most characterized bacteriocin within this group. The production of pediocin PA-1 is associated with a 9.4 kbp plasmid (pSRQ11) in the producing organism, *P. acidilactici* PAC1.0. A 5.6 kbp fragment of this plasmid, when introduced into *Escherichia coli*, produced the bacteriocin (Marugg *et al.*, 1992). A bacteriocin designated pediocin AcH, produced by *P. acidilactici* LB42-923 and production associated with a 8 877 bp plasmid designated pSMB74, was found to be identical to pediocin PA-1 (Bukhtiyarova *et al.*, 1994). Other bacteriocins that are part of this group include curvacin A (Tichaczek *et al.*, 1993), leucocin A (Hastings *et al.*, 1991), sakacin P (Tichaczek *et al.*, 1994), mundticin (Bennik, *et al.*, 1998), enterocin A (Aymerich *et al.*, 1996), bavaricin A (Larsen *et al.*, 1993), piscicolin 126 (Jack *et al.*, 1996), mesentericin Y105 (Héchard, *et al.*, 1992a, b), carnobacteriocin B2 (Quadri *et al.*, 1994), and bavaricin MN (Kaiser and Montville, 1996).

3.2.1.2.2 Class IIb: Two-peptide complexes

The activity of these bacteriocins depends on the complementary activity of two peptides. Examples include lactacin F and lactacin X (Fremaux *et al.*, 1993; Muriana and Klaenhammer, 1991), lactococcin G (Nissen-Meyer *et al.*, 1992), plantaricin Sa and b (Jiménez-Diaz *et al.*, 1995; Stephens *et al.,* 1998), thermophilin 1 and 2 (Marciset *et al.*, 1997), acidocin J1132a and b (Tahara *et al.*, 1996), plantaricin J and K (Anderssen *et al.*, 1998; Diep *et al.*, 1996), lactobin A (Contreras *et al.*, 1997) and plantaricin E and F (Anderssen et al., 1998; Diep *et al.*, 1996). Some two-peptide bacteriocins such as lactococcin G and lactococcin M need both peptides for activity, while one or both peptides of plantaricin S, lactacin F and thermophilin 13 are active. The combined effect of the two peptides of these bacteriocins is much greater than the total activity calculated from the individual effect of these peptides (Cintas *et al.*, 1998).

Table 3.2. Amino acid sequences of Class IIa bacteriocins with anti-*Listeria* activity containing the YGNGV consensus sequence

Bacteriocin	Leader peptide[1]	Mature peptide	Reference
Acidocin A	MISMISSHQKTLTDKELALISGG	KTYYGTNGVHCTKKSLWGKVRLKNVIPGTLCRKQSLPIKQDLKILLGWATGAFGKTFH	Kanatani *et al.*, 1995a
Bavaricin A		KYYGNGVHXGKHSXTVDWGTAIGNIGNNAAANXATGXNAGG	Larsen *et al.*, 1993
Bavaricin MN		TKYYGNGVYXNSKKXWVDWGQAAGGIGQTVVXGWLGGAIPGK	Kaser and Montville, 1996
Bifidocin B		KYYGNGVTCGLHDCRVDRGKATCGIINNGGMWGDIG.	Yildirim *et al.*, 1999
Carnobacteriocin B2[2]	MNSVKELNVKEMKQLHGG	VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	Quadri *et al.*, 1994)
Carnobacteriocin BM1[3]	MKSVKELNKKEMQQINGG	AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	Quadri *et al.*, 1994)
Curvacin A[4]	MNNVKELSMTELQTITGG	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	Tichaczek *et al.*, 1993
Divercin V41	MKNLKEGSYTAVNTDELKSINGG	TKYYGNGVYCNSKKCWVDWGQASGCIGQTVVGGWLGGAIPGKC	Métivier *et al.*, 1998
Enterocin A	MKHLKILSIKETQLIYGG	TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPGKC	Aymerich *et al.*, 1996
Leucocin A[5]	MNNMKPTESYEQLDNSALEQVVGG	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNFW	Hastings *et al.*, 1991
Leucocin Ta11a	MNNMKSADNYQQLDNNALEQVVGG	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	Felix *et al.*, 1994
Mesentericin Y 105[6]	MTNMKSVEAYQQLDNQNLKKVVGG	KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW	Fleury *et al.*, 1996
Mundticin		KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWSK	Bennik *et al.*, 1998
Pediocin PA-1[7]	MKKIEKLTEKEMANIIGG	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC	Marugg *et al.*, 1992
Piscicocin V1a[8]		KYYGNGVSCNKNGCTVDWSKAIGIIGNNAAANLTTGGAAGWNKG	Bhugaloo-Vial *et al.*, 1996
Plantaricin C19		KYYGNGLSCSKKGCTVNWGQ AFSCGVNRVATAGHGK	Atrih *et al.*, 1993
Sakacin P[9]	MEKFIELSLKEVTAITGG	KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK	Tichaczek *et al.*, 1994

[1]Leader peptide where available

[2]Carnobacteriocin B2 = Carnocin CP52 (Herbin *et al.*, 1997)

[3]Carnobacteriocin BM1 = Piscicolin V1b (Bhugaloo-Vial *et al.*, 1996) = Carnocin CP51 (Herbin *et al.*, 1997)

[4]Curvacin A = Sakacin A (Holck *et al.*, 1992)

[5] Leucocin A = Leucocin A-TA33a (Papathanasopoulos *et al.*, 1998)

[6]Mesentericin Y105 = Mesentericin 52A (Revol-Junelles *et al.*, 1996)

[7]Pediocin PA-1 = Pediocin AcH (Motlagh *et al.*, 1992)

[8]Piscicocin V1a = Piscicolin 126 (Jack *et al.*, 1996)

[9]Sakacin P = Sakacin 674 (Holck *et al.*, 1994b)

3.2.1.2.3 Class IIc: The *sec*-dependent bacteriocins

Some bacteriocins do not possess a double-glycine leader peptide, but are synthesized with a *sec*-type N-terminal leader sequence, leading to secretion and processing via the *sec* pathway (Nes *et al.*, 1996). These type of bacteriocins include divergicin A (Worobo *et al.*, 1995), enterocin P (Cintas *et al.*, 1997), acidocin B (Leer *et al.*, 1995), lactococcin 972 (Martínez *et al.*, 1996; 1999) and bacteriocin 31 (Tomita *et al.*, 1996).

3.2.1.2.4 Class IId: Unclassified small heat-stable non-lanthionine bacteriocins

Bacteriocins that do not meet the criteria of the previous sections within the Class II bacteriocins are included in this class. Moll et al. (1999) included carnobacteriocin A (Worobo *et al.*, 1994), enterocin B (Casaus *et al.,* 1997), enterocin I (Floriano *et al.*, 1998), enterocin L50 (Cintas *et al.*, 1998), curvaticin FS47 (Garver and Muriana, 1994), lactococcin A (Holo *et al.*, 1991), acidocin 8912 (Kanatani *et al.*, 1995b) and lactococcin B (Van Belkum *et al.*, 1992) in this group.

3.2.1.3 Class III: Large heat-labile bacteriocins

These bacteriocins are more than 30 kDa in size. The bacteriocin helveticin J is representative of this group. The operon of the bacteriocin has been cloned, and expressed in *Lactobacillus acidophilus* (Fremaux and Klaenhammer, 1994).

3.3 BACTERIOCINS PRODUCED BY LACTIC ACID BACTERIA

3.3.1 Bacteriocins produced by *Lactobacillus* spp.

Numerous bacteriocins produced by *Lactobacillus* spp. have been reported, dating as far back as 1947 (De Vuyst, 1994a; Grossowics *et al.* 1947). These antimicrobial substances often did not meet the basic criteria for bacteriocins and several of these bacteriocins have not been fully characterized. Those bacteriocins that have been characterized usually belong to Class I or Class II (Table 3.1). Helveticin J and caseicin 80 belong to Class III (Table 3.1).

Several bacteriocins produced by unidentified *Lactobacillus* spp. have been reported. Lewus *et al.* (1991) and Ahn and Stiles (1990) reported the production of bacteriocins by

several *Lactobacillus* spp. isolated from meat. Kelly *et al.* (1996b) isolated several bacteriocin-producing *Lactobacillus* spp. from meat, fish and dairy products.

3.3.1.1 Bacteriocins produced by obligate homofermentative *Lactobacillus* spp.

Lactacin F (=lacticin F), produced by *Lactobacillus johnsonii* VPI11088 [originally identified as *L. acidophilus* 11088 (NCK88)] is a small heat resistant bacteriocin that inhibits several *Lactobacillus* spp. and *E. faecalis*. The production of lactacin F is pH dependent, with maximum levels obtained in MRS broth maintained at pH 7 (Allison *et al.*, 1994; Fremaux *et al.*, 1993; Muriana and Klaenhammer, 1987, 1991). Molecular analysis of the bacteriocin genes indicated that the bacteriocin is dependent on two peptides, lactacin F and lactacin X, for maximum activity (Fremaux *et al.*, 1993). Similarly, *L. acidophilus* JCM1132 produces the two-peptide acidocin J1132 (Tahara *et al.*, 1996). Acidocin A, produced by *L. acidophilus* TK9201, a starter organism for fermented milk, is active against several closely related lactic acid bacteria and *Listeria monocytogenes* (Table 3.2) (Kanatani *et al.*, 1995a). Acidocin B is a plasmid-encoded bacteriocin produced by *L. acidophilus* strain M46, and is active against *L. monocytogenes, Clostridium sporogenes, Brochothrix thermosphacta, Lactobacillus fermentum* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, but inactive against most other *Lactobacillus* spp. (Leer *et al.* 1995; Ten Brink *et al.*, 1994). Lactacin B is produced by *L. acidophilus* N2 (Barefoot and Klaenhammer, 1983), and acidocin 8912 by *L. acidophilus* strain TK8912 (Kanatani *et al.*, 1995b). Kiliç *et al.* (1996) reported the bacteriocin production of several *L. acidophilus* and *L. delbrueckii* strains. Lacticin A and B are produced by *L. delbrueckii* subsp. *lactis* (Toba *et al.*, 1991).

Bacteriocins produced by *Lactobacillus helveticus* include lactocin 27, produced by strain LP27 (Upreti and Hinsdill, 1975), helveticin J, produced by strain 481 (Joerger and Klaenhammer, 1986) and helveticin V-1829, produced by strain V-1829 (Vaughan *et al.*, 1992).

Bacteriocins produced by *Lactobacillus amylovorus* include lactobin A, produced by strain LMG P-13139 isolated from corn steep liquor (Contreras *et al.*, 1997) and amylovorin L471 produced by strain DCE471 (Callewaert *et al.*, 1999). Salivaricin A is produced by *L. salivarius* 20P3 (Ross *et al.*, 1993), salivaricin B by *L. salivarius* M7 (Ten Brink *et al.*, 1994) and salivacin 140, a pH dependent bacteriocin, is produced by *L. salivarius* subsp. *salicinius* T140 (Arihara *et al.*, 1996). Gassericin A is produced by *Lactobacillus gasseri* LA39 (Kawai *et al.*, 1998).

Only lactacin F (Class IIb), acidocin J1132 (Class IIb), acidocin A (Class IIa), acidocin B

(Class IIc), acidocin 8912 (Class IId) and helveticin J (Class III) have been characterized (Table 3.1).

3.3.1.2 Bacteriocins produced by facultative heterofermentative *Lactobacillus* spp.

Numerous plantaricins have been described in the literature, and have also been reviewed by Olasupo (1996). The dearth of bacteriocins recorded in the literature as being produced by *Lactobacillus pentosus* could probably be attributed to the loss of species status of this organism for a decade until 1987 (Zanoni *et al.*, 1987).

Plantaricin ST31 (Todorov *et al.*, 1999) is produced by *Lactobacillus plantarum* ST31 isolated from sourdough. The bacteriocin inhibits strains of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Bacillus* and certain food-borne pathogens such as *Staphylococcus aureus*. Plantaricin ST31 is inactivated by the proteolytic enzymes protease IV, protease VIII, trypsin and pronase, is active in the pH range 3-8 and heat-stable (100°C for 10 min). The detergents SDS, Tween 20 and Tween 80 have no effect on the bacteriocin activity. Activity is inhibited by urea. Production of the bacteriocin is first detected in early logarithmic phase, with maximum inhibitory activity observed in the stationary phase of the producing organism. The total mass of the bacteriocin as determined by electrospray mass spectrometry is 2755.63 Da. Twenty amino acid residues have been sequenced: KRKKHRXQVYNNGMPTGMYR. No plasmids were detected, indicating that the gene encoding bacteriocin production is located on the chromosome.

Plantaricin C19 (Atrih *et al.*, 1993) is produced by *L. plantarum* C19 isolated from fermented cucumbers. Plantaricin C19 is active against several Gram-positive pathogenic and spoilage bacteria, but the activity against lactic acid bacteria is weak or nonexistent. The bacteriocin is produced during the logarithmic phase, is stable at pH 2-6, heat-stable (95°C for 60 min), and sensitive to proteolytic enzymes. The size of the peptide is about 3.5 kDa, with the sequence of the first six amino acids being KYYGNG.

Plantaricin C (González, *et al.*, 1994), produced by *L. plantarum* LL441, isolated from Cabrales cheese, is inhibitory to several strains of lactobacilli, leuconostocs, pediococci and *Streptococcus thermophilus*. Some food spoilage bacteria are also inhibited. Production of plantaricin C is detected during exponential growth, with maximum inhibitory activity at the beginning of the stationary phase. Plantaricin C is sensitive to pronase, trypsin and α-chymotrypsin, but is not affected by pepsin, proteinase K, α-amylase or lipase. The bacteriocin is resistant to treatments with methanol, chloroform and acetonitrile, heat-stable

(100°C for 60 min, 121°C for 15 min), and stable at acid and neutral pH. The sequence of the first 11 amino acids of plantaricin C is KKTKKNXSGDI. The mode of action of plantaricin C is bactericidal (González *et al.*, 1996). Turner *et al.* (1999) recently identified plantaricin C as a lantibiotic.

L. plantarum C-11, isolated from fermented cucumbers (Daeschel *et al.*, 1990), produces two two-peptide bacteriocins, plantaricin EF and plantaricin JK (Anderssen *et al.*, 1998). Plantaricin A, previously incorrectly identified as the bacteriocin responsible for inhibitory activity by *L. plantarum* C11 (Nissen-Meyer *et al.*, 1993), induces the production of these two two-peptide bacteriocins (see also Section 3.4.2.5).

Plantaricin UG1 (Enan *et al.*, 1996) is produced by *L. plantarum* UG1 isolated from dry sausage. The bacteriocin is able to inhibit several strains of lactobacilli and lactococci, and certain foodborne pathogens, such as *Bacillus cereus, L. monocytogenes, C. sporogenes* and *Clostridium perfringens*. Plantaricin UG1 is sensitive to α-chymotrypsin, trypsin, proteinase K and pronase E, but treatment with lipase, acetone, chloroform, diethyl ether, ethyl alcohol, hexane, isopropanol and toluene does not affect its activity. The bacteriocin is stable at pH 4.5-7 and is produced in mid-logarithmic phase of growth. Plantaricin UG1 is between 3 and 10 kDa, and chromosomally encoded.

Plantacin B (West and Warner, 1988) is produced by *L. plantarum* NCDO 1193. This bacteriocin inhibits the growth of various strains of *L. plantarum*, *L. mesenteroides* NCDO 8015 and *Pediococcus damnosus* NCDO1832. Plantacin B is sensitive to the enzymes pronase, pepsin, trypsin and α-chymotrypsin, lipase and α-amylase. Since the molecule cannot be isolated in liquid media, the mode of action and size of the protein has not been determined and no DNA or fermentation studies have been reported.

Plantaricins S and T are produced by *L. plantarum* LPCO10, isolated from green olive fermentations (Jiménez-Diaz *et al.*, 1993). Plantaricin S is inhibitory towards several *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus Micrococcus* and *Propionibacterium* spp. and towards *E. faecalis*, and *Clostridium tyrobutyricum*. Plantaricin S is resistant to heat (100°C for 60 min) at pH 4, 6 and 7. Lysozyme does not affect the bacteriocin, but treatment with the enzymes α-amylase, dextranase, lipase A, phospholipase C, α-chymotrypsin, trypsin, ficin, pronase E, proteinase K, thermolysin and subtilopeptidase causes inactivation of the peptide. Plantaricin S is produced during the logarithmic phase of growth. A second bacteriocin, plantaricin T, is secreted once the producing organism reaches the stationary phase of growth. Plantaricin T exhibits the same heat resistance as plantaricin S, but is not inactivated by α-amylase or lipase A. Plantaricin T also exhibits a lower level of

inhibition against the various organisms tested than plantaricin S. Plantaricin S is 2.5 kDa in size, while plantaricin T is slightly smaller. The genetic determinants for both bacteriocins do not appear to be plasmid encoded.

Plantacin 154 (Kanatani and Oshimura, 1994) is produced by *L. plantarum* LTF 154 isolated from fermented sausage. Plantacin 154 inhibits various *Lactobacillus* spp. such as *L. acidophilus*, *Lactobacillus casei*, *L. fermentum* and *L. plantarum*, *Pediococcus* spp. such as *P. acidilactici* and *Pediococcus pentosaceus*, *Streptococcus* spp. such as *Streptococcus lactis* and *S. thermophilus*, *E. faecalis* and *Propionibacterium* spp. such as *Propionibacterium acidipropionici*, *Propionibacterium jensenii* and *Propionibacterium theonii*. Other Gram-positive bacteria (*B. cereus, Bacillus licheniformis*, *B. subtilis and S. aureus*) and the Gram-negative bacteria *E. coli* and *Salmonella typhimurium* are not inhibited by plantacin 154. The antibacterial activity is stable during heat treatment at 80°C and boiling for 30 min. Treatment of plantacin 154 with proteolytic enzymes causes inactivity, suggesting that plantacin 154 is proteinaceous. The mode of action has not been reported. The molecular mass estimated by SDS-PAGE is 3.0 kDa or less. Bacteriocin-deficient mutants obtained after treatment of cells with acriflavin, coincided with the loss of a plasmid of 9.5 mDa, designated pLP1542. The bacteriocin-deficient mutants are immune to plantacin 154, suggesting that the genes coding for immunity are located on the chromosome.

Plantaricin KW30 (Kelly *et al.*, 1996a) is produced by *L. plantarum* strain KW30 isolated from fermented corn (Kaanga Wai). The inhibition spectrum of plantaricin KW30 is restricted to other lactobacilli. Plantaricin KW30 inhibits the growth of one out of five (1/5) *Lactobacillus brevis*, 0/4 *Lactobacillus paracasei* subsp. *paracasei*, 0/2 *L. pentosus*, 5/6 *L. plantarum*, 0/3 *L. lactis* subsp. *lactis* and 0/3 *Leuconostoc citreum* strains isolated from Kaanga Wai. Bacterial strains isolated from other sources (not published) and that are sensitive to plantaricin KW30 includes *L. delbrueckii* subsp. *lactis* and 5/8 *L. plantarum* strains. Proteinase type XIV, α-chymotrypsin, thermolysin, trypsin, and proteinase K inactivate plantaricin KW30, but antibacterial activity is not affected by lipase A, α-amylase and lysozyme. The surfactants SDS, N-lauryl sarcosine, Triton X-100, Tween 20 and Tween 80 increase the bacteriocin titre. This suggests that plantaricin KW30 exists as a multimeric form which can be dispersed to release more active units. Activity of the bacteriocin remains stable over a pH range of 2-10, but is lost after incubation at pH 12. The bacteriocin is resistant to boiling water for up to 60 min, but all activity is lost after autoclaving.

Plantaricin-149, produced by *L. plantarum* NRIC 149 and isolated from pineapple (Kato *et al.*, 1994), inhibits strains of *L. plantarum, L. delbrueckii, L. helveticus, L. casei, L. fermentum, L. mesenteroides, P. acidilactici, Pediococcus cerevisiae, Enterococcus hirae* and *L. lactis*.

The bacteriocin is inhibited by proteinase K, pronase, papain, pepsin, pancreatin and trypsin and is heat resistant (115°C for 10 min). Triton X-100 and Tween 80 do not affect plantaricin-149, but loss of activity is observed after treatment with SDS. Further studies have indicated that the genes coding for bacteriocin production are chromosomal. The sequence of the first 22 N-terminal amino acids of the bacteriocin is YSLQMGATAIKQVKKLFKKKGG (Kato *et al.*, 1994).

Plantaricin LC74, produced by *L. plantarum* LC74 isolated from crude goat's milk, inhibits strains of *L. plantarum, L. brevis, Lactobacillus buchneri, Leuconostoc paramesenteroides* and *Bacillus stearothermophilus*. (Rekhif *et al.*, 1994). The bacteriocin is sensitive to several proteases and stable at neutral and acidic pH. The detergents Triton X-100, Brij 35, SDS, as well as urea and β-mercaoptoethanol have no effect on the activity. Plantaricin LC74 is produced in the exponential phase of growth, and appears to be less than 5 kDa in size.

Plantaricin SA6, produced by *L. plantarum* SA6 isolated from fermented sausage, exhibits antimicrobial activity against several strains of the lactic acid bacteria *L. plantarum, L. brevis, L. buchneri, L. paramesenteroides, L. mesenteroides* and *Listeria grayi* (Rekhif *et al.*, 1995). The bacteriocin is inactivated by the proteolytic enzymes lysozyme, proteinase K, lipase, α-amylase, but not by urea and β-mercaptoethanol. The activity of the bacteriocin is more stable at acidic pH (pH 2-6) than at pH 8-12. The size of the bacteriocin is 3.4 kDa.

Plantaricin F, produced by *L. plantarum* BF001 isolated from spoiled catfish fillets, can only be detected after growth of the producing organism on solidified medium, or after a 50-fold concentration of liquid medium (Fricourt *et al.*, 1994, Paynter *et al.*, 1997). Strains of several genera of lactic acid bacteria are sensitive to plantaricin F. Foodborne pathogens such as *L. monocytogenes, S. aureus, Pseudomonas aeroginosa* and salmonellas are also inhibited. The bacteriocin is heat-stable (100°C for 30 min) and stable at acidic pH. Activity is lost at pH 7. Plantaricin F appears to be produced in the early stationary phase of growth.

Plantaricin D is produced by *L. plantarum* BFE 905 isolated from "Waldorf" salad (Franz *et al.*, 1998). This bacteriocin has a narrow spectrum of inhibition, being antagonistic against only one strain of *Lactobacillus sakei*, one strain of *L. plantarum* and several *L. monocytogenes* strains. The bacteriocin is inactivated by several proteolytic enzymes, is heat resistant and active at pH 2 to 10, although activity decreases at pH 8.

Schillinger and Lücke (1989) isolated various bacteriocin-producing lactobacilli from fresh meat and different meat products. Antagonism as a result of hydrogen peroxide or acetic acid was minimized by using anaerobic culture conditions. *Lactobacillus plantarum* strains Lb 75 and Lb 592 inhibit *L. sakei* strains Lb 68, Lb 693, Lb 699, Lb 790, *Lactobacillus curvatus* Lb 730, *Lactobacillus divergens* Lb 836, *L. monocytogenes* strains 8732 and 17a, but does not

inhibit *L. plantarum* Lb 828.

Okereke and Montville (1991a) investigated the ability of several lactic acid bacteria to produce bacteriocins and inhibit *Clostridium botulinum* spores at refrigeration and abuse (15 and 35°C) temperatures in the presence of various concentrations of sodium chloride as used in cured meats. Abuse temperatures were included to simulate temperature abuse conditions of minimally processed refrigerated meat products. *Lactobacillus plantarum* Lb 75 and *L. plantarum* Lb 592 produces inhibition zones at 15°C and 30°C, but not at 4°C or 10°C. Plantaricin BN, produced by *L. plantarum* BN forms inhibition zones at 4, 10, 15 and 30 °C, is resistant to pepsin, pronase E and trypsin, but sensitive to proteinase K. *Lactobacillus plantarum* strain Lb 75 is resistant to pepsin and trypsin, but sensitive to proteinase K, chymotrypsin and pronase E. *Lactobacillus plantarum* strain Lb 592 is resistant to all proteases tested, except proteinase K and chymotrypsin (Lewus *et al.*, 1991; Okereke and Montville, 1991b).

Garriga *et al.* (1993) isolated several bacteriocin-producing *L. plantarum* strains from fermented sausages obtained from different manufacturers at different times of ripening. Six of the 22 isolates of *L. plantarum* (designated CTC 242, CTC 244, CTC 272, CTC 305, CTC 306, CTC 316) produce antagonistic activity against two or more of the indicator strains (*L. plantarum* ATCC 8014, *L. plantarum* DSM 20174, *L. curvatus* NCDO 2739, *L. curvatus* (Researchers' own collection) and *L. sakei* DSM 20017). *Lactobacillus plantarum* strains CTC 242, CTC 244, CTC 305, CTC 316 inhibit *L. plantarum* ATCC 8014. *Lactobacillus plantarum* DSM 20174 is inhibited by all six isolates. *Lactobacillus curvatus* NCDO 2739 is not inhibited. A laboratory strain of *L. curvatus* is inhibited by CTC 242, CTC 244, CTC 306, and *L. sakei* DSM 20017 is inhibited by CTC 242, CTC 244, CTC 272. Further studies on *L. plantarum* CTC 305 and *L. plantarum* CTC 306 have indicated inhibition of *L. monocytogenes* 17a (Schillinger and Lücke, 1989) and *E. faecalis* (laboratory strain). Both compounds from *L. plantarum* CTC 305 and CTC 306 are resistant to heat of 100°C for 20 min, and sensitive to the enzymes trypsin, pepsin, proteinase K and Nagarse (*B. subtilis* protease). Both isolates have a bactericidal mode of action against *L. monocytogenes* 17a. The molecular weight of the compounds is larger than 10000 Da. Loss of activity and immunity after curing experiments do not coincide with loss of any plasmids, indicating chromosomal coding of the bacteriocins.

Plantaricin NA is produced by a *L. plantarum* sp. isolated from vegetable origin (Olasupo, 1998). The bacteriocin inhibits *L. monocytogenes*, and is inactivated by proteolytic enzymes, heat-resistant and active over a pH range of 2-10.

Olukoya *et al.* (1993) reported the production of plantaricin K, produced by *L. plantarum*

DK9 isolated from "fufu", a fermented cassava product, and pentocin D, produced by *L. pentosus* DK7, isolated from "ogi", a fermented maize product. The antagonistic effect of these two compounds disappears after treatment with trypsin. Both bacteriocins lose some activity after heat treatments at 80°C for 30 min. Both bacteriocins inhibit strains of *Lactobacillus*, *Leuconostoc* and *Enterobacter*.

Lactobacillus plantarum TMW 1.25 produces plantaricin 1.25α and plantaricin 1.25β, which are 5979 and 5203 Da in size, respectively. Partial sequencing of the α fraction is not homologous to any known bacteriocins, while the β peptide displays strong homology to the N-terminal of brevicin 27 (Remiger *et al.*, 1999).

Olasupo *et al.* (1997) found that the bacteriocinogenic *L. casei* starter organism for the production of an African fermented maize product "ogi" improves the shelflife of this product. Caseicin 80, a bacteriocin produced by *L. casei* B 80, was reported by Rammelsberg *et al.* (1990). The bacteriocin is sensitive to heat, with a narrow spectrum of inhibition and the apparent size of caseicin 80 is between 40 and 42 kDa. The bacteriocin has not been purified. Lactocin 705 is produced by *L. casei* CRL 705 isolated from dry sausages (Vignolo *et al.*, 1995).

Curvacin A (Table 3.2), a bacteriocin produced by *L. curvatus* LTH1174 isolated from, and a possible starter culture for, fermented sausage (Tichaczek *et al.*, 1992). This bacteriocin inhibits several strains of *Lactobacillus*, *L. monocytogenes* and *E. faecalis.* Proteinase K and trypsin inhibit the bacteriocin, but not pepsin, BSA or RNAse. The inhibitory effect of the bacteriocin is only slightly affected by heat treatment, but no activity remains after autoclaving. The bacteriocin is produced in the late logarithmic phase of growth. Other bacteriocins produced by *L. curvatus* include curvaticin FS47, produced by strain FS47 isolated from meat (Garver and Muriana, 1994), and curvaticin 13, produced by strain SB13 (Sudirman *et al.*, 1993).

Several bacteriocins produced by *L. sakei* have been reported, including sakacin M (Sobrino *et al.* 1992) and lactocin S (Mortvedt and Nes, 1990). Garriga *et al.* (1993) reported bacteriocin production by *L. sakei* CTC 372. Hugas *et al.* (1995) reported the inhibition of *Listeria* spp. by *L. sakei* CTC 494. Schillinger and Lücke (1989) reported the production of antimicrobial compounds by six *L. sakei* strains isolated from meat. Schillinger *et al.* (1991) reported the control of *L. monocytogenes* by *L. sakei* Lb706.

Sakacin P (Table 3.2), a bacteriocin produced by *L. sakei* LTH673, has similar characteristics to curvaticin A, although sakacin P appears more active (Tichaczek *et al.*, 1992). Sakacin 674 (Table 3.2), produced by *L. sakei* Lb674 inhibits the growth of several *Lactobacillus* spp. and *L. monocytogenes* (Holck *et al.*, 1994b). Subsequent studies have

shown that sakacins P and 674 are identical (Hühne *et al.*, 1996). Sakacin A (Table 3.2), produced by *L. sakei* Lb706 (Holck *et al.*, 1992) is identical to curvacin A. Bavaricin MN (Table 3.2), originally reported to be produced by *Lactobacillus bavaricus* MN (Lewus and Montville, 1992), is produced by *L. sakei* MN (Kaiser and Montville, 1996). Bavaricin A (Table 3.2), produced by *L. bavaricus* MI401, has a bactericidal mode of action against several *L. monocytogenes* strains (Larsen *et al.*, 1993).

Numerous bacteriocins produced by the facultative heterofermentative *Lactobacillus* spp. have been reported as discussed in this chapter, but only a few have been fully characterized. Two bacteriocins, plantaricin C and lactocin S are Class I lantibiotics, while plantaricin EF, plantaricin JK and plantaricin S are Class II two-peptide bacteriocins. The data available on plantaricin C19 suggests that this bacteriocin may be a Class IIa bacteriocin. Curvacin A (=sakacin A), sakacin P (=sakacin 674) and bavaricin MN are Class IIa bacteriocins as depicted in Table 3.2.

3.3.1.3 Bacteriocins produced by obligate heterofermentative *Lactobacillus* spp.

Considering the amount of species within this group (19), the lack of published data on bacteriocins produced by obligate heterofermentative *Lactobacillus* spp. is surprising. It may be speculated that this group is industrially not as important as the previous two groups, and that this group is usually associated with food spoilage rather than food fermentation.

Several bacteriocins produced by *L. brevis* have been reported. The bacteriocin brevicin is produced by *L. brevis* 37 (Rammelsberg and Radler, 1990). Brevicin 27 is produced *by L. brevis* strain SB27. This bacteriocin inhibits mainly strains of closely related *L. brevis and L. buchneri*. The protein is about 5200 Da in size and protein sequencing of the first 25 N-terminal amino acids revealed a high proprotion of lysine and hydrophobic amino acids (Benoit *et al.*, 1997). Brevicin 286 is produced by *L. brevis* VB286 isolated from vacuum-packaged meat. This bacteriocin is inactivated by several proteolytic enzymes and heat-stable (Coventry *et al.*, 1996).

Bacteriocins produced by other species within this group include fermenticin, produced by *L. fermentum* (DeKlerk and Smit, 1967) and reutericin 6, produced by *Lactobacillus reuteri* LA6 (Kabuki *et al.*, 1997).

3.3.2 Bacteriocins produced by *Carnobacterium* spp.

Several bacteriocins produced by *Carnobacterium* spp. have been reported. Most of these

bacteriocins have been fully characterized and belong to the Class II group of bacteriocins as described in Section 3.2.1 and Table 3.1. Carnocin U149 is a Class I lantibiotic, while divergicin 750 does not fit into any of the currently described groups. The production of bacteriocins by several *Carnobacterium* spp. isolated from meat, particularly *Carnobacterium piscicola* and *Carnobacterium divergens* has been reported (Ahn and Stiles, 1990; Lewus *et al.*, 1991).

Carnocin U149 is a 4635 Da bacteriocin produced by *C. piscicola*, isolated from fish (Stoffels *et al.*, 1992). *Carnobacterium piscicola* LV17B, isolated from meat, was found to produce carnobacteriocins BM1, B2 and A (Ahn and Stiles, 1990; Quadri *et al.*, 1994). Carnobacteriocins BM1 and B2 (Table 3.2) are active against various *Carnobacterium* spp., *Listeria innocua, L. monocytogenes, E. faecium* and *E. faecalis*. Worobo *et al.* (1994) reported the production of carnobacteriocin A by *C. piscicola* LV17A. This relatively heat and solvent resistant bacteriocin differs from most other small hydrophobic peptides since activity is easily lost. The bacteriocin is produced during the early logarithmic phase of growth, and therefore is thought to have an ecological advantage in mixed fermentations. The amino acid sequence of the prepeptide of this bacteriocin is identical to that of piscicolin 61 (Holck *et al.*, 1994a).

Herbin *et al.* (1997) reported the production of carnocin CP5, produced by *C. piscicola* CP5, isolated from French mold-ripened cheese. Purification of the bacteriocin indicated that two anti-listerial bacteriocins, carnocin CP51 and carnocin CP52 are produced. Carnocin CP51 shares homologies with carnobacteriocin BM1, and carnocin CP52 is similar to carnobacteriocin B2 (Table 3.2). The two producing bacteria are, however, not identical, since strain CP5 has four plasmids and strain LV17 three plasmids. Furthermore, the bacteriocin carnobacteriocin A is not produced by strain CP5 and its genetic determinants could not be detected with PCR. The two strains were isolated from different food products.

Two bacteriocins, piscicocin V1a (4416 Da) and piscicocin V1b (4526), produced by *C. piscicola* V1, isolated from fish, were purified and characterized (Bhugaloo-Vial *et al.*, 1996; Pilet *et al.*, 1995). Both bacteriocins inhibit various Gram-positive bacteria, including *L. monocytogenes*, although piscicocin V1a is more active than piscicocin V1b (Table 3.2).

Piscicolin 126, a heat resistant bacteriocin produced by *C. piscicola* isolated from spoiled ham, inhibits several species of *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Listeria* and *S. thermophilus* (Jack *et al.*, 1996). The amino acid sequence of this bacteriocin is identical to piscicocin V1a (Table 3.2).

Holck *et al.* (1994a) reported the production of a bacteriocin produced by *C. piscicola* LV61 isolated from meat, called piscicolin 61. This bacteriocin inhibits several *Carnobacterium, Lactobacillus, Leuconostoc, Pediococcus, Enterococcus* and *Listeria* spp.

Divergicin 750 (Table 3.2), a bacteriocin produced by *C. divergens*, inhibits species of *Carnobacterium*, *Enterococcus* and *Listeria*, as well as *C. perfringens* (Holck *et al.*, 1996).

Worobo *et al.* (1995) described divergicin A, produced by *C. divergens* LV13, isolated from meat. Divercin V41 (Table 3.2) is a bacteriocin produced by *C. divergens* V41, isolated from fish viscera (Métivier *et al.*, 1998; Pilet *et al.*, 1995).

3.3.3 Bacteriocins produced by *Enterococcus* spp.

Numerous bacteriogenic enterococci have been reported (De Vuyst, 1994b). Most of the bacteriocins described in the literature are produced by *E. faecium* and *E. faecalis*. These bacteriocins are more diverse than those produced by *Carnobacterium* spp., and several bacteriocins such as enterococcin Sf25 (Reichelt *et al.*, 1984), enterocin 81 (Ennahar *et al.*, 1998), enterocin 01 (Olasupo *et al.*, 1994), enterocin I (Floriano *et al.*, 1998), enterocin L50A and B (Cintas *et al.*, 1998), enterocin EJ97 (Gálvez *et al.*, 1998), enterocin AS-48 (Gálvez *et al.*, 1989), enterocins 1071A and B (Balla *et al.*, 2000) and enterocin 012 (Jennes *et al.*, 2000) either do not belong in any of the currently described groups, or have not been characterized to the extent that they can be classified (Section 3.2.1 and Table 3.1). Other enterocins described belong to either the Class I or Class II groups of bacteriocins.

Arihara *et al.* (1991) isolated six strains of *E. faecalis* that showed inhibitory activity towards *L. monocytogenes*. Other bacteriocins produced by *E. faecalis* include enterocin EJ97, produced by *E. faecalis* EJ97 isolated from municipal waste water (Gálvez *et al.*, 1998), cytolysin (Gilmore *et al.*, 1994), bacteriocin 31 (Tomita *et al.*, 1996) and enterocin AS-48 (Gálvez *et al.*, 1989), which is identical to enterocin 4 (Joosten *et al.*, 1996). Balla *et al.* (2000) reported the characterization of enterocin 1071A and enterocin 1071B, two bacteriocins produced by *E. faecalis* BFE 1071, isolated from minipig faeces. These two peptides showed 64 and 61% homology with the α and β peptides of lactococcin G (Section 3.3.4), respectively.

Reichelt *et al.* (1984) described the production of enterococcin Sf25, produced by *Streptococcus faecium* (= *E. faecium*) strain 25 isolated from human sources. Torri Tarelli *et al.* (1994) isolated bacteriocin-producing strains of *E. faecium* (strains 7C5 and CNRZ EFM4) and *E. faecalis* strains (X1, X2 and X3) from dairy sources.

Enterocin A, produced by *E. faecium* DPC1146, was originally designated enterocin 1146 (O'Keeffe *et al.*, 1999). This bacteriocin is identical to enterocin A (Table 3.2) produced by *E. faecium* CTC492, which was isolated from fermented Spanish sausage (Aymerich *et al.* 1996). *Enterococcus faecium* CTC492 also produces enterocin B (Nilsen *et al.*, 1998). *Enterococcus faecium* T136, also isolated from fermented Spanish sausage, produces enterocin A and B as

well (Casaus *et al.*, 1997). Enterocin B is also produced by *E. faecium* BFE 900 isolated from black olives (Franz *et al.*, 1999). Enterocin 81, produced by *E. faecium* WHE 81, isolated from Muster cheese, shows a narrow spectrum of inhibition against *Enterococcus* and *Listeria* spp. (Ennahar *et al.*, 1998). Farias *et al.* (1996) isolated a pediocin-like enterocin CRL 35 from Argentinean cheese. The N-terminal amino acid sequence is KYYGNGVTLNKXGXSVNXXXA. Other bacteriocins produced by *E. faecium* include enterocin 01, produced by strain NA01 isolated from "wara" (Olasupo *et al.*, 1994), enterocin P, produced by strain P13 isolated from a Spanish dry-fermented sausage (Cintas *et al.*, 1997), enterocin I, produced by strain 6T1a, isolated from a Spanish green olive fermentation (Floriano *et al.*, 1998) is identical to enterocin L50, produced by *E. faecium* L50 (Cintas *et al.*, 1998).

Bennik *et al.* (1998) reported the characterization of a bacteriocin, mundticin, produced by a strain of *Enterococcus mundtii*, associated with vegetables. Jennes *et al.* (2000) described enterocin 012, produced by *Enterococcus gallinarum* strain 012, isolated from the duodenum of ostrich.

3.3.4 Bacteriocins produced by *Lactococcus* spp.

A variety of bacteriocins are produced by *Lactococcus* spp. These include the three Class I lantibiotics nisin (Teuber, 1995), lacticin 481 (Piard *et al.* 1990), and lacticin 3147 (Ryan *et al.*, 1996), lactococcin G, a Class IIb two peptide bacteriocin (Nissen-Meyer *et al.*, 1992), and lactococcins A, B, and M, which are produced by various strains of *L. lactis*.

Nisin is a lantibiotic produced by many strains of *L. lactis* subsp. *lactis*. The host range of nisin includes most Gram-positive bacteria such as staphylococci, enterococci, pediococci, lactobacilli, leuconostocs, listerias, corynebacteria, *Mycobacterium tuberculosis*, and germinating spores of bacilli and clostridia (Teuber, 1995). Nisin is the first bacteriocin to be granted GRAS status and is used commercially in various food products as a preservative (Teuber, 1995). Nisin Z, produced by *L. lactis* subsp. *lactis* strain NIZO 22186, is a natural variant of nisin A (Mulders *et al.*, 1991).

Lactococcins A, B, and M are produced by *L. lactis* subsp. *cremoris* 9B4. These bacteriocins are inhibitory towards other lactococci only (Teuber, 1995, Van Belkum *et al.*, 1991; 1992). *Lactococcus lactis* subsp. *diacetylactis* DPC938 produces three bacteriocins identical to lactococcins A, B and M (Morgan *et al.*, 1995). The *L. lactis* subsp. *diacetylactis* DPC938 plasmid encoding these bacteriocins is larger than that reported by Van Belkum *et al.* (1989) for lactococcins A, B and M produced by *L. lactis* subsp. *cremoris* 9B4 (Morgan *et al.*,

1995). Lactococcin A is also produced by *L. lactis* subsp. *cremoris* LMG 2130 (Holo *et al.*, 1991) and *L. lactis* subsp. *diacetylactis* WM4 (Stoddard *et al.*, 1992).

Piard *et al.* (1990) reported the production of lacticin 481, produced by *L. lactis* CNRZ 481. This small (5500 Da) heat resistant bacteriocin inhibited most *Lactococcus* spp. tested as well as some *Lactobacillus*, *Leuconostoc* and *Clostridium* spp. Maximum production was obtained at a maintained pH of 5.5.

Lactococcin G, produced by *L. lactis* LMG 2081, is a two-peptide bacteriocin that inhibits various lactic acid bacteria and different clostridia (Nissen-Meyer *et al.*, 1992).

The lantibiotic lactococcin DR, produced by *L. lactis* subsp. *lactis* ADRIA 85LO30 (Rince *et al.*, 1994) is identical to lacticin 481. *Lactococcus lactis* subsp. *lactis* IPLA 972, isolated from home-made cheese, produces lactococcin 972 (Martínez *et al.*, 1996). Other bacteriocins produced by *Lactococcus* spp. include lacticin 3147, produced by *L. lactis* DPC3147 (Ryan *et al.*, 1996), and lactostrepsin, produced by *L. lactis* subsp. *cremoris* strain 202 (Zajdel *et al.*, 1985).

3.3.5 Bacteriocins produced by *Leuconostoc* and *Weissella* spp.

Several bacteriocins produced by *Leuconostoc* spp. have been described. Most of these bacteriocins belong to the Class IIa group of bacteriocins, such as mesentericin Y105 (Héchard *et al.*, 1992a), leucocin A-UAL 187 (Hastings and Stiles, 1991), and leucocin C-TA33c (Papathanasopoulos *et al.*, 1997; 1998). Leuconocin S (Lewus *et al.*, 1992) and carnocin LA54A (Keppler *et al.*, 1994) are two of the few bacteriocins that are sensitive to α-amylase. Mesentericin 52A (Revol-Junelles *et al.*, 1996) is identical to mesentericin Y105 (Héchard *et al.*, 1992a), and leucocin A-TA33a (Papathanasopoulos *et al.*, 1997; 1998) is identical to leucocin A-UAL 187 (Hastings and Stiles, 1991). Several characterized bacteriocins in this group cannot be classified in the currently described groups, such as mesentericin 52B (Revol-Junelles *et al.*, 1996) and leucocin B-TA33b (Papathanasopoulos *et al.*, 1997; 1998).

Ahn and Stiles (1990) and Lewus *et al.* (1991) reported the production of bacteriocins produced by several *Leuconostoc* spp. isolated from meat. Leuconocin J is produced by *Leuconostoc* sp. J2, isolated from Korean Kimchi (Choi *et al.*, 1999). Leucocin H is a two-peptide bacteriocin produced by *Leuconostoc* MF215B (Blom *et al.*, 1999). Kelly *et al.* (1996b) isolated several bacteriocin-producing *Leuconostoc* spp. from meat, fish and dairy products.

Carnocin LA54A, a 4 kDa, heat resistant bacteriocin produced by *Leuconostoc carnosum* LA54A, isolated from meat, is sensitive to α-amylase as well as other proteolytic enzymes,

suggesting a proteinaceous and a carbohydrate moiety (Keppler *et al.*, 1994). Other bacteriocins produced by *L. carnosum* include leucocin F10, produced by strain F10 isolated from meat (Parente *et al.*, 1996), leucocin B-TA11a, produced by strain TA11a isolated from meat (Felix *et al.*, 1994), and carnosin, isolated from vacuum packaged Vienna-type sausages (Van Laack *et al.*, 1992).

Mesentericin Y105, produced by *L. mesenteroides* strain Y105, inhibits *L. monocytogenes* and is a 37 amino acid heat resistant bacteriocin active over a pH range of 4 to 8.5 (Fleury *et al.*, 1996; Héchard, *et al.*, 1992a, b). Papathanasopoulos *et al.* (1997) reported the production of three bacteriocins, leucocin A-TA33a, leucocin B-TA33b and leucocin C-TA33c. Leucocin A-TA33a is identical to leucocin A-UAL 187 (Papathanasopoulos *et al.*, 1998). Other bacteriocins produced by *L. mesenteroides* include mesentericin 52A and mesentericin 52B produced by *L. mesenteroides* subsp. *mesenteroides* FR52 (Revol-Junelles *et al.*, 1996) and dextranicin 24, produced by *L. mesenteroides* subsp. *dextranicum* J24 (Revol-Junelles and Lefebvre, 1996).

Hastings and Stiles (1991) reported the production of a bacteriocin-like substance, leucocin A-UAL 187 (= leucocin A) produced by *Leuconostoc gelidum* UAL 187 isolated from vacuum-packaged meat (Table 3.2). The producer organism grows well at refrigeration temperatures, but not at 35°C. The bacteriocin is resistant to heat and inhibited by protease and trypsin. The bacteriocin is active against several *Leuconostoc, Lactobacillus, Pediococcus,* and *Carnobacterium* spp., *E. faecalis* and *L. monocytogenes* (Hastings *et al.*, 1991).

Leuconocin S, an α-amylase sensitive bacteriocin (Lewus *et al.*, 1992) and leucocin C-LA7a (Hastings *et al.*, 1996) is produced by *Weissella paramesenteroides*.

3.3.6 Bacteriocins produced by *Pediococcus* spp.

Pediocin PA-1, which belongs to the Class IIa group of bacteriocins as discussed in Chapter 3, has been extensively researched. Gonzalez and Kunka (1987) reported the production of pediocin PA-1 by *P. acidilactici* PAC1.0. Pediocin PA-1 is active against a wide spectrum of Gram-positive lactic acid bacteria as well as *L. monocytogenes (*Pucci *et al.* 1988). Pediocin PA-1 also inhibits *L. monocytogenes* in fermented semidry sausage (Berry *et al.,* 1990) and fresh meat (Nielsen *et al.*, 1990). The bacteriocin is produced by two *Pediococcus parvulus* strains isolated from minimally processed vegetables (Bennik *et al.*, 1997). A bacteriocin produced by a *P. acidilactici* strain isolated from commercial starter cultures by Nieto Lozano *et al.* (1992) is identical to pediocin PA-1. Pediocin AcH, produced

by a *P. acidilactici* isolated from fermented sausage (Bhunia *et al.*, 1988) is identical to pediocin PA-1 (Marugg *et al.*, 1992).

Pediocin 5 is produced by *P. acidilactici* UL5, originally identified as *L. mesenteroides* UL5 (Daba *et al.*, 1991; 1994, Huang *et al.*, 1996). Pediocin PD-1, is produced by *P. damnosus* NCFB 1832 (Green *et al.*, 1997). Pediocin A is produced by *P. pentosaceus* FBB61 (Piva and Headon, 1994). Pediocin L50 has been renamed enterocin L50 (Cintas *et al.*, 1998).

3.3.7 Bacteriocins produced by *Streptococcus* spp.

Tagg (1992) reported the production of numerous bacteriocin-like inhibitory substances by streptococci, which at the time were still identified using the Lancefield groupings. The potential use of mutacins, produced by mutans streptococci, in the prevention of dental caries has been reported (Chikindas *et al.*, 1997; Novák *et al.*, 1994). Lewus *et al.* (1991) reported the production of a bacteriocin by a *Streptococcus* sp. isolated from meat. Bacteriocin production by several *S. thermophilus* strains have been reported (De Vuyst, 1994c). These include thermophilin 13 produced by *S. thermophilus* Sfi13 (Marciset *et al.*, 1997), thermophilin 347, produced by strain 347 isolated from yoghurt (Villani *et al.*, 1995), and thermophilin T produced by strain ACA-DC0040, isolated from "feta" cheese (Aktypis *et al.*, 1998).

3.3.8 Bacteriocins produced by *Bifidobacterium* spp.

Bifidobacteria are one of the predominant groups of bacteria in the human intestinal tract and are thought to have several advantageous effects on the health of the host, but little work has been done on the production of bacteriocins by this group of organisms (Yildirim and Johnson 1998). Bifidocin B, produced by *Bifidobacterium bifidum* NCFB 1454 has a wide antimicrobial spectrum, inhibiting several species of *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Listeria* and *Pediococcus*. *Staphylococcus*, *Clostridium* and *Streptococcus* spp. were not inhibited (Yildirim and Johnson, 1998). The molecular mass of this bacteriocin was determined to be 3.3 kDa. The bacteriocin was heat-resistant, inactivated by trypsin, α-chymotrypsin, papain, proteases and pepsin, and activity was retained at pH values of 2 to 12. Yildirim *et al.* (1999) reported the protein sequence of bifidocin B (Table 3.2). Production of the bacteriocin was associated with an 8 kbp plasmid.

3.4 GENETICS OF CLASS II BACTERIOCIN PRODUCTION

3.4.1 Gene location

Bacteriocins may be either chromosomally or plasmid-encoded, for example, enterocin A (Aymerich *et al.*, 1996) and sakacin P (Tichaczek *et al.*, 1994) are chromosomally encoded, while curvacin A (Tichaczek *et al.*, 1992), leucocin A (Hastings and Stiles, 1991), mesentericin Y105 (Héchard *et al.*, 1992a), bifidocin B (Yildirim *et al.*, 1999) and pediocin PA-1/AcH (Marugg *et al.*, 1992; Bukhtiyarova *et al.*, 1994) are plasmid-encoded. Plasmids associated with bacteriocin production vary considerably in size. Some plasmids are known to carry the genetic determinants for several bacteriocins (Jack *et al.*, 1995). Where more than one bacteriocin is produced, the bacteriocins can be plasmid (carnobacteriocin B2) and chromosomally (carnobacteriocin BM1) encoded (Quadri *et al.,* 1995).

3.4.2 Genetic organization

The general genetic structure leading to synthesis of cationic bacteriocins usually encompasses four genes that encode the functions required for production of extracellular antibacterial activity (Nes *et al.*, 1996). These are the structural gene which encodes the prebacteriocin, an immunity gene, a gene which encodes an ABC-transporter and an accessory gene that is also necessary for bacteriocin externalization. These genes are usually organized in one or two operons (Nes *et al.*, 1996), with an upstream promoter area. The genetic structure of several Class IIa bacteriocins has been analysed, including pediocin PA-1 (Marugg *et al.*, 1992), mesentericin Y105 (Fremaux *et al.*, 1995), leucocin A (Van Belkum and Stiles, 1995), divercin V41 (Métivier *et al.*, 1998), sakacin P (Hühne *et al.*, 1996), acidocin A (Kanatani *et al.*, 1995a), sakacin A (Axelsson and Hölck, 1995) and enterocin A (O'Keeffe *et al.*, 1999). For several bacteriocins, including the plantaricins of *L. plantarum* C11, divercin V41, sakacin A, sakacin P and enterocin A, regulatory genes involved in bacteriocin production have also been identified. These regulatory systems are discussed in Section 3.4.2.5.

The DNA sequence of the pediocin PA-1 operon revealed four open reading frames (ORF's), designated *pedA*, *pedB*, *pedC* and *pedD*, with an upstream promoter area (Fig. 3.1). *pedA* encodes pre-pediocin PA-1, the structural gene of with its leader peptide, a protein of 62 amino acids. *pedB* encodes the immunity protein, consisting of 112 amino acids. *pedC* encodes an accessory protein of 174 amino acids found to be necessary for protein

externalization, while *pedD* encodes an ABC transporter protein of 724 amino acids (Marugg *et al.*, 1992).

Fig. 3.1. Schematic representation of the pediocin PA-1 operon (Marugg *et al.*, 1992). The promoter (•) and filled arrows indicate the individual genes and the direction of transcription.

The gene clusters of mesentericin Y105 and leucocin A are very similar and are arranged in two operons (Fig. 3.2). *mesY* the gene encoding the 61 amino acid structural gene of mesentericin Y105, and m*esI* encoding the immunity gene which consists of 113 amino acids, are preceded by a promoter and followed by a terminator, indicating an operon. A second operon-like structure consisting of ORF's *mesC*, *mesD* and *mesE* is transcribed in the opposite direction. *mesD* encodes an ABC transporter of 722 amino acids. *mesE* encodes an accessory factor consisting of 457 amino acids. The function of the protein encoded by *mesC* has not been determined (Fremaux *et al.*, 1995).

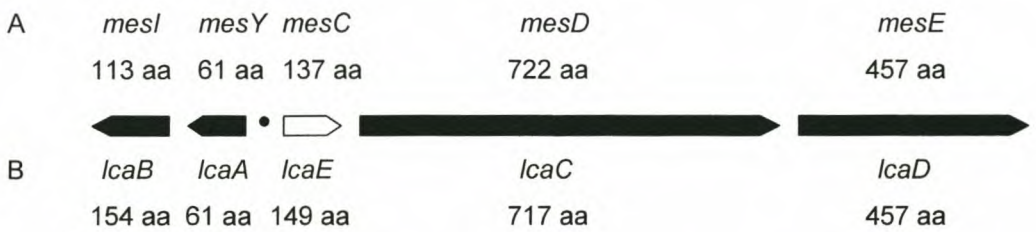

Fig. 3.2. Schematic representation of the (A) mesentericin Y105 (*mesIY* and *mesCDE*) (Fremaux *et al.*, 1995) and (B) leucocin A (*lcaAB* and *lcaECD*) operons (Van Belkum and Stiles, 1995). The promoter (•) and filled arrows indicate the individual genes and the direction of transcription.

Leucocin A has a similar genetic arrangement. *lcaA* encodes a 61 amino acid structural protein, and *lcaB* encodes the 154 amino acid immunity gene of leucocin A. *lcaE* encodes a protein of unknown function consisting of 149 amino acids. LcaE shows 85% similarity to MesC. LcaC encodes an ABC transporter that consists of 717 amino acids, with 99% similarity to MesD. LcaD is an accessory factor consisting of 457 amino acids, which shows

96% similarity to MesE (Van Belkum and Stiles, 1995).

The chromosomal DNA fragment identified as the divercin V41 gene cluster consists of the genes ORFA, *dvnA*, *dvnT1*, *dvnT2*, *dvn1*, *dvnR* and *dvnK*, which encodes the structural gene, ABC transporter, immunity protein, a second immunity protein, response regulator and histidine kinase, respectively (Fig. 3.3). No homology was found between ORFA and any previously described protein. Predicted structural conformation of the protein implied that it could form a transmembrane helix, suggesting that the protein could be anchored in the cytoplasmic membrane (Métivier *et al.*, 1998).

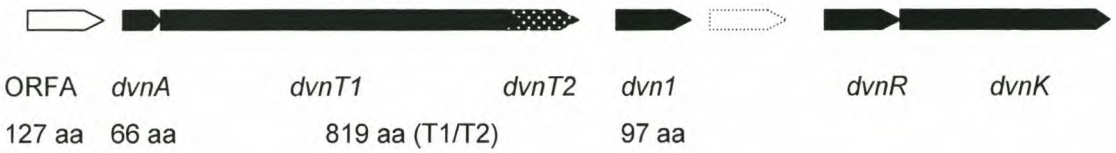

Fig. 3.3. Schematic representation of the gene cluster of divercin V41 (Métivier *et al.*, 1998). Filled arrows indicate the individual genes and direction of transcription. The dashed box encodes an ORF whose product is similar to the C-terminus of an ATP-transporter.

Two genes were identified for acidocin A (Fig. 3.5). *acdA* encodes the structural gene of acidocin A, while it was suggested that ORF2 encodes an immunity protein (Kanatani *et al.*, 1995a).

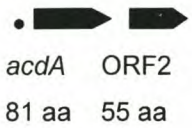

Fig. 3.5. Schematic representation of the gene cluster of acidocin A (Kanatani *et al.*, 1995a). The promoter (•) and filled arrow indicate the individual genes and direction of transcription.

Six ORF's were identified in the DNA sequence of the chromosomal fragment encoding sakacin P (Fig. 3.4). These were identified as *sppK*, encoding a histidine kinase protein consisting of 448 amino acids, *sppR*, encoding a response regulator of 248 amino acids, *sppA*, encoding the structural gene of sakacin P (61 amino acids), *spiA*, encoding a putative immunity protein of 98 amino acids. *sppT* and *sppE* encode the transporter proteins, which consist of 718 and 458 amino acids, respectively (Hühne *et al.*, 1996). The function of ORF1 was not determined, but the N-terminal residues of the putative protein encoded by this gene

showed significant similarities to the leader sequences of Class II bacteriocins, indicating a possible role in the sakacin P system.

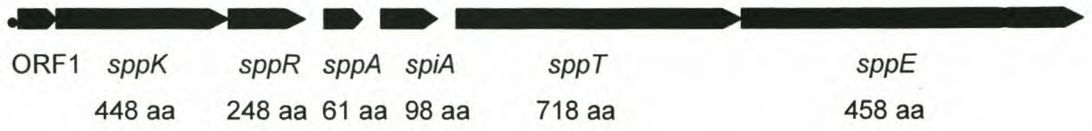

Fig. 3.4. Schematic representation of the gene cluster of sakacin P (Hühne *et al.*, 1996). The promoter (•) and filled arrows indicate the individual bacteriocin genes and the direction of transcription.

Ten ORF's were identified on the plasmid DNA fragment encoding sakacin A production (Fig. 3.6) (Axelsson and Hölck, 1995). ORF1, encodes a bacteriocin-like protein. *saiA* encodes the 51 amino acid immunity protein. *sapA* encodes the 59 amino acid structural protein of sakacin A. ORF's 2, 3 and 4, *sapK*, *sapR*, *sapT* and *sapE* are transcribed in the opposite direction. ORF's 2 and 3 overlap each other. ORF4 encodes a bacteriocin-like protein that shares homology with plantaricin A, a bacteriocin-like induction factor for plantaricins EF and JK (Anderssen *et al.*, 1998). *sapK* encodes a 432 amino acid histidine kinase protein. *sapR* encodes a 247 amino acid response regulator. *sapT,* encodes a 719 amino acid protein, and *sapE* encodes a 461 amino acid protein. SapT and SapE are the transporter proteins.

Between *sapA* and *sapK*, and overlapping ORF's 2 and 3, is the insertion element IS1163. This insertion element is also found in the vicinity of the genetic determinants of lactocin S, a lantibiotic produced by *L. sakei* L45 (Skaugen and Nes, 1994). This transposable element occurs in *L. sakei* and is a member of the IS3 family of insertion sequences that are found in a wide range of bacteria (Fayet *et al.*, 1990). In the sakacin A genetic structure, the IS element is flanked by two inverted repeat regions LIR (left inverted repeat) and RIR (right inverted repeat). The inverted repeat LIR was found to be essential for expression of the bacteriocin and for immunity, while the inverted repeat RIR was essential for bacteriocin production but not for immunity. It was speculated that ORF's 2 and 3 probably encodes a fusion protein (Axelsson and Hölck, 1995). Although sakacin A is identical to curvacin A, the IS1163 element and inverted repeat sequences are not present in the curvacin A DNA sequence (Axelsson and Hölck, 1995).

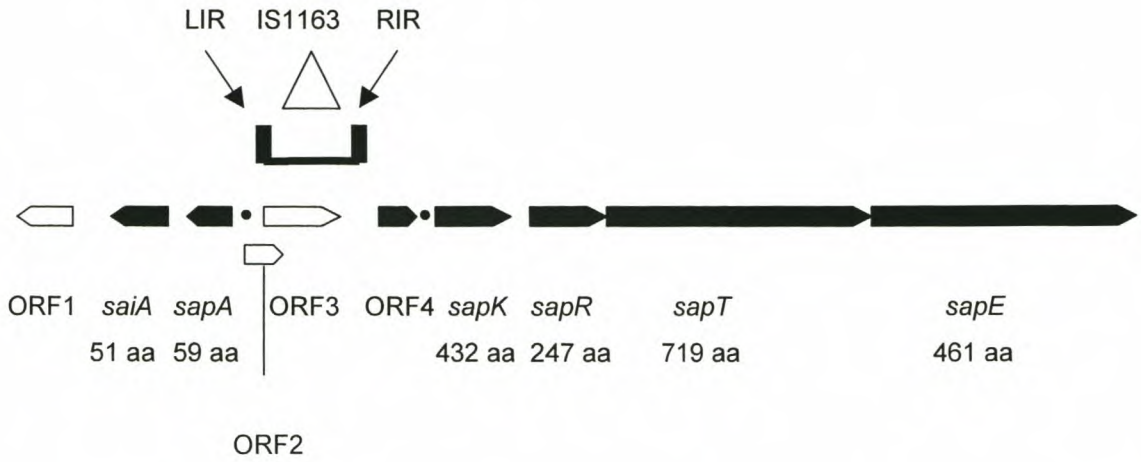

Fig. 3.6. Schematic representation of the gene cluster of sakacin A (Axelsson and Hölck, 1995). The promoter (•) and filled arrows indicate the individual genes and the direction of transcription.

O'Keeffe *et al.* (1999) characterized the genetic arrangement of enterocin A produced by *E. faecium* strain DPC1146. The enterocin A gene cluster comprises 12 ORF's (Fig. 3.7). *entA*, encoding the structural protein, *entI*, encoding the immunity protein, *entF*, an induction factor, *entK*, a histidine kinase protein and *entR*, a response regulator, are followed downstream by ORF's 3, 2 and1, which are oriented in the opposite direction. No function could be determined for ORF's 1 and 3. ORF2 showed homology to components of lactococcin M and lactacin F. Downstream, again oriented in the opposite direction from ORF's 3, 2 and 1 are *entT*, encoding an ABC transporter, *entD*, encoding an accessory factor, and ORF4, which possibly encodes a serine protease that may play a roll in the degradation of signal peptides in the cell membrane. No function could be determined for ORF5 (O'Keeffe *et al.*, 1999).

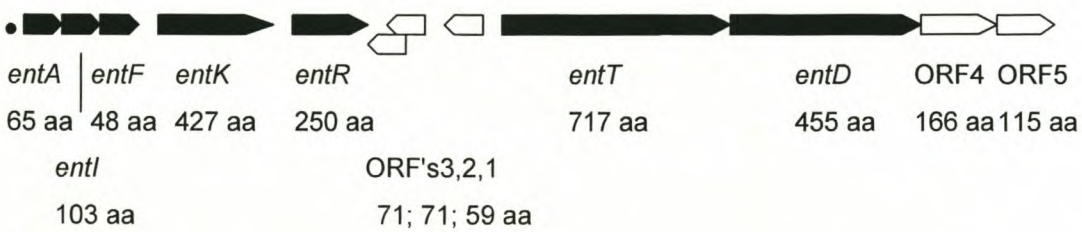

Fig. 3.7. Schematic representation of the gene cluster of enterocin A (O'Keeffe *et al.*, 1999). The promoter (•) and filled arrows indicate the individual genes and direction of transcription.

It has not been determined whether the genetic structure of the enterocin A locus of *E. faecium* DPC1146 (O'Keeffe *et al.*, 1999) is identical to the genetic structures of enterocin A loci of strain T136 (Casaus *et al.*, 1997) and strain CTC492 (Aymerich *et al.*, 1996). The latter two strains also produce a second bacteriocin, enterocin B.

The genetic organization of several other Class II bacteriocins have been determined. These include the Class IIb two-peptide bacteriocins, lactacin F and plantaricins EF and JK, the Class IIc *sec*-dependent bacteriocin enterocin P and the Class IId unclassified bacteriocin lactococcin A.

Analysis of the two-peptide lactacin F DNA region revealed a small operon that encodes three ORF's, called lafA, lafX and ORFZ. LafA was identified as the structural gene for lactacin F. Expression of both genes lafA and lafX is necessary for bacteriocin production, while ORFZ is a putative immunity protein (Fremaux *et al.*, 1993).

Lactobacillus plantarum C11 produces six bacteriocin-like structures, PlnA, PlnE, PlnF, PlnJ, PlnK, and PlnN. The genes encoding these bacteriocin-like structures, are arranged in five operons, *plnABCD*, *plnEFI*, *plnJKLR*, *plnMNOP* and *plnGHSTUV*. *plnEF* and *plnJK* encode two two-peptide bacteriocins. *plnI*, *plnL*, *plnM* and *plnP* probably encode immunity proteins. *plnB*, *plnC*, and *plnD* encode proteins involved in signal transduction, and *plnG* and *plnH* encode secretion and processing proteins. No function could be determined for the protein encoded by *plnN* (Anderssen *et al.*, 1998).

Genetic characterization of the DNA sequence encoding the *sec*-dependent enterocin P revealed two ORF's. The first ORF encodes a 71 amino acid protein containing an N-terminal *sec*-dependent leader sequence. The second ORF encoded an 88 amino acid putative immunity protein (Cintas *et al.*, 1997).

Analysis of the DNA fragment encoding the production and immunity of lactococcin A, a Class IId bacteriocin, showed four ORF's. These genes occurred in the order *lcnC*, *lcnD*, *lcnA* and *lciA*. *lcnA* and *lciA* encoded the structural and immunity genes, respectively. LcnC, a protein of 716 amino acids and LcnD, a 474 amino acid protein are the ABC transporter and accessory proteins (Stoddard *et al.*, 1992).

3.4.2.1 The structural prebacteriocin gene

The structural gene encodes a prebacteriocin, called a precursor or prepeptide. These prepeptides have always been thought to be biologically inactive, and contain an N-terminal leader sequence and a C-terminal propeptide which is cleaved from the N-terminal leader sequence to form a mature, antimicrobial peptide (Jack *et al.*, 1995; Kolter and Moreno, 1992).

All Class II bacteriocins are produced as precursors with an N-terminal extension (Van Belkum and Stiles, 1995). Most of the leader peptides differ from typical signal secretion peptides that direct polypeptides into *sec*-dependent secretion pathways (Jack *et al.*, 1995).

The function of leader peptides appear to be the prevention of biological activity of the bacteriocin while still in the producer cell, and to provide a recognition signal for the ABC transporter (Nes *et al.*, 1996; Kolter and Moreno, 1992). Leader peptides may prevent activity of prebacteriocins by increasing the solubility of prebacteriocins in water, causing the peptides to partition into the aqueous phase rather than into the membrane. Leader peptides may also interact with mature peptides and thus reduce their affinity for membranes. Recently, Ray *et al.* (1999) found the precursor of pediocin AcH to be 80% as active as the mature peptide, suggesting that the leader peptide has little effect on the function of mature domains. This indicates that producer cells with active prebacteriocins need other mechanisms to protect themselves from the prebacteriocin. Suggested mechanisms include the limitation of prebacteriocins to bind successfully to the putative receptor of pediocin AcH, limited membrane insertion activity due to the reverse orientation of the membrane electrochemical potential inside the cell, and neutralization of the prebacteriocin by immunity proteins. Since some bacteriocins require disulfide bonds for activity, the cystein thiol groups may be maintained in a reduced state, resulting in inactivity of the prebacteriocin. In this case, the question arises why it is necessary for the leader peptide to be cleaved during secretion. A possible explanation is that the prebacteriocin is more susceptible to proteases produced by target cells (Ray *et al.*, 1999).

The N-terminal leader peptides of Class IIa bacteriocins are referred to as double-glycine leader peptides. These peptides have two glycine residues at the C-terminus before the cleavage site. Other consensus elements include conserved hydrophobic and hydrophilic regions. The minimum length of the leader peptide of non-lanthionine bacteriocins appears to be 14 amino acids, while the length of the mature bacteriocins identified to date varied from 30 to more than 100 residues (Nes *et al.*, 1996).

3.4.2.2 The immunity gene

The immunity gene encodes a protein that protects the producer organism from its own mature bacteriocin (Nes *et al.*, 1996). Potential immunity proteins have been identified next to, or downstream from, all bacteriocin structural genes studied. Immunity genes not directly associated with the bacteriocin cluster have also been identified (Eijsink *et al.*, 1998). Variation in the presence and expression of these genes may account for the large variation in

sensitivity displayed by lactic acid bacteria towards bacteriocins. Immunity proteins range in sizes from 51 to 150 amino acids. While significant homology exists among the structural genes of the pediocin-like bacteriocins, this trend does not occur with immunity genes, although some resemblances do occur (Aymerich *et al.*, 1996; Moll *et al.*, 1999). The mechanism of action of immunity proteins is not currently understood, but could entail the shielding of a receptor, prevention of pore formation, pore blocking or bacteriocin degradation (Moll *et al.*, 1999).

Several immunity proteins have been identified. Deletion analysis and overexpression of the *pedB* gene of the pediocin PA-1 operon in *P. acidilactici* were conducted to confirm that PedB was the immunity gene of pediocin PA-1 (Venema *et al.*, 1995). Similarly, the confirmation of LcaB as the immunity protein of leucocin A was confirmed by deletion analysis (Van Belkum and Stiles, 1995).

Although the bacteriocins carnobacteriocin B2 and carnobacteriocin BM1 which are produced by the same organism, share significant amino acid homology, the immunity gene of carnobacteriocin B2 does not confer immunity to carnobacteriocin BM1 (Quadri *et al.*, 1995).

3.4.2.3 The transporter gene

Bacteriocins, similar to other molecules synthesized in the cytoplasm of bacteria and secreted, need to cross one or more membranes to reach their destination. This transport is facilitated via the general *sec* signal secretion pathway, or by using a dedicated export system (Fath and Kolter, 1993; Wickner *et al.*, 1991).

Bacteriocins containing the double-glycine type leader sequences (G-G) are translocated by a dedicated export system identified as ABC (*A*TP-*b*inding *c*assette) transporters (Fath and Kolter, 1993; Nes *et al.,* 1996). The gene encoding the bacteriocin ABC transporter is usually part of the bacteriocin operon, or can be found on an operon near the vicinity of the bacteriocin operon (Nes *et al.,* 1996). ABC transporters facilitate the secretion of a wide range of products in both prokaryotic and eucaryotic organisms. These products include periplasmic permeases (bacterial importers), which transport oligopeptides, amino acids, sugars, phosphate, metal ions and vitamins, eukaryotic exporters, which transport lipophylic drugs, peptides and pigments, and bacterial exporters, which transport molecules such as large protein toxins, small peptide antibiotics, polysaccharides, antibiotics, and possibly heme molecules (Fath and Kolter, 1993).

The bacteriocin ABC transporters have a dual function, facilitating both the removal of the leader peptide from its substrate and the transport of the substrate across the cytoplasmic

membrane (Håvarstein *et al.*, 1995). Bacteriocin ABC-transporters contain three domains on the same polypeptide, consisting of a cytoplasmic N-terminal proteolytic domain, a hydrophobic integral membrane domain, and a cytoplasmic C-terminal ATP-binding domain (Fig.3.8) (Håvarstein *et al.*, 1995; Nes *et al.*, 1996). Two polypeptides appear to be required for the bacteriocin ABC transporter to be functional (Håvarstein *et al.*, 1995).

A unique feature of bacteriocin ABC transporters is that they carry an N-terminal extension of approximately 150 amino acids, the proteolytic domain, which appears to be involved in the processing of the bacteriocins (Nes *et al.,* 1996). Two conserved motifs, the cysteine motif (QX4D/ECX2AX3MX4Y/FGx4I/L) and the histidine motif (HY/FY/VVX10I/LXDP) have been identified in the proteolytic domain and appear to be necessary for translocation (Håvarstein *et al.*, 1995). Håvarstein *et al.* (1995) hypothesized that the proteolytic domain of the double-glycine leader bacteriocins binds the bacteriocin precursor. The processing site is part of the transporter, which indicates that the processes of cleavage and translocation are integrated, and that the leader peptide serves as a recognition signal for the transmembrane transport process of the bacteriocin (Nes *et al.,* 1996; Van Belkum *et al.*, 1997). Studies on the ABC transporter of pediocin PA-1 suggest that the N-terminal part of PedD is required for cleavage and that this process can be uncoupled from secretion (Venema *et al.*, 1995). Franke *et al.* (1996) proposed a model of LcnD, a protein involved with the transport of several bacteriocins from *L. lactis*, where the N-terminal part of the protein is located intracellularly and one transmembrane helix spans the cytoplasmic membrane.

The membrane spanning domains (MSD's) consist of six membrane spanning segments (Fig. 3.8). ABC transporters (or traffic ATPases) use ATP hydrolysis as a source of energy required for translocation and have a highly conserved ATP-binding cassette (Fath and Kolter, 1993). The conserved ATP-binding motif can be found in all the ABC transporters of the bacterial export subfamily.

The removal of the leader peptide from its substrate and the subsequent translocation of the bacteriocin across the cytoplasmic membrane effectively prevent the mature and active bacteriocins from remaining in the cytoplasm (Håvarstein *et al.*, 1995).

Some bacteriocins do not possess a double-glycine leader peptide, but are synthesized with a *sec*-type N-terminal leader sequence, leading to secretion and processing via the *sec* pathway (Nes *et al.*, 1996). Secretion via the *sec*-dependent pathway requires that the product be a protein with an N-terminal signal sequence (Fath and Kolter (1993). The Class IIc bacteriocins divergicin A (Worobo *et al.,* 1995) and enterocin P (Cintas *et al.*, 1997) are secreted in this manner. McCormick *et al.* (1996) reported the successful secretion of carnobacteriocin B2, which is usually exported by an ABC-transporter, using the signal peptide

of divergicin A, a *sec*-dependent peptide. Similarly heterologous expression of mesentericin Y105 using both the dedicated transport system and the *sec* pathway has been reported (Biet *et al.*, 1998).

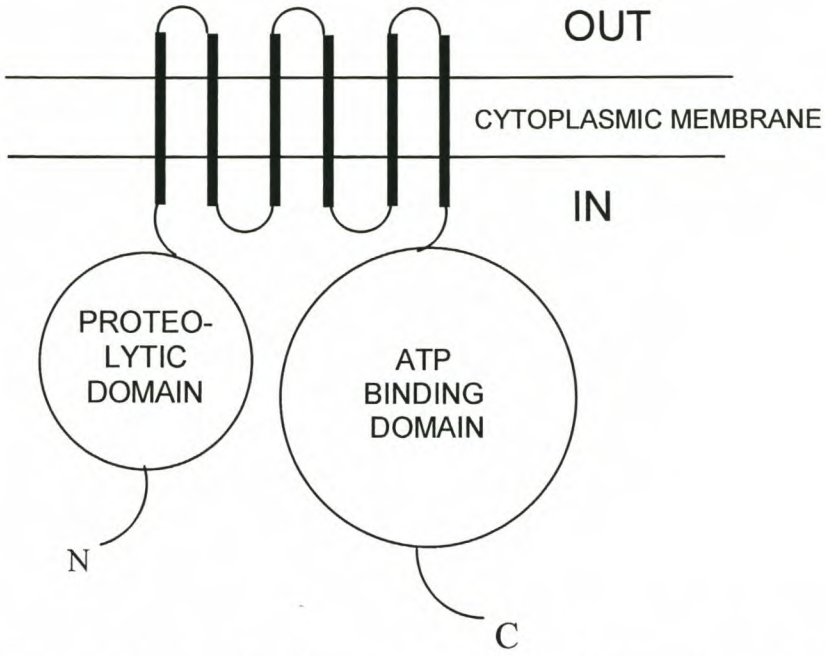

Fig. 3.8. ABC translocator with the N-terminal proteolytic domain, 6 membrane spanning domains and the ATP-binding domain in the C-terminal of a single polypeptide. (Redrawn from Håvarstein *et al.*, 1995).

3.4.2.4 The accessory protein

Several studies have indicated the presence of an additional gene within bacteriocin operons, called the accessory protein (also accessory factor), that is required for the ABC-transporter dependent translocation process. These additional factors have been identified in several Gram-negative systems to be needed when the secreted product is destined for immediate release into the extracellular medium (Fath and Kolter, 1993). It is hypothesized that the accessory factor is anchored in the inner membrane and spans the periplasm, probably connecting the inner and outer membranes to facilitate the export of products through both membranes of Gram-negative bacteria. In Gram-positive bacteria, the function of the accessory factor is unclear, since the secreted product only needs to cross one membrane (Fath and Kolter, 1993; Nes *et al.*, 1996). Accessory factors involved in bacteriocin secretion include pedC, of the pediocin PA-1 operon (Franke *et al.*, 1996; Marugg *et al.*, 1992; Venema *et al.*, 1995).

3.4.2.5 Regulation of bacteriocin synthesis

Regulatory genes associated with the gene clusters of several bacteriocins produced by lactic acid bacteria have been reported (Diep *et al.*, 1994; Nes *et al.*, 1996). The autoregulatory system usually consists of three components, a response regulator (RR) gene, a sensor histidine protein kinase (HK) gene and an induction factor (Nes *et al.*, 1996). Histidine kinase proteins, which are associated with the cytoplasmic membrane, respond to environmental signals, which are communicated to the response regulators. The response regulators transcribe and regulate the expression of target operons (Diep *et al.*, 1995). Activation of the histidine kinase by the induction factor appears to be cell-density-dependent, with a secreted peptide pheromone (induction factor) functioning as the input signal for a specific sensor component of the regulatory system in a quorum-sensing manner (Kleerebezem *et al.*, 1997; Nes *et al.*, 1996). Although induction factors are structurally similar to bacteriocins, they are usually considerably shorter and lack antimicrobial activity (Kleerebezem *et al.*, 1997; Nes *et al.*, 1996).

Nisin induces the transcription of its own structural gene and downstream genes by signal transduction, acting as an extracellular signal for the NisK sensor histidine kinase (Brurberg *et al.*, 1997; Kuipers *et al.*, 1995). The genes of the inducer molecule (nisin) do not occur in the same transcriptional unit as the two-component signal transduction system (Brurberg *et al.*, 1997).

The mechanism of signal transduction for the plantaricins of *L. plantarum* C11 have been extensively researched (Anderssen *et al.*, 1998; Diep *et al.*, 1994; 1996). Plantaricin A, a bacteriocin-like peptide, induces transcription of several bacteriocin operons of *L. plantarum* C11. The gene encoding plantaricin A occurs in an operon, *plnABCD* (see also Section 3.4.2) but unlike other bacteriocin operons, none of the other genes in this operon encode an immunity protein (Diep *et al.*, 1995).

EntF is a 41 amino acid peptide with a G-G leader peptide which is necessary for enterocin A and B production by the producing strain *Enterococcus faecium* CTC492 (Nilsen *et al.*, 1998). Similarly, sakacin P production is dependent on an induction factor, which also induces the immunity protein as well as itself (Brurberg *et al.*, 1997; Eijsink *et al.,* 1996; Hühne *et al.*, 1996). Signal transducing systems have also been identified for sakacin A (Axelsson and Holck, 1995) and carnobacteriocins B2 and BM1 (Quadri *et al.*, 1997). For sakacin A, orf4 (Fig. 3.6) encodes the putative precursor of a 23 amino acid peptide, termed Sap-Ph. Sap-Ph has been identified as a pheromone that regulates bacteriocin production (Diep *et al.*, 2000). It has also been shown that the production of sakacin A is temperature sensitive, with

production reduced or abolished at temperatures of 33.5-35°C, while production occurred at 25-30°C. Whether the sensitivity to temperature plays a role in the initiation of bacteriocin production is not known (Diep *et al.*, 2000).

3.5 MODE OF ACTION OF CLASS II BACTERIOCINS

Although the mode of action of lantibiotics, particularly nisin, have been studied in detail, much less is known about the interaction between Class II bacteriocins and the membranes of target organisms.

The Class II bacteriocins demonstrate a bactericidal mode of action against other closely related organisms. These bacteriocins dissipate the proton motive force by disrupting the transmembrane potential and/or the pH gradient of sensitive cells (Montville and Bruno, 1994). Pediocin PA-1, mesentericin Y105 and lactococcin A permit the efflux of relatively large molecules (Chikindas *et al.*, 1993; Maftah *et al.*, 1993; Van Belkum *et al.*, 1991). Two-peptide bacteriocins appear to form relatively specific pores, dissipating the transmembrane potential. Lactococcin G dissipates the membrane potential, which causes the selective efflux of potassium ions from sensitive cells (Moll *et al.*, 1996). Lactococcin G activity is dependent on the extracellular pH (Moll *et al.*, 1998). Plantaricin E/F dissipates the pH gradient, causing a pH increase (Moll *et al.*, 1999). Acidocin J1132 permits efflux of molecules such as glutamate (Tahara *et al.*, 1996). Lactacin F induces the efflux of potassium ions and phosphate (Abee *et al.*, 1994. Acidocin J1132 (Tahara *et al.*, 1996) and thermophilin 13 (Marciset *et al.*, 1997) immediately dissipate the pH gradient.

It is proposed that bacteriocin mediated transmembrane ion flow results in cytotoxic effects, causing a drop in the intracellular pH and inhibiting enzymatic processes. An influx of cytotoxic sodium ions and a depletion of ATP due to futile cycles are caused by ion gradient dissipation. Dissipation of the proton motive force and the transmembrane potential arrest processes dependent on these gradients (Bruno and Montville, 1993; Moll *et al.*, 1999).

Bacteriocins form pores in the membranes of target cells (Abee, 1995; Abee *et al.*, 1995). It is hypothesized that the mode of action involves various steps such as binding, insertion and pore formation (Montville and Chen, 1998). Binding of the bacteriocin to the target membrane is necessary for subsequent insertion and pore formation. Although the interaction of a receptor-like factor has been implicated for pediocin PA-1 (Chikindas *et al.*, 1993) and leucocin A (Fregeau Gallagher *et al.*, 1997), a protein receptor does not appear to be essential for binding. . Studies by Breukink *et al.* (1999) have indicated that nisin specifically interacts with the membrane-anchored cell wall precursor Lipid II. Chen *et al.* (1997b) suggested that the

binding step primarily involved electrostatic interactions between positive areas of amino acid groups in the bacteriocin and negatively charged phospholipid groups in the target membrane. Jack *et al.* (1995) also implied that anionic cell surface molecules in the cell wall of Gram-positive bacteria might play a role in the initial interaction with cationic bacteriocins. Analysis of chimers that consist of pediocin-like peptides, indicated that the C-terminal part of the molecule is responsible for target specificity (Fimland *et al.*, 1996). A C-terminal fragment of pediocin PA-1 inhibited the activity of pediocin PA-1 peptide, indicating that this fragment competed with the intact peptide for binding sites on the target membrane (Fimland *et al.*, 1998). Kaiser and Montville (1996) observed that the C-terminal of bavaricin MN was probably essential for membrane interaction. Lactococcin G, a two-peptide Class IIb bacteriocin also showed strong membrane interaction, with the two peptides being capable of binding to the membrane independently (Hauge *et al.*, 1998).

Bacteriocins are unstructured in an aqueous solution, but have the ability to form α-helical structures when exposed to structure promoting solvents, or when mixed with anionic phospholipid membranes (Moll *et al.*, 1999). Studies on the three-dimensional structure of leucocin A (37 amino acids) has shown that the peptide exists as a random coil in water, but two domains can be identified in lipophilic media. These domains are an amphiphilic α-helix from residues 17-31, and a three-stranded antiparallel β-sheet from residues 2-16, that is linked by a disulfide bridge (Fregeau Gallagher *et al.*, 1997). It is hypothesized that the highly conserved N-terminal of the Class IIa bacteriocins contributes to membrane binding. This allows the low homologous C-terminals to transform from random conformations to defined secondary structures, which are essential for pore formation (Montville and Chen, 1998).

Specific amino acids play a role in the antimicrobial activity of Class IIa bacteriocins. The presence of cysteins in the structure of these bacteriocins with subsequent modification of pairs of cysteine residues to form disulfide bridges affects the activity of bacteriocins (Miller *et al.*, 1998). Comparative studies by Eijsink *et al.* (1998) showed that pediocin PA-1 and enterocin A, which both contain two disulfide bonds, were more active than sakacin P and curvacin A, which contain only one disulfide bond. Pediocin PA-1 lost its activity completely when its sulfide bonds were reduced with dithiothreitol (Chikindas *et al.*, 1993).

Aromatic amino acids are also involved with antimicrobial activity. Removal of tryptophan from the C-terminus of mesentericin Y105 (Fleury *et al.*, 1996), substitution of phenylalanine with serine in carnobacteriocin B2 (Quadri *et al.*, 1997) and substitution of tryptophan with arginine in pediocin PA-1 (Miller *et al.*, 1998) resulted in reduction of activity of these bacteriocins. Loss of activity when small fragments of the N-terminal or the C-terminal are removed suggests that the whole sequence of the bacteriocin is necessary for activity (Fleury

et al., 1996; Miller *et al.*1998).

Two models are proposed for pore-formation by the Class II bacteriocins. It is thought that the bacteriocins may form a barrel-stave-like bundle of α-helical peptides upon membrane insertion causing the formation of a pore (Fig. 3.9) (Moll *et al.*, 1999). The presence of a helix-breaking amino acid residue in the middle of their sequence may facilitate the insertion of the peptide into the membrane from an initial surface bound state. The hydrophilic faces of a bundle of amphiphatic α-helical peptides form the inner wall of the water-filled pore. The outer hydrophobic side of the helical bundles is oriented towards the fatty acyl chains of the membrane lipids (Moll *et al.*, 1999).

Alternatively, a carpet-like model could explain membrane pore formation. Single peptide molecules might be orientated parallel to the membrane surface and interfere with the membrane bilayer organization without forming a peptide aggregate. Once sufficient peptides are present, temporary membrane collapse due to a strong phospholipid mobilizing activity occurs, resulting in a local and transient permeability (Moll *et al.*, 1999). Homblé *et al.* (1998) suggested that the negative charge of the membrane lipids confer cation selectivity to such pores.

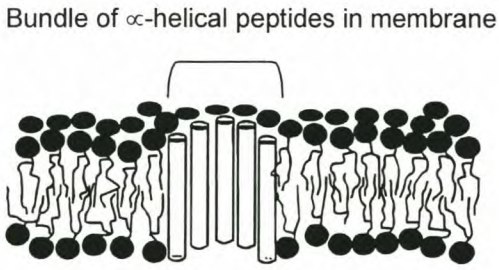

Fig. 3.9. The barrel-stave method as hypothesized. Reproduced from Moll *et al.* (1999).

The mode of action of lactococcin 972, a *sec*-dependent bacteriocin, appears to differ from other bacteriocins, since cell wall biosynthesis is the primary target and not the plasma membrane. Lactococcin 972 inhibits septum formation in susceptable lactococci, causing deformation and gross structural changes that lead to cell death (Martínez *et al.*, 2000). Further studies on the primary target of lactococcin 972 may lead to the creation of a new subgroup within the Class II group of bacteriocins (Martínez *et al.*, 2000).

3.6 FUTURE PROSPECTS FOR BACTERIOCIN APPLICATION AND RESEARCH

Several questions about bacteriocins remain unanswered:

Although the use of bacteriocins produced by lactic acid bacteria is efficient for the inhibition of organisms such as *C. tyrobutyricum* in cheese, the application of bacteriocins to dairy products requiring lactic acid starter cultures may be limited, due to the inhibition by bacteriocins of various lactic acid bacteria. The use of bacteriocins proved successful in the preservation of meat and fish products (Abee *et al.*, 1995). Various factors influence the activity of bacteriocins in food, such as pH, proteolytic enzymes, lipid content, solid or liquid systems, salt content and temperature (Abee *et al.*, 1995; Gänzle *et al.*, 1999). Bacteria may also become resistant to bacteriocins (Abee *et al.*, 1995; Gravesen *et al.* 2000; Maisnier-Patin and Richard, 1996). To date several factors such as inactivation, low production, genetic instability and regulation of bacteriocins by starter cultures, have made cultures producing bacteriocins not widely successful in food fermentation processes (Nes *et al.*, 1996).

Improvement in production efficiency, stability and activity of bacteriocins could be achieved by protein engineering (Nes *et al.*, 1996). The solubility of nisin, for example, is strongly dependent on pH and ionic strength, with the highest solubility observed at low pH and low ionic strength. Similarly optimal stability is observed at pH 3. Using site-directed mutagenesis, Rollema *et al.* (1995) found that the solubility and chemical stability of constructed nisin Z mutants could be significantly improved, while the antimicrobial spectrum and activity remained similar.

The mechanism of the immunity gene and the existence and identity of bacteriocin receptors remain unresolved (Moll *et al.*, 1999; Nes *et al.*, 1996).

How bacteriocin production is activated is explained by two models (Nes *et al.*, 1996). If induction factors are produced in low amounts, the gradual accumulation of induction factors will take place during growth until it exceeds the concentration required to induce the start of bacteriocin production. Alternatively, the production of the induction factor could be balanced at a concentration just below that required for induction. Nutritional, physical or chemical changes in the environment may also trigger a temporarily increased production of induction factors, which would lead to induction of bacteriocin production (Nes *et al.*, 1996).

The observation that bacteriocin-like substances do not always have antimicrobial activity, but may function as induction signals for gene expression, points to a wider function of bacteriocin peptides and raises questions about their biological function, as well as their evolutionary history that is still unexplored (Nes *et al.*, 1996).

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CHAPTER 4

EVALUATION OF NUMERICAL ANALYSIS OF RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)-PCR AS A METHOD TO DIFFERENTIATE *LACTOBACILLUS PLANTARUM* AND *LACTOBACILLUS PENTOSUS*

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Evaluation of Numerical Analysis of Random Amplified Polymorphic DNA (RAPD)-PCR as a Method to Differentiate *Lactobacillus plantarum* and *Lactobacillus pentosus*

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Abstract. *Lactobacillus plantarum* and *Lactobacillus pentosus* grouped into one protein profile cluster at $r \geq 0.70$, separate from *Lactobacillus casei*, *Lactobacillus sake* and *Lactobacillus curvatus*. Similar sugar fermentation reactions were recorded for representative strains of *L. plantarum* and *L. pentosus*. Representative strains, including the type of each species, were selected from the different protein profile clusters and their genetic relatedness determined by using numerical analysis of random amplified polymorphic DNA (RAPD)-PCR. The type strains of *L. plantarum* (ATCC 14917^T) and *L. pentosus* (NCFB 363^T) displayed different RAPD profiles and grouped into two independent clusters, well separated from *L. casei*, *L. curvatus* and *L. sake*. Numerical analysis of RAPD-PCR proved a reliable and accurate method to distinguish between strains of *L. plantarum* and *L. pentosus*.

The species *Lactobacillus plantarum* consists of a genetically heterogeneous collection of strains that are difficult to differentiate from *Lactobacillus pentosus* by simple physiological tests [3, 9]. Pentose-fermenting strains of streptobacteria were originally classified as *L. pentosus* [8]. However, the name *L. pentosus* (Fred, Peterson and Anderson) was not listed in the Approved Lists of Bacterial Names [12] and strains that fermented pentose sugars were classified as *L. plantarum* [9]. Dellaglio et al. [3] were the first to show that a group of pentose-fermenting strains of *L. plantarum*, some of which were originally classified as *L. pentosus* by Fred et al. [8], are genotypically not closely related to *L. plantarum*. Extensive DNA-DNA hybridization studies [13] confirmed the results reported by Dellaglio et al. [4] and the name *L. pentosus* was revived.

DNA similarity studies are the most reliable method to differentiate *L. pentosus* from *L. plantarum*. However, DNA-DNA hybridizations are tedious to perform and often involves the use of radioactive nucleotides. RAPD-PCR analysis, on the other hand, is a safe, rapid and

accurate technique that can be performed in most laboratories. The technique proved valuable in differentiating *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus amylovorus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri* and *Lactobacillus johnsonii* [7].

In this study we evaluated numerical analysis of RAPD-PCR profiles as a method to distinguish between *L. plantarum* and *L. pentosus*.

Materials and Methods

Bacterial strains and growth conditions. The strains included in this study are listed in Fig. 1. Strains with CTC numbers were received from M. Hugas (Institut de Recerca i Tecnologia Agroalimentàries, Monells, Spain). Strains CTC 309, CTC 306, CTC 381, and CTC 378 were originally classified as *L. plantarum*; CTC 287 as *Lactobacillus casei* subsp. *pseudoplantarum*; CTC 368, CTC 372, CTC 376, CTC 13 and CTC 41 as *Lactobacillus sake*; and CTC 253 and CTC 285 as *Lactobacillus curvatus*. Strains with LD numbers were received from F. Dellaglio (Istituto Policattedra, Universitá degli Studi di Verona, Verona, Italy) and were designated as *L. sake* (LD-1 and LD-23) and *L. curvatus* (LD-4, LD-3, LD-15, LD-8, LD-17, LD-16, LD-14, and LD-2). Strains without alphabetical numbers were isolated from sorghum beer. Reference strains were from the American Type Culture Collection (ATCC), the National Collection of Food Bacteria (NCFB), and the Deutsche Sammlung von Mikroorganismen (DSM).

Numerical analysis of total soluble cell protein patterns. Cultures were grown in MRS broth [5] for 18h at 30°C. Preparation of cell extracts, polyacrylamide gel electrophoresis, densitometry, normalization of densitometric tracings, and numerical analysis of normalized electropherograms were done as described by Dicks et al. [6].

Carbohydrate fermentations. Carbohydrate fermentation reactions were determined using the API 50 CHL system (La Balme Les Grottes, Montalieu Vercieu, France). Results were recorded after 48 h at 30°C.

RAPD-PCR analysis. DNA was isolated according to the method of Dellaglio et al. [2]. Polymerase chain reactions (PCR) were performed in duplicate. Each reaction of 25µl contained 50mM KCl, 10mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 0.1% Triton X-100, 200µM of each dNTP, 5 picamoles of a single 10 base primer (Operon Kit L, Operon Technologies, Alameda), 40 ng of genomic DNA, and 2.5 units of Taq Polymerase (Advanced Biotechnologies, West Hampstead, England). Four single primers [GGCATGACCT (OPL-01),

TGGGCGTCAA (OPL-02), GACTGCACAC (OPL-04), and ACGCAGGCAC (OPL-05)] were used. DNA amplification was performed in a Biometra Trio-thermoblock. The cycling program used was 45 cycles of 94°C (1 min), 36°C (1 min), and 72°C (2 min). Final incubation was performed at 72°C for 5 min, followed by cooling to 4°C, until samples were retrieved. Amplification products were analyzed by electrophoresis in 1.4% agarose gels using TAE buffer [10]. Lambda DNA, digested with EcoR1 and HindIII (Boehringer Mannheim), was used as molecular weight marker.

Numerical analysis of RAPD-PCR profiles. The four RAPD-PCR gels were individually photographed and printed to the same size. An overhead transparency was placed on each of the gel photographs and divided into 24 vertical columns (one column for each lane) and several horizontal rows (one row for each DNA band on the gel). A value of 1 was allocated to a block containing a DNA band. A value of 0 (zero) was allocated to a block without a DNA fragment. The data was analyzed using the CLUSTER program of the SAS Institute Inc. [11]. Dendrograms were constructed from the normalized average linkage cluster analysis. Distances between clusters were expressed in R^2-values.

Results and Discussion

Twenty-eight strains of *L. plantarum* and *L. pentosus*, including the type strains (ATCC 14917^T and NCFB 363^T, respectively) grouped into one protein profile cluster at $r \geq 0.70$, separate from *Lactobacillus casei* and *Lactobacillus paracasei* (cluster II), *L. sake* (cluster III), and *L. curvatus* in cluster IV (Fig. 1). The overall protein profiles of *L. plantarum* and *L. pentosus* corresponded well, confirming that the species are phenotypically closely related.

Similar sugar fermentation reactions were recorded for representative strains of *L. plantarum*, with little difference in the profiles between these strains and the type strain of *L. pentosus*, NCFB 363^T (Table 1). D-Xylose was fermented by *L. pentosus* NCFB 363^T, but not by strains of *L. plantarum*, confirming the results reported by Kandler and Weiss [9] and Zanoni et al. [13]. Strains of *L. plantarum* included in our study were unable to ferment glycerol (Table 1) and are in this respect different from the type strain of *L. pentosus* (NCFB 363^T). However, two out of 14 strains of *L. plantarum* studied by Zanoni et al. [13] fermented glycerol. The fermentation of glycerol can thus not be used to differentiate *L. plantarum* from *L. pentosus*. The inability of *L. pentosus* to ferment α-methyl-D-mannoside [13] was confirmed (Table 1). However, this characteristic is not unique to *L. pentosus*. Four out of eight strains of *L. plantarum* which we have studied (Table 1), and six out of 14 strains of *L. plantarum*

studied by Zanoni et al. [13], were unable to utilize α-methyl-D-mannoside.

The RAPD profiles of representative strains selected from protein profile clusters I - IV (Fig. 1) are shown in Fig. 2. Six RAPD-PCR profile clusters were obtained at $R^2 \geq 0.49$ (Fig. 3). Twelve of the 15 strains of *L. plantarum*, including the type strain (ATCC 14917^T), grouped in cluster I at $R^2 \geq 0.50$. Three strains of *L. plantarum* (CTC 306, CTC 381 and CTC 378) grouped in cluster II at $R^2 \geq 0.95$. Two strains of *L. pentosus* (NCFB 1194 and NCFB 363^T) grouped at $R^2 = 0.88$ in cluster IV, well separated from strains of *L. plantarum*. Strains of *L. casei*, *L. curvatus* and *L. sake* grouped into three well-defined clusters.

Lactobacillus paracasei NCFB 151^T, NCFB 2713 and DSM 20006, and *L. casei* LHS and ATCC 334 formed a tight cluster at $r \geq 0.80$ (Fig. 1). The RAPD-PCR profiles of *L. paracasei* NCFB 151^T and *L. casei* ATCC 334 corresponded well (Fig. 2) and they grouped at $R^2 = 0.60$ (Fig. 3). These results confirmed our previous findings [4], that is, that strains of *L. paracasei* are genotypically closely related to *L. casei* ATCC 334, one of the original strains of *L. casei*.

The protein profiles of strain CTC 13 and CTC 41, received as *L. sake*, corresponded well and grouped with strains of *L. curvatus* in one protein profile cluster at $r \geq 0.80$ (Fig. 1). However, the RAPD-PCR profile of strain CTC 13 is very similar to that obtained for *L. sake* ATCC 2714^T (Fig. 2) and they formed a tight cluster at $R^2 \geq 0.70$ (Fig. 3). The classification of strains CTC 13 and CTC 41 as *L. sake* is confirmed.

Strain CTC 287, received as *L. casei* subsp. *pseudoplantarum*, grouped with strains of *L. plantarum* and *L. pentosus* at $r \geq 0.70$ in cluster I, separate from strains of *L. casei* and *L. paracasei* (Fig. 1). The classification of strain CTC 287 as *L. casei* subsp. *pseudoplantarum* is questioned.

Numerical analysis of RAPD-PCR profiles proved valuable in the differentiation of strains of *L.* plantarum and *L. pentosus*, which grouped in the same protein profile cluster and displayed similar sugar fermentation reactions. Distinctive differences were recorded in the RAPD-PCR profiles of *L. plantarum*, *L. pentosus*, *L. casei*, *L. curvatus* and *L. sake*. Groupings obtained by numerical analyses of RAPD-PCR profiles and total soluble cell protein patterns are summarized in Table 2.

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Table 1. Differential carbohydrate fermentation reactions among strains of *Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus sake* and *Lactobacillus curvatus* [a]

Carbohydrate	*L. plantarum*								*L. pentosus* [b]	*L. casei*		*L. paracasei* [c]	*L. sake*	*L. curvatus*
	81	780	413 I	412	759	423	285	ATCC 14917^T	NCFB 363^T	ATCC 334	ATCC 393^T	NCFB 151^T	NCFB 2714^T	NCFB 2739^T
Adonitol	-	-	-	-	-	-	-	-	-	-	-	d	-	-
Amidon	+	-	-	-	+	+	+	-	-	-	-	d	-	-
Amygdalin	+	+	+	+	+	+	+	+	+	-	+	+	-	+
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	d	-	-
L-Arabinose	+	-	-	-	-	+	+	+	+	-	-	-	+	+
D-Arabitol	-	-	+	-	-	-	-	-	d	-	-	d	-	-
Arbutin	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	d	-	-	d	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	d	-	-
β-Gentiobiose	+	+	+	+	+	+	+	+	+	-	+	d	+	+
Gluconate	-	-	+	+	+	-	-	-	+	-	-	d	-	+
Glycerol	-	-	-	-	-	-	-	-	+	+	-	d	-	-
Glycogen	+	-	-	-	+	+	+	-	-	-	-	ND	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	d	-	-
Inulin	-	-	-	-	-	+	+	-	-	+	-	d	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	d	+	+
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	d	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Melezitose	+	+	+	-	+	+	+	+	d	-	-	+	-	+
Melibiose	+	+	+	+	+	+	+	+	+	-	-	ND	+	+
α-Methyl-D-glucoside	-	-	-	-	-	-	-	-	d	+	-	d	-	-
α-Methyl-D-mannoside	-	-	-	-	+	+	+	+	-	-	-	-	-	-
D-Raffinose	+	+	+	-	+	+	+	+	+	-	-	ND	-	+
Rhamnose	-	-	-	-	-	-	-	-	d	-	-	-	-	-
Ribose	+	+	-	+	+	+	+	+	+	+	+	d	+	+
Sorbitol	+	+	+	+	+	-	-	+	+	-	+	d	-	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	+	d	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	-	d	+	+
D-Tagatose	-	-	-	-	-	-	-	-	-	+	+	+	-	-
Trehalose	+	-	+	+	+	+	+	+	+	+	+	+	+	+
D-Turanose	+	-	-	-	+	-	-	+	d	+	+	d	-	+
D-Xylose	-	-	-	-	-	-	-	-	+	-	-	-	-	-

[a] +, positive reaction; -, negative reaction; d, variable reaction; ND, not determined.
All strains fermented N-acetyl-D-glucosamine, cellobiose, D-fructose, galactose, D-glucose, maltose, D-mannose, salicin and hydrolized esculin.
None of the strains fermented L-arabitol, erythritol, L-fucose, 2-keto-gluconate, 5-keto-gluconate, β-methyl-D-xyloside, xylitol and L-xylose.

[b] From Zanoni *et al.* [13].

[c] From Collins *et al.* [1].

Table 2. Genotypic relatedness among *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus sake*, and *Lactobacillus curvatus*

Strain	Protein cluster[a]	RAPD-PCR cluster[b]
L. plantarum		
81, 780, 413 l, 412, 759, 423,	I	I
285, CTC 309, ATCC 14917T,	I	I
ATCC 8014, NCFB 340, NCFB 965,	I	I
415, 413d, 410, 448, 453, 780,	I	ND[c]
427, CTC 287, 447, 411, 414,	I	ND
CTC 306, CTC 381, CTC 378,	I	II
L. pentosus		
NCFB 1194, NCFB 363T	I	IV
L. casei		
ATCC 334,	II	III
LHS	II	ND
L. paracasei		
NCFB 151T,	II	III
NCFB 2713, DSM 20006	II	ND
L. sake		
NCFB 2714T, CTC 376,	III	VI
LD-1, CTC 368, CTC 372,	III	ND
CTC 13	IV	VI
LD-23, CTC 41	IV	ND
L. curvatus		
NCFB 2739T, LD-14	IV	V
LD-4, LD-3, LD-15, LD-8, CTC 253,	IV	ND
LD-17, LD-16, CTC 285, LD-2	IV	ND

[a] From Fig. 1.

[b] From Fig. 3.

[c] ND, not determined.

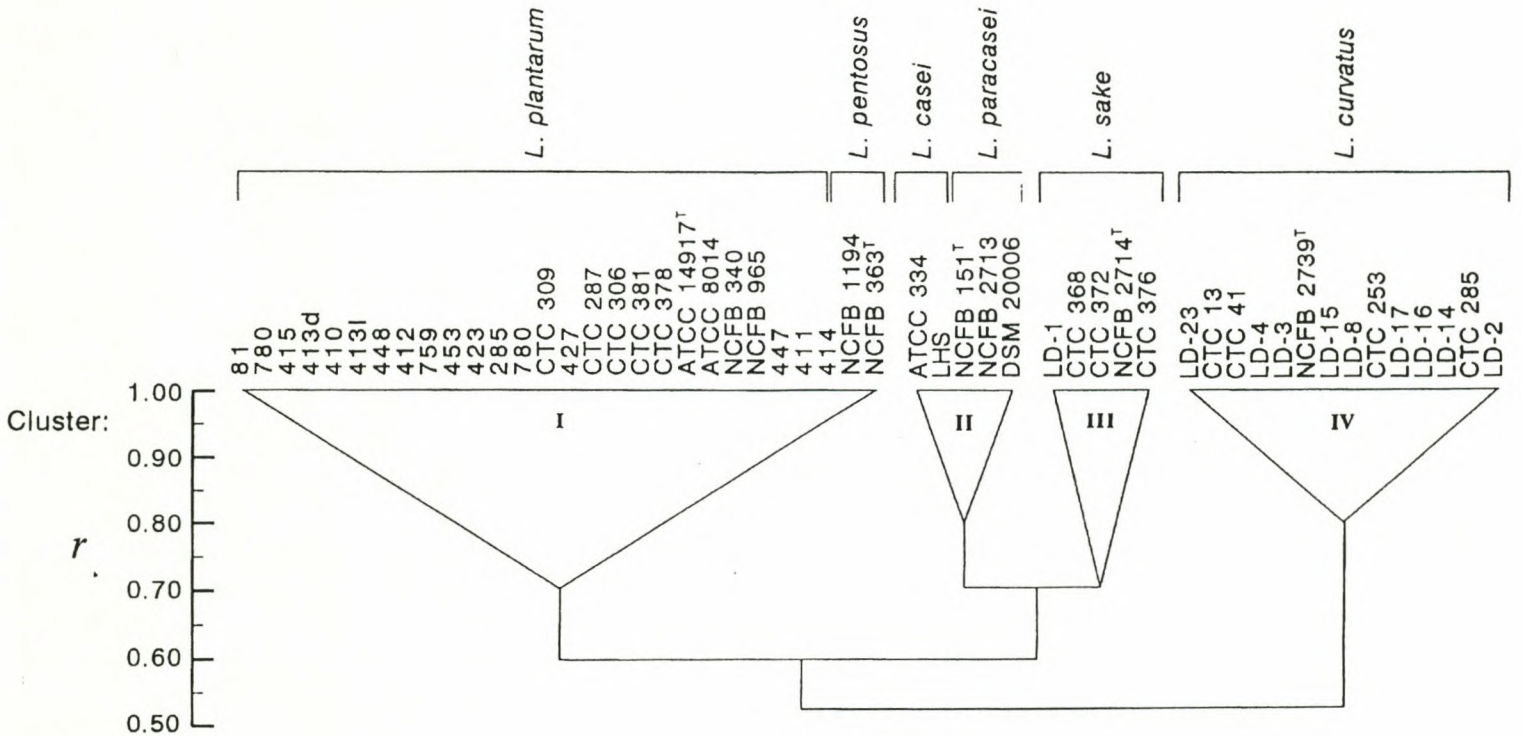

Fig. 1. Simplified dendrogram showing the clustering of *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus sake* and *Lactobacillus curvatus* obtained by numerical analysis of total soluble cell protein patterns. The methods used were described by Dicks et al. [6]. Clustering was by the unweighted average pair group method.

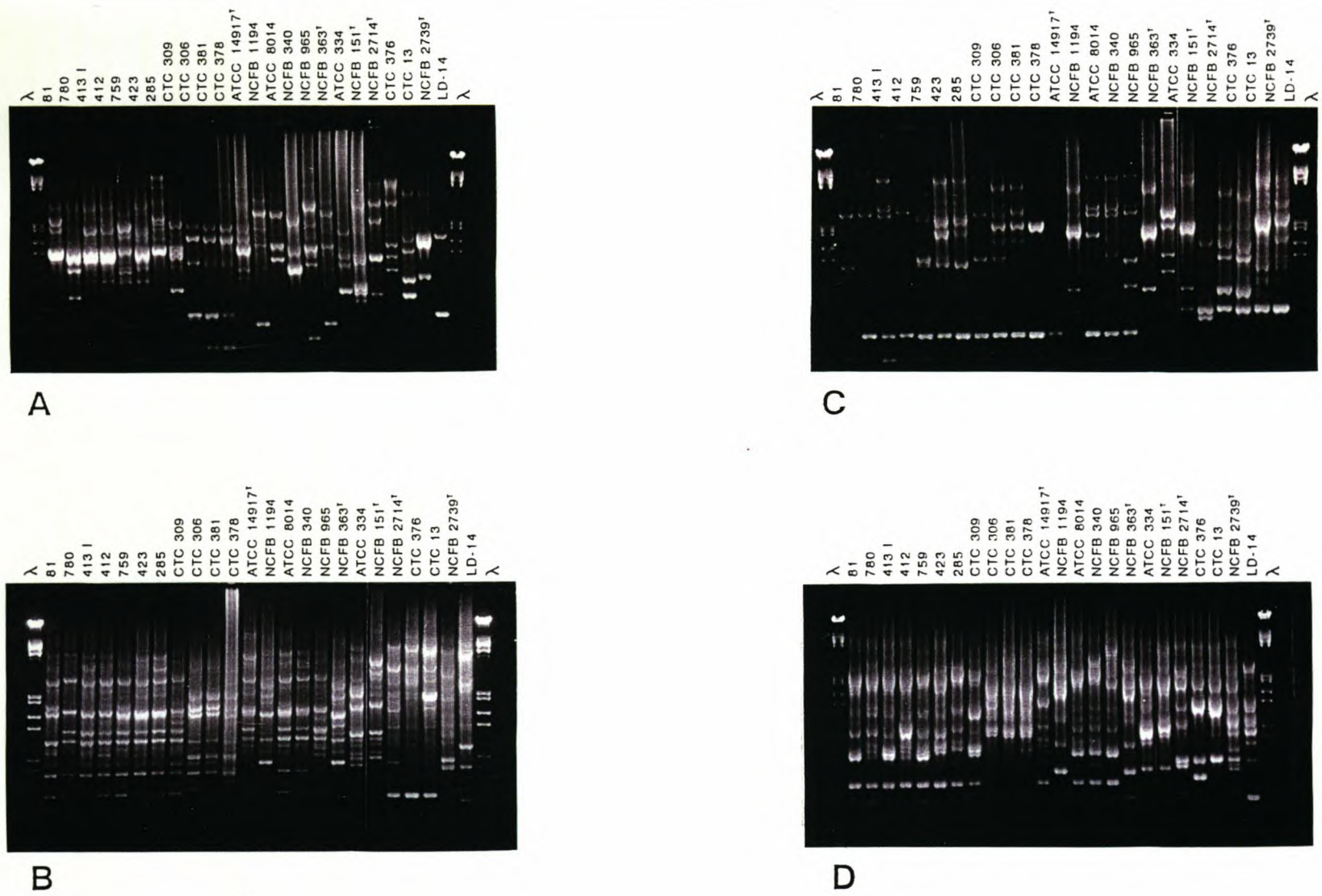

Fig. 2. DNA fragments obtained after RAPD-PCR of the genomic DNA of *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus paracasei*, *Lactobacillus casei*, *Lactobacillus sake* and *Lactobacillus curvatus*. Four different primers were used. A: Primer OPL-1 (GGCATGACCT), B: Primer OPL-2 (TGGGCGTCAA), C: Primer OPL-4 (GACTGCACAC), and D: Primer OPL-5 (ACGCAGGCAC).

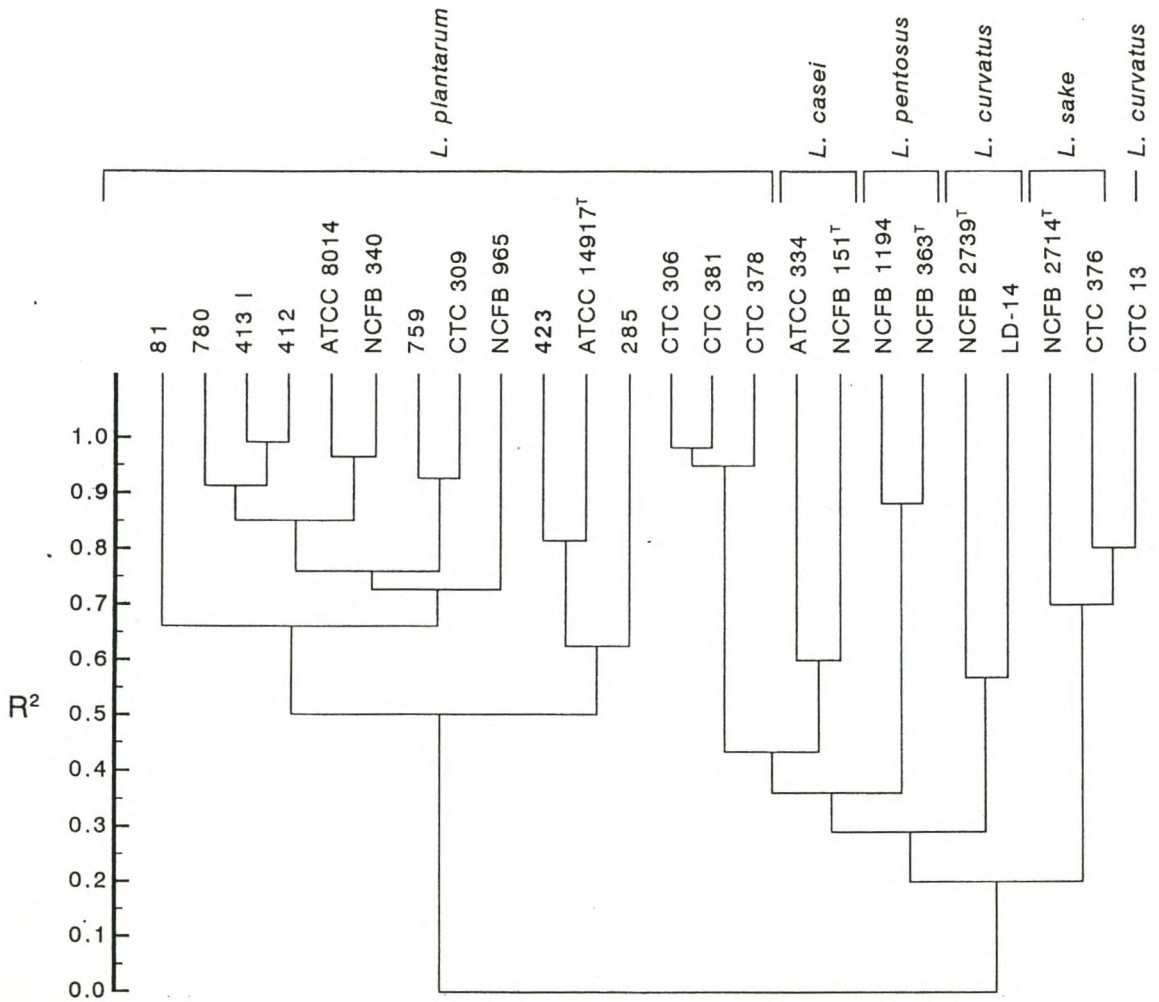

Fig. 3. Dendrogram showing the clustering of *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus pentosus*, *Lactobacillus curvatus* and *Lactobacillus sake* obtained by numerical analysis of RAPD-PCR profiles (Fig. 2). Clustering was by the normalized average linkage analysis. Distances between clusters are expressed in R^2-values.

CHAPTER 5

ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF PLANTARICIN 423, A BACTERIOCIN PRODUCED BY *LACTOBACILLUS PLANTARUM*

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Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*

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C.A. VAN REENEN, L.M.T. DICKS AND M.L. CHIKINDAS. 1998. *Lactobacillus plantarum* 423, isolated from sorghum beer, produces a bacteriocin (plantaricin 423) which is inhibitory to several food spoilage bacteria and food-borne pathogens, including *Bacillus cereus, Clostridium sporogenes, Enterococcus faecalis, Listeria* spp. and *Staphylococcus* spp. Plantaricin 423 is resistant to treatment at 80°C, but loses 50% of its activity after 60 min at 100°C and 75% of its activity after autoclaving (121°C, 15 min). Plantaricin 423 remains active after incubation at pH 1-10 and is inactivated when treated with pepsin, papain, α-chymotrypsin, trypsin and Proteinase K. Plantaricin 423 was partially purified and its size estimated at 3·5 kDa, as determined by tricine-SDS-PAGE. The mechanism of activity of plantaricin 423 is weakly bactericidal, as determined against *Oenococcus oeni* (previously *Leuconostoc oenos*). High DNA homology was obtained between the plasmid DNA of strain 423 and the pediocin PA-1 operon of *Pediococcus acidilactici* PAC1·0, suggesting that plantaricin 423 is plasmid-encoded and related to the pediocin gene cluster.

INTRODUCTION

Bacteriocins produced by lactic acid bacteria have received considerable attention during recent years for their possible use as preservatives in food, with a resultant reduction in the use of chemical preservatives. *Lactobacillus plantarum* is one of the most important lactic acid bacteria used for the production of fermented meat, grass and vegetable products (Ruiz-Barba *et al.* 1991; Kato *et al.* 1994). Various bacteriocins produced by *Lact. plantarum* have been described, i.e. plantaricin A (Daeschel *et al.* 1990; Nissen-Meyer *et al.* 1993; Diep *et al.*

1994), plantacin B (West and Warner 1988), plantaricin C (González *et al.* 1994), plantaricin C19 (Atrih *et al.* 1993), plantaricin F (Fricourt *et al.* 1994), plantaricin S (Jiménez-Díaz *et al.* 1993, 1995), plantaricin T (Jiménez-Díaz 1993), plantaricin LC74 (Rekhif *et al.* 1994), plantaricin SA6 (Rekhif *et al.* 1995), plantaricin 149 (Kato *et al.* 1994), plantacin 154 (Kanatani and Oshimura 1994), plantaricin 406 (Larsen *et al.* 1993), plantaricin UG1 (Enan *et al.* 1996), plantaricin KW30 (Kelly *et al.* 1996), and plantaricins produced by *Lact. plantarum* strains BN, LB75, LB592, CTC 305 and CTC 306 (Schillinger and Lücke 1989; Lewus *et al.* 1991; Okerere and Montville 1991a, 1991b; Garriga *et al.* 1993).

Four distinct classes of bacteriocins have been identified on the basis of biochemical and genetic characterization: (I) lantibiotics, (II) small heat-stable, non-lanthionine peptides, (IIa) *Listeria*-active peptides, (IIb) poration complexes consisting of two peptides for activity, (IIc) thiol-activated peptides, (III) large heat-labile proteins, and (IV) complex bacteriocins (Klaenhammer 1993). A number of bacteriocins produced by *Lactobacillus* spp. are inhibitory to *Listeria* spp., i.e. acidocin A (Kanatani *et al.* 1995), curvacin A (Tichaczek *et al.* 1992), sakacin A (Schillinger *et al.* 1991; Holck *et al.* 1992), sakacin 674 (Holck *et al.* 1994) and enterocin A (Aymerich *et al.* 1996). These bacteriocins share conserved regions in their amino acid sequence with pediocin PA-1 and bacteriocins produced by other genera of lactic acid bacteria, referred to as pediocin-like bacteriocins (Aymerich *et al.* 1996). Various plantaricins have been reported to inhibit *Listeria* spp. (Atrih *et al.* 1993; Garriga *et al.* 1993), but their relatedness to the class IIa bacteriocins has, to the authors' knowledge, not been established. Plantaricins A, S, C and 149 have been completely characterized (Jiménez-Díaz *et al.* 1995), but none of these are classified as *Listeria*-active peptides and they do not show DNA homology with any of the pediocins described.

Several bacteriocin-producing strains have been isolated from traditional South African fermented beverages. The antimicrobial peptide produced by one of these strains, later identified as *Lact. plantarum* (Van Reenen and Dicks 1996), inhibits the growth of a number of food spoilage bacteria, including the pathogen *Listeria monocytogenes*. This paper reports on the spectrum of antimicrobial activity, production, characteristics, isolation and mode of activity of plantaricin 423. The genetic relatedness of this bacteriocin to pediocin PA-1 is also discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus plantarum 423 was isolated from sorghum beer. The indicator strains used in

this study are listed in Table 1. The strains were from ATCC (American Type Culture Collection, Rockville, MD, USA), LMG (Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium), NCFB (National Collection of Food Bacteria, Reading, UK), NCTC (National Collection of Type Cultures, Colindale, UK) and our own culture collection (Department of Microbiology, University of Stellenbosch, Stellenbosch, South Africa). All lactic acid bacteria were grown in MRS broth (Biolab Diagnostics, Midrand, South Africa), except *Oenococcus oeni* (previously *Leuconostoc oenos*, Dicks *et al.* 1995) and the malolactic bacteria starter cultures of *O. oeni* (*Leuc. oenos*, Lallemand SA, St Simon, France and Viniflora oenos, Christian Hansen's Laboratory, Hørsholm, Denmark), which were grown in acidic grape medium (Dicks *et al.* 1990). All other strains were cultured in BHI broth (Biolab), except *Clostridium* spp. which were grown in DRCM medium (Merck, Darmstadt, Germany) and *Propionibacterium* spp., which were cultured in GYP medium (glucose 5g l^{-1}, yeast extract 3g l^{-1}, peptone 10g l^{-1}, meat extract 10g l^{-1}, NaCl 5 g l^{-1}).

Inhibitory activity

Lactobacillus plantarum 423 was inoculated into MRS broth (Biolab) and incubated at 30°C without aeration until mid-logarithmic phase of growth ($O.D._{.600}$=1·4). The antimicrobial activity of plantaricin 423 was tested against the organisms listed in Table 1. An aliquot of 10 µl cell-free culture supernatant fluid was spotted onto an agar plate (0·7 % w/v agar) seeded with active growing cells of the test organism (approximately 10^6 cells ml^{-1}). Plates were incubated at the optimal growth temperature of the test organism, as indicated in the respective culture collection catalogues. A clear zone of inhibition of at least 2 mm in diameter was recorded as positive.

Isolation and purification of plantaricin 423

Lactobacillus plantarum 423 was grown in MRS broth (Biolab) at 30°C until mid-logarithmic phase ($O.D._{.600}$=1·4). The cells were harvested and the bacteriocin isolated from the cell-free supernatant as described by Green *et al.* (1997). After evaporation in the Rotavapor (Büchi, Labortechnik, Flawil, Switzerland), the pH of the sample was adjusted to 7·0 with 0·5 N NaOH and desalted by filtration through a 3 kDa cut-off dialysis membrane. The desalted sample was lyophilized, resuspended in sterile distilled water, and stored at -20°C.

Further purification of plantaricin 423 (sample of 300 µl (approximately 650 µg)) was performed by reverse phase FPLC (Pharmacia LKB LCC-501 Plus), using a PepRPC HR 5/5 column (Pharmacia). The method described by Green *et al.* (1997) was used. Fractions of

0·70 ml were collected, lyophilised, resuspended in sterile distilled water and stored at -20°C.

Size determination

The molecular size of plantaricin 423 was determined by separation of partially purified, concentrated (lyophilised) cell-free supernatant fluid and the protein fraction obtained after reverse phase FPLC by using tricine-SDS-PAGE (Schägger and von Jagow 1987). A low molecular weight protein marker with sizes ranging from 2·35-46 kDa (Amersham International, UK) was used. The gels were fixed and one half stained (Schägger and von Jagow, 1987), except that Coomassie Blue R250 (Saarchem, Krugersdorp, South Africa) was used instead of Serva Blue G. The position of the active plantaricin 423 peptide was determined by overlaying the other half of the gel (not stained and extensively pre-washed with sterile distilled water) with cells of *Listeria innocua* LMG 13568 (approximately 10^6 ml^{-1}), embedded in Brain Heart Infusion (BHI) agar (0·7 % agar w/v).

Sensitivity to heat, pH and proteolytic enzymes

Crude extract samples of plantaricin 423 (cell-free supernatant fluid of the producer strain, 10 x concentrated by lyophilization) were used in these tests. *Listeria monocytogenes* LM1 was used as indicator organism. Aliquots of plantaricin 423 were exposed to heat treatments of 40, 60, 80 and 100°C for 10, 30 and 60 min, respectively, and 121°C for 15 min. The samples were then tested for antimicrobial activity, as described before. In a separate experiment samples of plantaricin 423 were adjusted to pH values ranging from 1 to 10, incubated at 37°C for 30 min, neutralised to pH 7, and tested for bactericidal activity. Resistance of plantaricin 423 to proteolytic enzymes was determined by incubation of the bacteriocin samples in the presence of Proteinase K (10 U mg^{-1} plantaricin 423), pepsin (1250 U mg^{-1} plantaricin 423), papain (15 U mg^{-1} plantaricin 423), α-chymotrypsin (45 U mg^{-1} plantaricin 423), and trypsin (55 U mg^{-1} plantaricin 423) at 37°C for 1 h. After incubation the enzymes were heat-inactivated (3 min at 100°C) and tested for antimicrobial activity.

Mode of action

Partially purified plantaricin 423 was added (0·25 µg ml^{-1}) to mid-logarithmic growth phase cells of *O.oeni* 19CI in 200 ml acidic grape medium. Concentrated MRS medium was added to a control flask. Changes in the turbidity of the cultures were recorded at an O.D. of 600 nm and the number of colony-forming units (cfu) was determined by plating the samples on acidic grape agar (Dicks *et al.* 1990).

Isolation of DNA

Total DNA was isolated according to the method of Dellaglio *et al.* (1973). Plasmid DNA was isolated using the method described by Burger and Dicks (1994), after which the DNA was further purified by CsCl density gradient centrifugation (Sambrook *et al.* 1989). The DNA was separated on an agarose gel, according to the method described by Sambrook *et al.* (1989). Lambda DNA digested with *Eco*RI and *Hind*III (Promega, Madison, USA) was used as molecular weight marker.

Southern blot hybridizations

Southern blot hybridizations were performed as described by Sambrook *et al.* (1989). Total and plasmid DNA were hybridised with probes made from the 5·6 kb *Eco*RI/*Sal*I fragment of the pSRQ220 plasmid encoding pediocin PA-1 (Marugg *et al.* 1992), the 5 kb *Hind*III fragment of plasmid pGH3 encoding plantaricin A (Diep *et al.* 1994), and the 0·5 kb *Eco*RI/*Xba*I fragment of plasmid pCE36 containing the *nisA* gene (A.M. Ledeboer, personal communication).

Isolation of bacteriocin-deficient mutants

Curing experiments were conducted as described by Ruiz-Barba *et al.* (1991). *Lactobacillus plantarum* 423 was incubated with various concentrations of novobiocin (0·125 - 8 µg ml^{-1}) and incubated for 72 h. Those cultures which displayed growth at the highest concentration of novobiocin were serially diluted with sterile saline and plated out on MRS agar plates. After 48h of incubation at 30°C, the colonies were replica plated and the original plates overlaid with *Lactobacillus sake* DSM 20017. After 24 h of incubation at 30°C, the colonies were checked for loss of plantaricin 423 production.

RESULTS

Plantaricin 423 is active against *Bacillus cereus*, *Clostridium sporogenes, Enterococcus faecalis*, several *Lactobacillus* spp., (including *Lact. plantarum*), *O. oeni, L. innocua, L. monocytogenes, Ped. acidilactici, Ped. pentosaceus, Propionibacterium acidipropionici, Propionibacterium* sp., *Staphylococcus carnosum*, *Streptococcus thermophilus,* and the malolactic starter cultures of *O. oeni* (*Leuc. oenos*, Lallemand SA and Viniflora oenos, Christian Hansen's Laboratory), as indicated in Table 1.

Isolation of plantaricin 423 with Amberlite XAD-1180 and separation on a tricine-SDS-PAGE gel yielded an active peptide band corresponding in size to 3·5 kDa (Fig. 1b,c,d). Further separation of the Amberlite fraction by reverse phase FPLC yielded one active peak (Fig. 2). Separation of this fraction by tricine-SDS-PAGE yielded a peptide band of 3·5 kDa (Fig. 1c), corresponding to the peptide band obtained after the Amberlite XAD-1180 separation (Fig. 1b).

Plantaricin 423 is resistant to heat treatments of 40, 60, 80 and 100°C for 10 and 30 min, respectively. However, 50% activity was lost after 60 min at 100°C, and 75% activity was lost after the bacteriocin was autoclaved (121°C for 15 min). Incubation in buffers at pH values ranging from 1 to 10 had no effect on the activity of plantaricin 423. Plantaricin 423 is sensitive to pepsin, papain, α-chymotrypsin and trypsin and Proteinase K. No rest-activity of plantaricin 423 was obtained after treatment with these proteolytic enzymes.

The addition of plantaricin 423 to active growing cells of *O. oeni* 19Cl (at 36 h of growth) resulted in a slow decrease in the number of viable cells (2·5 x 10^6 to 1 x 10^6 cfu ml^{-1}) over a period of 24 h, after which the cell count stabilized for the remainder of incubation (Fig. 3). The optical density readings of *O. oeni* 19Cl increased for 6 h after the addition of plantaricin 423, after which it stabilized for the remainder of the incubation period (Fig. 3).

Southern blot hybridizations revealed DNA homology between the plasmid DNA of *Lact. plantarum* 423 and probes made from the 5·6 kb *Eco*RI/*Sal*I fragment of the pSRQ220 plasmid encoding pediocin PA-1. No DNA homology was obtained with probes from the 5 kb *Hind*III fragment of plasmid pGH3 encoding plantaricin A, or the 0·5 kb *Eco*RI/*Xba*I fragment of plasmid pCE36 containing the *nisA* gene.

Curing with novobiocin yielded two mutants of *Lact. plantarum* 423, designated 423/1 and 423/4, which both lost a plasmid of approximately 6 kbp (Fig. 4) and the ability to produce plantaricin 423.

DISCUSSION

The spectrum of antimicrobial activity of plantaricin 423 (Table 1) and its heat stability (up to 30 min at 100°C) is similar to that reported for other plantaricins, e.g. plantaricin A (Daeschel *et al.* 1990), plantaricin C19 (Atrih *et al.* 1993), plantaricin S (Jiménez-Díaz *et al.* 1993), plantaricin 149 (Kato *et al.* 1994) and plantaricin SA6 (Rekhif *et al.* 1995). However, the strong bactericidal activity obtained against *L. innocua* and *L. monocytogenes* distinguishes plantaricin 423 from the other bacteriocins produced by *Lact. plantarum.* According to the

classification system proposed by Klaenhammer (1993), plantaricin 423 belongs to the group IIa (anti-*Listeria*) bacteriocins.

Separation of plantaricin 423 by Amberlite XAD-1180 suggests that the bacteriocin molecule is hydrophobic and, in this sense, similar to many other bacteriocins. Plantaricin 423 is in the region of 3·5 kDa in size, as determined by tricine SDS-PAGE. This also conforms to the classification of Klaenhammer (1993). Plantaricins that have been completely characterized are all smaller than 10 kDa (Nissen-Meyer *et al.* 1993; González *et al.* 1994; Kato *et al.* 1994; Jiménez-Díaz *et al.* 1995).

The slight decline in number of living cells of *O. oeni* 19Cl recorded over a period of 24 h after the addition of plantaricin 423 ($2{\cdot}5 \times 10^6$ to 1×10^6 cfu ml^{-1}) suggests that the mode of activity of the peptide is only weakly bactericidal (Fig. 3). This was supported by the slow increase in absorbency (O.D.) readings recorded for the first 6 h since the addition of plantaricin 423, before the readings stabilized. The stable O.D. readings recorded for the remaining period of incubation (Fig. 3) suggests that the cells of *O. oeni* 19Cl were not lysed.

DNA hybridization studies have shown homology between the plasmid DNA of *Lact. plantarum* 423 and the pediocin PA-1 operon. This suggests that plantaricin 423 belongs to the anti-*Listeria* group of pediocin-like bacteriocins. Various anti-*Listeria* bacteriocins have been described for *Lactobacillus* spp., such as acidocin A (Kanatani *et al.* 1995), curvacin A (Tichaczek *et al.* 1992), sakacin A (Schillinger *et al.* 1991; Holck *et al.* 1992), and sakacin 674 (Holck *et al.* 1994). Comparison of the amino acid sequences of these pediocin-like bacteriocins with similar bacteriocins produced by other lactic acid bacteria, such as pediocin PA-1, carnobacteriocin BM1, and leucocin A-UAL187 (Kanatani *et al.* 1995) revealed conserved regions which have been suggested to be important for the activity of the anti-*Listeria* class of bacteriocins (Lozano *et al.* 1992).

Based on the results obtained with the curing experiments, plantaricin 423 is encoded by a plasmid of approx. 6 kbp in size (Fig. 4). Pediocin-like bacteriocins may be either plasmid encoded (Gonzalez and Kunka 1987; Garriga *et al.* 1993; Quadri *et al.* 1994; Kanatani *et al.* 1995), or genomically encoded (Holck *et al.* 1994; Aymerich *et al.* 1996). It might even be that only certain of the genes encoding plantaricin 423 are located on the 6 kbp plasmid. The structural gene of plantaricin A was shown to be located on the chromosome (Diep *et al.* 1994). All the genes encoding the anti-*Listeria* bacteriocin sakacin P, on the other hand, are located on a 7.6 kbp chromosomal fragment (Hühne *et al.* 1996). The genes encoding the secretion and maturation of lactococcin A are located on the chromosome of *Lactocossus lactis* IL 1403 (Venema *et al.* 1996). The structural gene of carnobacteriocin BM1 is located on the chromosome. However, expression of this chromosomal bacteriocin

and its immunity function required the presence of a 61 kbp plasmid encoding the carnobacteriocin B2 structural gene (Quadri *et al.* 1994). The 6 kbp plasmid of *Lact. plantarum* 423 is currently being sequenced to determine the exact location of the structural gene of plantaricin 423.

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Table 1 Spectrum of antimicrobial activity of plantaricin 423

Organism	Strain	Result
Acetobacter aceti	ATCC 23746	-
Bacillus cereus	LMG 13569	+
Clostridium sporogenes	LMG 13570	+
Enterococcus faecalis	LMG 13566	+
Lactobacillus brevis	ATCC 11577	+
	ATCC 14869	+
Lactobacillus buchnerii	ATCC 4005T	+
Lactobacillus bulgaricus	LMG 13551	-
	ATCC 11482T	+
Lactobacillus casei	ATCC 334	-
	ATCC 393T	-
	LMG 13552	-
	T395	-
	LHS	-
Lactobacillus curvatus	LMG 13553	+
Lactobacillus fermentum	ATCC 23271	-
	LMG 13554	+
	ATCC 9338	+
Lactobacillus fructivorans	NCFB 2167	-
Lactobacillus helveticus	LMG 13555	+
Lactobacillus plantarum	ATCC 14917	+
	LMG 13556	+
	NCFB 1193	-
Lactobacillus reuteri	LMG 13557	-
	DSM 20016	+
Lactobacillus sake	LMG 13558	+
	DSM 20017	+
Leuconostoc mesenteroides subsp. *cremoris*	LMG 13562	-
	LMG 13563	-
Listeria innocua	LMG 13568	+
Listeria monocytogenes	LM1	+
Oenococcus oeni	ML 34	+
	19CI	+
Oenococcus oeni (= *Leuconostoc oenos*, Lallemand SA)	NLO-09	+
Oenococcus oeni (= Viniflora oenos, Christian Hansen's Laboratory)	DSM 7008	+
Pediococcus acidilactici	ATCC 12697	+
Pediococcus pentosaceus	LMG 13560	+
	LMG 13561	-
Propionibacterium acidipropionici	LMG 13572	+
Propionibacterium sp.	LMG 13573	+
Salmonella enteritidis	SE1	-
Staphylococcus carnosum	LMG 13567	+
Streptococcus mutans	NCTC 10449	-
Streptococcus thermophilus	LMG 13564	-
	LMG 13565	+

+, Sensitive to plantaricin 423.

-, Resistant to plantaricin 423.

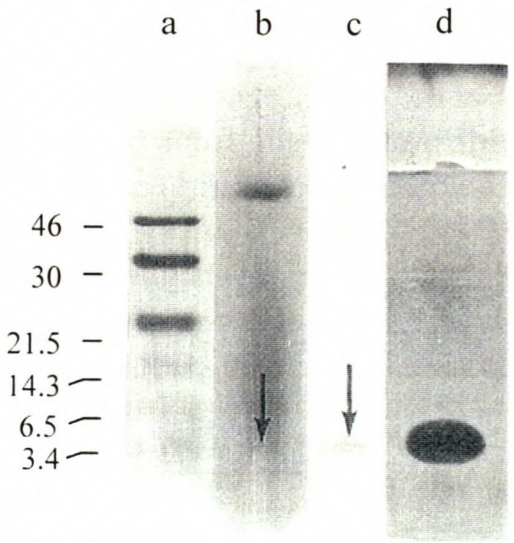

Fig. 1 Separation of plantaricin 423 by tricine-SDS-PAGE. (a) Rainbow protein molecular weight marker; (b) partially purified crude extract and (c) fraction after reverse phase FPLC, both gels stained with Coomassie brilliant blue R250; (d) gel overlaid with cells of *Listeria innocua* embedded in BHI agar (0·7% (w/v) agar). The active protein bands are indicated by the arrows

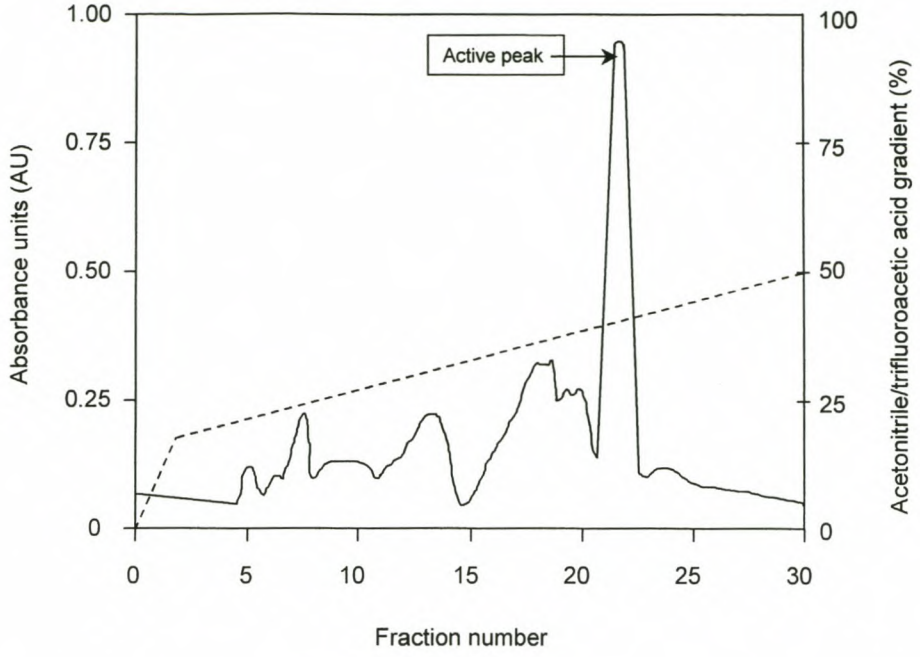

Fig. 2 Separation of plantaricin 423 by reverse phase FPLC. A gradient of 20-50% B (0·1% trifluoroacetic acid in acetonitrile) was used

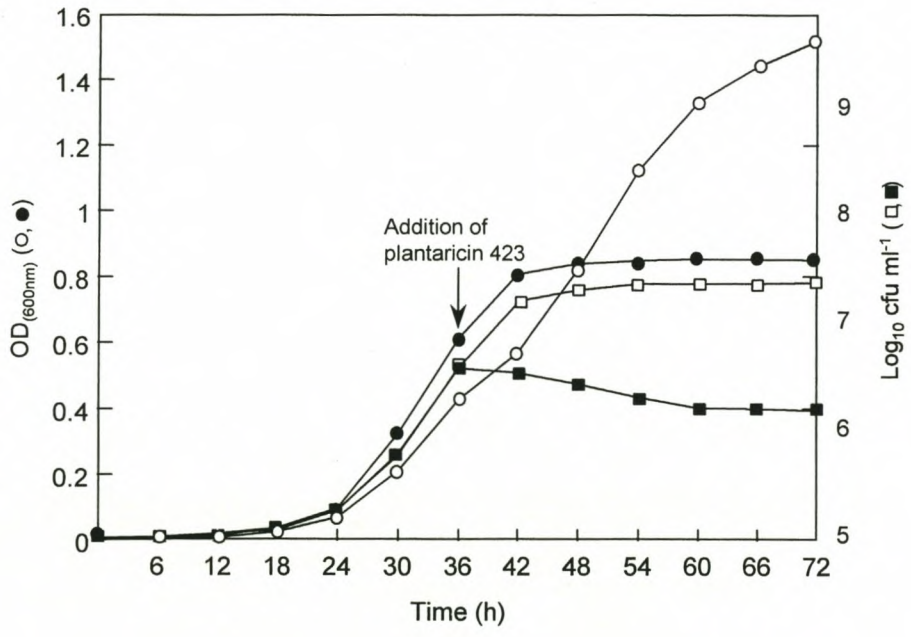

Fig. 3 The effect of plantaricin 423 on the growth of *Oenococcus oeni* 19Cl. (-○- and -●- = turbidity of the cells [measured at O.D. of 600 nm] growing in the absence and presence of plantaricin 423, respectively. -□- and -■- = cfu ml^{-1} counts in the absence and presence of plantaricin 423, respectively)

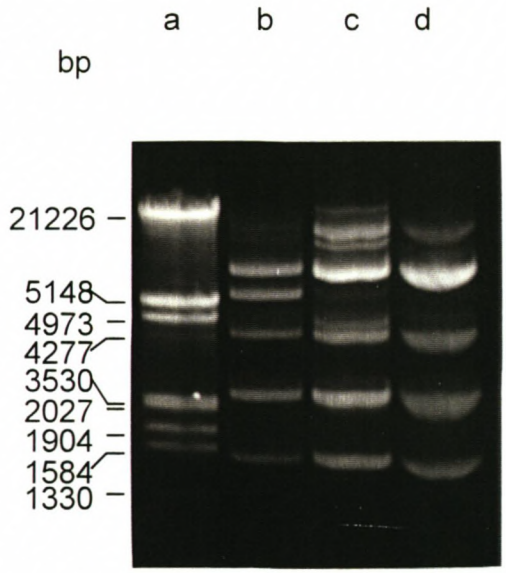

Fig. 4 Plasmid profiles of *Lactobacillus plantarum* 423. (a) Lambda DNA digested with *Eco*RI and *Hind*III (Promega, Madison, USA); (b) plasmid profile before curing; (c, d) plasmid profiles after curing with novobiocin (mutants 423/1 and 423/2, respectively)

CHAPTER 6

GENETIC CHARACTERIZATION OF BACTERIOCIN 423 AND EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

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Genetic characterization of bacteriocin 423 and expression in *Saccharomyces cerevisiae*

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ABSTRACT

Bacteriocin 423, a small heat stable bacteriocin, is produced by *Lactobacillus pentosus* 423. Nucleotide sequencing of the DNA of plasmid pBAC4 encoding bacteriocin production by *L. pentosus* 423, revealed an operon-like structure similar to that described for pediocin PA-1. Four open reading frames (*bacA*, *bacB*, *bacC* and *bacD*) were identified. *BacA* encodes a 56-amino acid prepeptide consisting of a 37-amino acid mature peptide with a 19 N-terminal leader peptide. *BacB* encodes a putative immunity protein with protein sequence similarities to several bacteriocin immunity proteins described in the databases. The *bacC* and *bacD* genes are virtually identical to *pedC* and *pedD* of pediocin PA-1. Although a part of the *pedB* gene of pediocin PA-1 also occurs in the bacteriocin 423 operon, this stretch of DNA does not appear to be part of any ORF. Bacteriocin 423 (KYYGNGVTCGKHSCSVNWGQAFSCSVSHLANFGHGKC as predicted by nucleotide and protein sequencing) was successfully expressed by *Saccharomyces cerevisiae*. Although inhibition zones were detected on agar plates, activity levels were very low, and no inhibitory activity could be detected in the unconcentrated supernatant of the transformed yeast.

INTRODUCTION

Bacteriocins of food related lactic acid bacteria have received much attention during the last decade for their possible use as natural preservatives in food and beverages. In food products, bacteriocins may replace or reduce the use of preservatives such as nitrates and nitrites. In wine, the use of bacteriocins as preservatives may reduce the use of sulfur dioxide (39). Bacteriocins that inhibit *Oenococcus oeni* may possibly also play a role in the control of malolactic fermentations in wine. Bacteriocins are ribosomally synthesized peptides that usually exhibit a narrow range of antimicrobial activity, affecting only organisms closely related to the producer. Bacteriocins are classified into four groups (28). Class I are lantibiotics, Class II are small heat-stable membrane-active peptides, Class III are large, heat-labile proteins and Class IV are complex proteins. Most of the bacteriocins characterized to date belong to Class II. Class II is subdivided into four different groups.

Class IIa bacteriocins share a YGNGV consensus sequence near their N-termini and several of these peptides have been fully characterized, such as pediocin PA-1 (30), mesentericin Y105 (16), leucocin A (42), divercin V41 (31), sakacin P (24), acidocin A (27), sakacin A (3) and enterocin A (4, 35). Class IIa bacteriocins are also referred to as anti-*Listeria* peptides, or pediocin-like peptides, after pediocin PA-1, the first characterized bacteriocin representative of this group. Class IIb are two-peptide bacteriocins, Class IIc are *sec*-dependent bacteriocins, and Class IId are Class II bacteriocins that do not belong to any of the three defined groups (34).

Bacteriocin 423, also referred to as plantaricin 423, is produced by *Lactobacillus plantarum* 423 (43). Strain 423 is, however, phylogenetically (based on 16S rRNA sequence analysis) more related to *L. pentosus* (Collins, M.D., Department of Food Science and Technology, University of Reading, Reading RG66AP, UK, personal communication). Bacteriocin 423 is a small (approximately 3.5 kDa) plasmid-encoded peptide that remains active after several heat treatments, loses activity when treated with proteolytic enzymes, and is stable over a pH range of 1 to 10. Several Gram-positive bacteria are inhibited by bacteriocin 423, including *Lactobacillus* spp., *Leuconostoc* spp., *O. oeni*, *Pediococcus* spp., *Enterococcus* spp. and *Propionibacterium* spp., and food-spoilage or pathogenic bacteria such as *Staphylococcus*, *Bacillus*, *Clostridium* and *Listeria* spp. (43). High DNA homology between plasmid pBAC4 (initially referred to as p423(4)) of the bacteriocin 423 producer organism and the operon of pediocin PA-1 has been reported (43).

In this article the primary structure and genetic organization of bacteriocin 423 is examined. Bacteriocin 423 was also successfully expressed in *S. cerevisiae*.

MATERIALS AND METHODS

Microbial strains and media: The organisms included in this study are listed in Table 1. The bacteriocin producer, *L. pentosus* 423, and the indicator organism, *Lactobacillus sakei* LMG 13558, were cultured in de Man-Rogosa-Sharpe (MRS) medium (Biolab Diagnostics, Midrand South Africa) at 30°C. *Listeria monocytogenes* was propagated in Brain Heart Infusion (BHI) medium (Biolab Diagnostics, Midrand South Africa) at 37°C. *Escherichia coli* DH5α was propagated in Luria-Bertani (LB) medium (tryptone 1% m/v, yeast extract 0.5% m/v, NaCl 1% m/v) at 37°C. *E. coli* transformants were grown in LB broth containing ampicillin (100 µg/ml). *S. cerevisiae* $\sum$1278 was propagated in YPD (yeast extract 1% m/v, peptone 2% m/v, glucose 2% m/v) medium at 30°C. Yeast transformants were grown in synthetic complete minimal medium (SC) containing 0.67% yeast nitrogen base without amino acids, 0.5% $(NH_4)_2SO_4$, 2% glucose and all the required

amino acids except uracil (2).

Inhibitory activity: The inhibitory activity of bacteriocin 423 was monitored by the spot-on-lawn method as described previously (43), using *L. monocytogenes* LM1 or *L. sakei* LMG 13558 as indicator strains. One arbitrary unit of bacteriocin 423 was defined as 10 µl of the highest dilution of protein sample yielding a zone of growth inhibition. Protein concentrations were determined spectrophotometrically (2).

Bacteriocin production and protein purification: The supernatant of an overnight *L. pentosus* 423 culture was clarified by centrifugation and the proteins precipitated with ammonium sulfate (75% w/v, final concentration). After centrifugation the precipitated proteins were resuspended in MilliQ water. The pH of the sample was adjusted to neutral with 1M NaOH before overnight dialysis using Spectrapor 1000 Da molecular weight cut off tubing (Spectrum Laboratories Inc., Gardena, California, USA). A 1:2 methanol:chloroform solution was added to the dializate (1:25 dilution) and stirred at 4°C for 1 hour. After centrifugation, the pellet was resuspended in 2 ml deionized water. To this sample, deionized water and 100 mM ammonium acetate was added (1:4:5) and then subjected to cation exchange chromatography (Spectragel fastflow SP Sepharose, Spectrum Laboratories Inc., Gardena, California, USA). The protein was eluted using a 0.1 to 0.8 M ammonium acetate step gradient. Fractions collected from the column at each of the ammonium acetate concentrates were pooled and tested for activity. The active pool was freeze-dried and stored at –20°C.

Electrospray mass spectrometry and amino acid sequencing: The active lyophilized sample was dissolved in 1:9 acetonitrile-water containing 0.01% formic acid and the mass of the purified peptide determined using a Quattro triple quadropole mass spectrometer (Micromass, Manchester, United Kingdom) as described (5). The peptide was sequenced using an automated Edman degradation protein sequencing apparatus (Applied Biosystems Procise 491, PE Biosystems SA [Pty] Ltd.).

DNA preparation and analysis: Plasmid DNA was isolated from *L. pentosus* 423 as described previously (43). The 9 kb plasmid, designated pBAC4, (previously incorrectly described as 6 kb), already established as carrying the bacteriocin genetic information (43), was separated from the four other plasmids by agarose gel electophoresis and purified using a Biotrap BT 1000 (Schleicher and Schuell) apparatus. Standard techniques were used for DNA restriction enzyme analysis and ligations (2). Partial *Sma*I DNA fragments and a 2000 bp *Hin*dIII fragment of pBAC4 were cloned and sequenced. The enzymes used were from Roche Molecular Chemicals, Mannheim, Germany. DNA was sequenced on an automatic sequencer (ABI Prism™ 377, PE Biosystems SA [Pty] Ltd.), using dye terminator chemistry (Biosystems, Warrington, England). To sequence the operon of bacteriocin 432, primers were designed to sequence upstream of the *Hin*dIII and *Sma*I

fragments homologous to sections of the *pedD* gene of pediocin PA-1, and to fill in gaps. The primers for sequencing were obtained from Genosys Biotechnologies (Europe) Ltd. (Pampisford, United Kingdom).

Sequence analysis: Computer alignment and comparison of the pBAC4 DNA sequence to that of pediocin PA-1 and other homologous sequences were facilitated using the Sequence Navigator (PE Biosystems SA [Pty] Ltd.), DNAMAN *for Windows*® (Lynnon Biosoft, Quebec, Canada) and BLAST (Basic Local Alignment Search Tool) programs.

Cloning of the structural gene and plasmid construction for expression in *S. cerevisiae*: The techniques used were similar to that described previously (39). The putative structural gene of the mature bacteriocin 423 protein was amplified by PCR. The two primers used for amplification were:
423A5' GTCGCCCGGGAAATACTATGGTAATGGGG and
423A3' GCGTCCCGGGTTAATTAGCACTTTCCATG. Both contained a *Sma*I site (underlined). Plasmid pBAC4 from *L. pentosus* 423 was used as template. The amplified product was cut with *Sma*I and cloned into the *Sma*I site of the pBluescript SK vector. Using *Sma*I, the PCR generated fragment was cloned into the filled in *Hin*dIII site of pPRL2 (Fig. 1). Plasmid PRL2 is the 6.11 kb single-copy yeast- *E. coli* shuttle vector YCplac111 (amp^r, leu2) (18), with the yeast alcohol dehydrogenase promoter and terminator, and mating pheromone α-factor secretion signal insert, as constructed previously (39). The *Sal*I-*Sma*I DNA fragment from pPRL2 containing $ADHI_P$- $MF\alpha1_S$-*bacA*-$ADHI_T$ was cloned into the multiple cloning site of the multicopy yeast-*E. coli* shuttle vector YEp352. This constructed plasmid (YEpBac423) was used to transform *S. cerevisiae* $\sum$1278.

Standard DNA manipulation, cloning of the DNA fragments, and *E. coli* and yeast transformation techniques were used (2).

RNA isolation and Northern blot analysis: Total yeast RNA was isolated and analyzed using standard techniques as described (2) and a Random Primer DNA Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany). The 114 bp *bac*A PCR fragment encoding bacteriocin 423 was used as probe.

Yeast bacteriocin activity assays: Yeast transformants were grown in SC^{-ura} overnight at 30°C. Twenty µl of cells, concentrated from a 10 ml overnight culture, was spotted onto SC^{-ura} plates, incubated for 3 days at 30°C, overlaid with a 1% (v/v) overnight culture of *L. monocytogenes* LM1, suspended in 0.7% BHI agar. After incubation at 37°C for 24h, the plates were examined for inhibition zones. Yeast cells transformed with YEp352 without the bacteriocin 423 gene cassette were used as negative controls.

Nucleotide sequence accession number: The DNA sequence was submitted to GenBank (Los Alamos, USA). Accession number: AF304384.

RESULTS

Characterization of bacteriocin 423: The total protein concentration, activity, specific activity and percentage recovery are given in Table 2. Electrospray mass spectrometry analysis indicated the presence of a single peptide of 3930.1 Da in the active sample collected from the SP-Sepharose gel (Fig. 2). Peptide sequencing revealed the 37 AA peptide KYYGNGVTXGXHSXSVNXGQAFSXSXSHLANFGHGKX. Positions X indicate ambiguous residues that could not be resolved. Comparison of the protein sequence to known protein sequences in the databases revealed significant homology to the Class IIa bacteriocins (Table 3).

Genetic analysis of bacteriocin 423: Bacteriocin 423 production was previously shown to be associated with the presence of an (approximately) 9 kbp plasmid, designated pBAC4. Southern hybridization using the operon of pediocin PA-1 as probe had also revealed extensive homology between the DNA encoding pediocin PA-1 and pBAC4 (43). In an attempt to clone sections of pBAC4, the DNA was incubated with several restriction enzymes. *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Pst*I, *Sac*I and *Sma*I failed to linearize the plasmid. *Sal*I cut the plasmid DNA once, but attempts to clone the complete plasmid into either pBR322 or pBluescript SK failed. *Hin*dIII and *Xba*I cut pBAC4 several times. The one end of a 2000 bp *Hin*dIII fragment and several *Sma*I fragments derived from partial restriction enzyme digestions were homologous to sections of *pedD*, the fourth gene in the pediocin PA-1 operon (30). From this result, reverse primers were used to sequence the original pBAC4 DNA upstream to locate the structural gene of bacteriocin 423. Sequencing was continued until a whole operon-like structure was identified (Fig. 3). The nucleotide sequence revealed significant homology to the pediocin PA-1 operon (Fig. 4). The presence of at least four open reading frames (ORF's) was revealed when the DNA was translated.

The first open reading frame (*bacA*) encodes a protein consisting of 56 amino acid residues followed by a TAA stop codon. Comparison of this sequence with the mature bacteriocin 423 peptide sequence showed that *bacA* was the structural gene with a 37-residue C-terminal corresponding to the amino acid sequence of bacteriocin 423 and a 19 or 18 residue N-terminal extension with a glycine-glycine (GG) cleavage site (Fig. 3). This N-terminal extension is identical to the N-terminal extension of pediocin PA-1, except for an extra M residue at the start of the ORF. Although computer analysis of the ORFs indicate that *bacA* starts at the first M-residue, DNA sequence comparison of the promoter area and ribosome binding site of pediocin PA-1 with the bacteriocin 423 DNA sequence indicates that b*acA* may start at the second M residue.

A second open reading frame (*bacB*) follows directly downstream of *bacA* encoding a protein consisting of 72 amino acid residues followed by a TAA stop codon. Comparison of

the DNA sequence with those in the databases did not reveal any homology. However, comparison of the protein sequence predicted by this ORF to previously submitted protein sequences revealed significant homology to the putative immunity proteins of leucocin A, mesentericin Y105, divercin V41 and enterocin A, indicating that this ORF could be the immunity protein of bacteriocin 423.

The third open reading frame (*bacC*), located 355 bp downstream of *bacB* codes for a protein which starts with a TTG initiation codon and consists of 174 amino acid residues followed by a TAG stop codon. Further downstream, a fourth open reading frame was located, consisting of 724 amino acid residues with a TAA stop codon. These two ORF's are virtually identical to the *pedC* and *pedD* genes of pediocin PA-1 (30), respectively, and may thus be necessary for crossmembrane translocation of bacteriocin 423. The sequences of the putative –35 and –10 regions are TTGACA and TAGAAT, respectively.

Although the bacteriocin 423 operon contains a DNA sequence identical to that which encodes the C-terminal of *pedB*, the immunity protein of pediocin PA-1, this stretch of DNA does not appear to be part of any predicted ORF in the bacteriocin 423 operon.

Heterologous expression of bacteriocin 423: A PCR fragment of 114 bp cloned into pBluescript SK and sequenced revealed an identical DNA sequence to that encoding the predicted mature peptide. This fragment was reisolated using *Sma*I and cloned in-frame into pPRL2 containing the yeast *ADHI* transcriptional regulatory sequences and the *MFα1* secretion signal (pBac423). The constructed $ADHI_P$- $MF\alpha1_S$-*bacA*-$ADHI_T$ cassette was isolated from pBac423 and ligated into the multicopy YEp352 vector. The final constructed plasmid, YEpBac423 was introduced into *S. cerevisiae*. *Saccharomyces cerevisiae* transformants grown on agar plates were overlaid daily after 2, 3 and 4 days of growth with soft agar containing *L. monocytogenes*. Clear zones of inhibition were observed around colonies of the yeast YEpBac423 transformants after 3 days. No zones could be detected around the negative controls (Fig. 5). Inhibitory activity displayed by yeast transformants was very low, with no inhibitory activity detected in the unconcentrated supernatant of the yeast transformants.

Northern blot analysis: Northern blot analysis of yeast RNA isolated from transformants indicated a transcript of approximately 700 nucleotides (Fig. 6).

DISCUSSION

L. pentosus produces a small heat-stable bacteriocin, designated bacteriocin 423 (43). This anti-listerial bacteriocin has a relatively wide spectrum of inhibition. Initial studies indicated a plasmid-encoded peptide of approximately 3.5 kDa in size. Here, data is presented on the partial nucleotide sequence of pBAC4, the plasmid implicated in

bacteriocin 423 production, and conclusions made based on the protein sequence, heterologous expression of bacteriocin 423 and computer comparisons of various nucleotide and protein sequences in the databases.

Characterization of bacteriocin 423: Several Class IIa bacteriocins have been isolated (Table 3). These bacteriocins all share the YGNGV consensus sequence in the N-terminal of the mature peptide, although the exact function of this consensus motif is not clear (9). The first 16 amino acid residues in the N-terminal of mature bacteriocin 423 is identical to that of pediocin PA-1, whereas the C-terminal of bacteriocin 423 differs considerably from pediocin PA-1 and other Class IIa bacteriocins (Table 3). The amino acid sequence of bacteriocin 423 appears to be most homologous to plantaricin C19 (1).

Studies on the primary and three-dimensional structures of Class IIa bacteriocins suggest that these peptides consist of two functional domains: a well-conserved hydrophilic N-terminal β-sheet domain and a more diverse hydrophobic or amphiphilic C-terminal α-helical domain. It is hypothesised that the N-terminal domain may mediate the initial unspecific binding of the bacteriocins to target cells through electrostatic interaction, while the C-terminal domain interacts with the hydrophobic part of the target membrane (13). Bacteriocin 423 may form an α-helix between residues 17 and 31 (Fig. 7) similar to those described for pediocin AcH (32), leucocin A (15) and mesentericin Y 105 (14).

Bacteriocin 423 has four cystein residues, similar to pediocin PA-1 (30), enterocin A (4) and divercin V41 (31). Most bacteriocins of the Class IIa group only have two cysteine residues. Pairs of cysteine residues form disulfide bonds that stabilize the structure of the peptide and affect the activity of bacteriocins (32). Studies have shown that enterocin A and pediocin PA-1 were more active than sakacin P and curvacin A, the latter both containing only one disulfide bond (11). Some studies also suggested that in those bacteriocins containing more than one disulfide bridge, thioester linkage might be necessary for activity (10). Protein mutants of pediocin AcH generated by PCR random mutagenesis showed that for pediocin AcH, all four cysteins were required for activity (32).

Genetic analysis of bacteriocin 423: Four genes are usually required for bacteriocin production. These genes include a structural gene which encodes a prebacteriocin, an immunity gene which protects the producer cell against the bacteriocin, a gene encoding an ABC transporter, and an accessory gene which is also required for bacteriocin secretion (34). Although the operons that encode for the production of the Class IIa bacteriocins possess all the necessary genetic information, organization of these genes may vary considerably.

The gene cluster of bacteriocin 423 is arranged in a single operon identical to the genetic arrangement of pediocin PA-1 (30). The gene *bacA* appears to encode a 56 amino

acid precursor of bacteriocin 423. This was concluded after comparison of the gene-derived bacteriocin with the direct sequence data of bacteriocin 423 and confirmed by the expression of the mature peptide in *S. cerevisiae*.

Downstream of *bacA*, a second gene, *bacB*, encoding a protein of 72 amino acids is located. The homology of the protein derived from the nucleotide sequence of this gene to the immunity genes of leucocin A (19), mesentericin Y105 (16), divercin V41 (31) and enterocin A (4), suggests that *bacB* is the putative immunity gene of bacteriocin 423. This protein shares no homology with the 112 amino acid immunity gene of pediocin PA-1 and is much smaller.

The 174 amino acid *bacC* and 724 amino acid *bacD* is located further downstream from *bacB*. The *bacC* gene is identical to *pedC*, the accessory gene of pediocin PA-1 and *bacD* shares 99 % homology with *pedD*, the ABC translocator of pediocin PA-1. Similarity of pediocin PA-1 to bacteriocin 423 does not appear to be that unusual. Leucocin A (42) and mesentericin Y105 (16) are different Class IIa bacteriocins, but the organization of the genetic determinants of these bacteriocins is identical. The gene clusters of these two bacteriocins are arranged in two operons, and the genes share between 85 and 99% homology.

Heterologous expression of bacteriocin 423: We conclude from the occurrence of inhibition zones around colonies of *S. cerevisiae* transformants that, like pediocin PA-1 (39), bacteriocin 423 can be used to develop a bactericidal yeast. As was found previously (39), activity could be detected on agar plates, but the level of bacteriocin production was low in the yeast supernatant. Schoeman *et al.* (39) postulated that the bacteriocin remained cell wall associated. Further research is needed to improve the antimicrobial activity of yeasts. Fermentation studies are to be conducted to optimize production and test the viability of bactericidal yeasts used in food and wine products.

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Table 1. Microbial strains and plasmids

Strain or plasmid	Characteristics	Reference
Bacterial strain		
L. pentosus 423	Bacteriocin 423 producer	(43)
L. sakei LMG 13558	Sensitive to bacteriocin 423	LMG[1]
L. monocytogenes LM1	Sensitive to bacteriocin 423	This laboratory[3]
E. coli DH5α		(2)
Yeast strain		
S. cerevisiae Σ1278	Wild-type haploid strain; ura3	(17)
Plasmid		
pBR322	Ap^r; Tc^r	(8)
pBluescript SK	Ap^r; LacZ	(2)
pSRQ220	pBR322 with 5.6-kbp insert containing the pediocin PA1 operon	(30)
pBAC4	wild type plasmid with bacteriocin 423 operon	(43)
YEp352	*bla*; URA3	(21)
pPRL2	*bla*; LEU2; Single-copy (YCplacIII) expression vector with $ADHI_P$-$MF\alpha1_S$-$ADHI_T$ insert[2]	(39)
pBac423	pPRL2 with bacteriocin 423 PCR fragment (*bacA*) insert	This study
YEpBac423	YEp352 with $ADHI_P$- $MF\alpha1_S$-*bacA*-$ADHI_T$ insert	This study

[1]LMG, Culture collection of the Laboratory of Microbiology, University of Gent, Belgium

[2]$ADHI_P$-$ADHI_T$, yeast alcohol dehydrogenase I promoter-terminator cassette; $MF\alpha1_S$, mating pheromone α-factor secretion signal

[3] Department of Microbiology, University of Stellenbosch, Stellenbosch, South Africa

Table 2. Purification of bacteriocin 423 from *Lactobacillus pentosus* 423

Fraction	Volume (ml)	Protein (mg/ml)	Activity (AU/ml)	Total[1] activity (AU)	Sp. act.[2] (AU/mg)	Fold increase in sp. act.	Yield[3] (%)
Supernatant	750	12.20	1.28×10^4	9.6×10^6	1.05×10^3	1	100
Precipitation $(NH_4)_2SO_4$	42	3.16	1.02×10^5	4.3×10^6	3.24×10^4	31	44.8
Methanol-chloroform extraction	2	1.95	2.06×10^5	4.1×10^5	1.05×10^5	100	0.04
Cation exchange	1	0.56	2.05×10^5	2.05×10^5	3.6×10^5	343	0.13

[1]Bacteriocin activity units/ml X volume (ml).

[2]Specific activity represents bacteriocin activity divided by protein concentration.

[3]% yield of protein.

Table 3. Comparison of bacteriocin 423 to other Class IIa bacteriocins (YGNGV sequence underlined)

Bacteriocin	Leader peptide[1]	Mature peptide	Reference
Bacteriocin 423	MMKKIEKLTEKEMANIIGG	KYYGNGVTCGKHSCSVNWGQAFSCSVSHLANFGHGKC	This study
Acidocin A	MISMISSHQKTLTDKELALISGG	KTYYGTNGVHCTKKSLWGKVRLKNVIPGTLCRKQSLPIKQDLKILLGWATGAFGKTFH	(27)
Bavaricin A		KYYGNGVHXGKHSXTVDWGTAIGNIGNNAAANXATGXNAGG	(29)
Bavaricin MN		TKYYGNGVYXNSKKXWVDWGQAAGGIGQTVVXGWLGGAIPGK	(26)
Bifidocin B		KYYGNGVTCGLHDCRVDRGKATCGIINNGGMWGDIG	(44)
Carnobacteriocin B2[2]	MNSVKELNVKEMKQLHGG	VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	(37)
Carnobacteriocin BM1[3]	MKSVKELNKKEMQQINGG	AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	(37)
Curvacin A[4]	MNNVKELSMTELQTITGG	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	(40)
Divercin V41	MKNLKEGSYTAVNTDELKSINGG	TKYYGNGVYCNSKKCWVDWGQASGCIGQTVVGGWLGGAIPGKC	(31)
Enterocin A	MKHLKILSIKETQLIYGG	TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPGKC	(4)
Leucocin A[5]	MNNMKPTESYEQLDNSALEQVVGG	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNFW	(19)
Leucocin Ta11a	MNNMKSADNYQQLDNNALEQVVGG	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	(12)
Mesentericin Y 105[6].	MTNMKSVEAYQQLDNQNLKKVVGG	KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW	(14)
Mundticin		KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWSK	(6)
Pediocin PA-1[7]	MKKIEKLTEKEMANIIGG	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC	(30)
Piscicocin V1a[8]		KYYGNGVSCNKNGCTVDWSKAIGIIGNNAAANLTTGGAAGWNKG	(7)
Plantaricin C19		KYYGNGLSCSKKGCTVNWGQAFSCGVNRVATAGHGK	(1)
Sakacin P[9]	MEKFIELSLKEVTAITGG	KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK	(41)

[1]Leader peptide where available
[2]Carnobacteriocin B2 = Carnocin CP52 (20)
[3]Carnobacteriocin BM1 = Piscicolin V1b (7) = Carnocin CP51 (20)
[4]Curvacin A = Sakacin A (22)
[5]Leucocin A = Leucocin A-TA33a (36)
[6]Mesentericin Y105 = Mesentericin 52A (38)
[7]Pediocin PA-1 = Pediocin AcH (33)
[8]Piscicocin V1a = Piscicolin 126 (25)
[9]Sakacin P = Sakacin 674 (23)

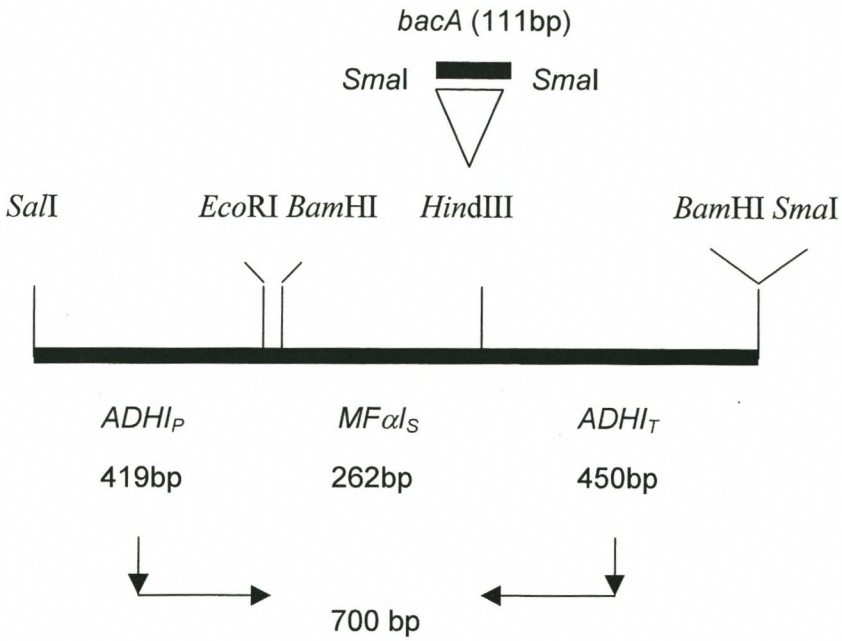
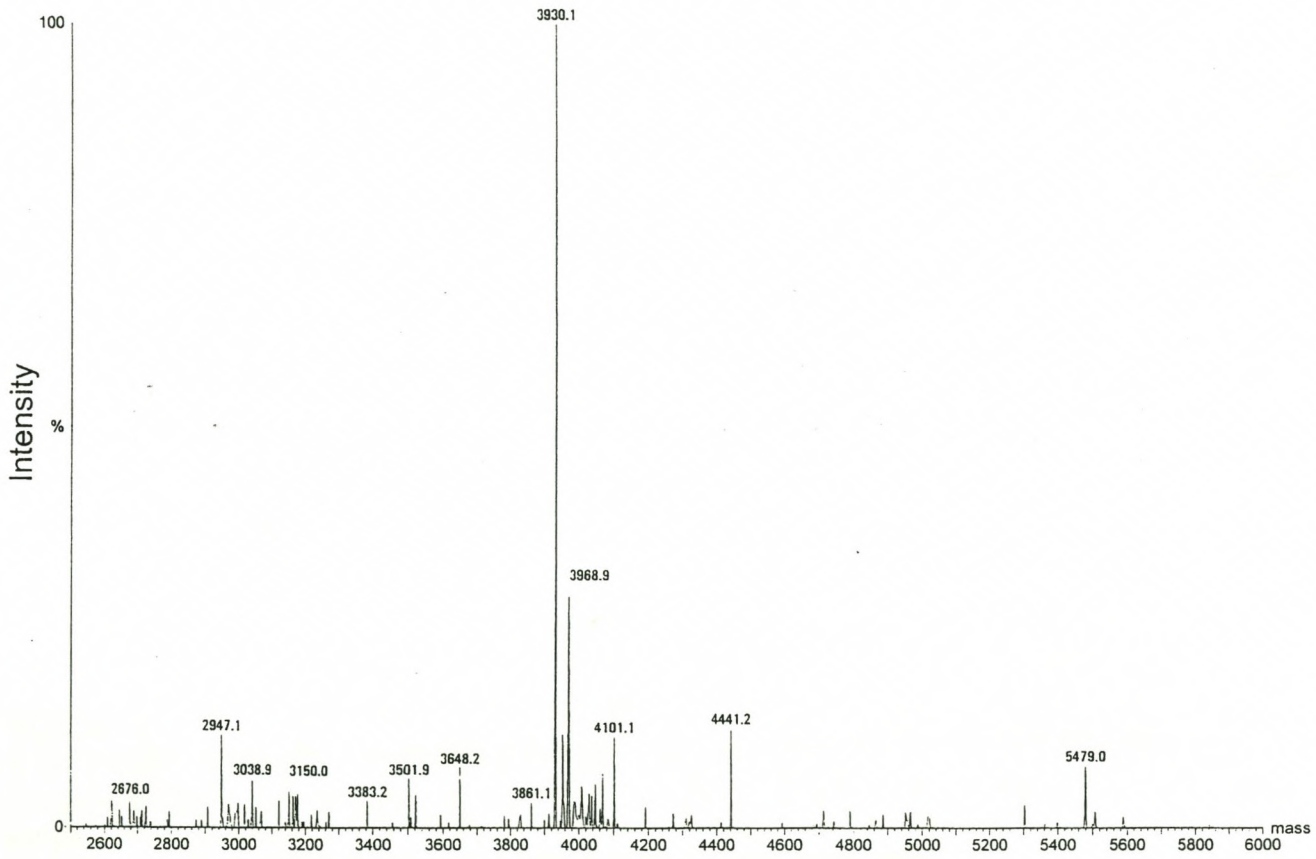


Fig. 1. Schematic representation of the yeast expression cassette used to express the bacteriocin 423 (*bacA*) gene in *S. cerevisiae*.



Mass

Fig. 2. Molecular mass of bacteriocin 423 calculated from the electrospray ionization-mass spectroscopy multiple charged spectra.

```
1       TCTAACTAATACTTGACATTTACATTGAGTAGGAACTAGAATGACCGCGTATTAAAAATGATTTAAGAAGAAGGAGATTTTTATGATGAA
                  -35                    -10                                RBS       M  M  K

91      AAAAATTGAAAAATTAACTGAAAAAGAAATGGCCAATATCATTGGTGGTAAATACTATGGTAATGGGGTTACTTGTGGTAAACATTCCTG
         K  I  E  K  L  T  E  K  E  M  A  N  I  I  G  G  K  Y  Y  G  N  G  V  T  C  G  K  H  S  C

181     CTCTGTTAACTGGGGCCAAGCATTTTCTTGTAGTGTGTCACATTTAGCTAACTTCGGTCATGGAAAGTGCTAATTAATTAATGGGGGGGA
         S  V  N  W  G  Q  A  F  S  C  S  V  S  H  L  A  N  F  G  H  G  K  C  *

271     TTATCTTTGGCTGAAGATACTCGCAATATGGCTGAGCAGTACGTAACTGAACTATATAAAAAATTAAAGTCTCGAGATTCAAAAACATCT
                              M  A  E  Q  Y  V  T  E  L  Y  K  K  L  K  S  R  D  S  K  T  S

361     GGCCTTTTAGATATTTTAGATGTTCTTATCCAAGTTCAAAAAAACTTATCAACGGTGAAAAACCCTGAGGCATTAGTAAATCGTTGCGTT
         G  L  L  D  I  L  D  V  L  I  Q  V  Q  K  N  L  S  T  V  K  N  P  E  A  L  V  N  R  C  V

451     CAATATATATACGTAGTGTTGCCATCAAAGACAAATTATATTTTCCTCCAGCAGAAGAAAATATAATTATTAATTTAGAAGTTATTGGCC
         Q  Y  I  Y  V  V  L  P  S  K  T  N  Y  I  F  L  Q  Q  K  K  I  *

541     AAAAAGCAGGTTGGAACGGTAGCTATATGGCTGATTTTAGTGATAAATCACAGTTTTATAAACTTTCAGAATCAATCCCACACCATTAGA

631     TTTGAGCTTCCACTATATTAGTATTTAGCCTGATAGATGGTGAACGACAAGATTGCGTAGTCTACAATAAGATTGCTAGTCAAGCGGTAG

721     TAGATAAGTTACATTTTACTGCCGAAGAAACCAAAGTTCTAGCAGCCATCAATGAATTGGCGCATTCTCAAAAAGGGTGGGGCGAGTTTA

811     ACATGCTAGATACTACCAATACGTGGCCTAGCCAATAGTACTGATAAAGGGGATATTGTAGTTGTCTAAGAAATTTTGGTCAAATATCTT
                                                    RBS          L  S  K  K  F  W  S  N  I  F

901     TTTAGCATTAGGCGTCTTTCTTGCTTTTGCAGGAGTTGCTACCATATCGGTGAGTGCTGACAGTTCCGCTACTATAGAATCAAATACTAG
         L  A  L  G  V  F  L  A  F  A  G  V  A  T  I  S  V  S  A  D  S  S  A  T  I  E  S  N  T  S

991     CTCGAAAATCATCGATGGTGCAACTTATGAAGAAAACATCAAGGGCGTTATTCCTATTACGCTAACTCAATATTTGCATAAAGCTCAAAC
         S  K  I  I  D  G  A  T  Y  E  E  N  I  K  G  V  I  P  I  T  L  T  Q  Y  L  H  K  A  Q  T

1081    TGGAGAAAAATTTATTGTCTTTGTCGGGTTCAAGGAGTGTGTGCATTGTCGTAAATTTTCTCCAGTCATGAAACAGTACTTACAACAAAG
         G  E  K  F  I  V  F  V  G  F  K  E  C  V  H  C  R  K  F  S  P  V  M  K  Q  Y  L  Q  Q  S

1171    TCAGCATCCCATTTATTACTTAGACTATGGGAACAACGGGTCTTTCAGCATGGCTTCTCAAAAACAAATAACTGATTTCTATTCAACTTT
         Q  H  P  I  Y  Y  L  D  Y  G  N  N  G  S  F  S  M  A  S  Q  K  Q  I  T  D  F  Y  S  T  F

1261    TGCAACCCCCATGAGTTTTATGGGAACGCCAACTGTTGCCTTGCTCGATAATGGTAAGGTGGTATCAATGACCGCTGGTGATGATACCAC
         A  T  P  M  S  F  M  G  T  P  T  V  A  L  L  D  N  G  K  V  V  S  M  T  A  G  D  D  T  T

1351    TTTATCTGATTTACAACAGATTACTGCTGATTACAATAATCAGTAGTCACCTGGTTAATATGGTTTTGTAACCAATGTAAAAGGCGATGG
         L  S  D  L  Q  Q  I  T  A  D  Y  N  N  Q  *

1441    ATCTTTGAAATCGTCTTTTTTTATGCACAAATTTTAAAGATCGGTGGTTTGCTTATGTGGACTCAAAAATGGCACAAATATTATACAGCA
                                                    RBS          M  W  T  Q  K  W  H  K  Y  Y  T  A

1531    CAAGTTGATGAAAATGACTGTGGTTTAGCTGCACTAAATATGATCCTAAAATACTATGGCTCCGATTACATGTTGGCCCATCTTCGACAG
         Q  V  D  E  N  D  C  G  L  A  A  L  N  M  I  L  K  Y  Y  G  S  D  Y  M  L  A  H  L  R  Q
```

Fig. 3. Nucleotide sequence of the bacteriocin 423 gene cluster and deduced amino acid sequences (shown below the nucleotide sequence). Translational termination codons are depicted by asterisks. Predicted –10 and –35 promotor sites and putative RBS are underlined. GG cleavage site depicted by ↑ stop codon by *.

```
1621    CTTGCCAAAACAACTGCTGACGGTACAACTGTTTTGGGGCTTGTTAAAGCAGCAAAACACTTAAATTTAAATGCCGAAGCTGTGCGTGCT
        L  A  K  T  T  A  D  G  T  T  V  L  G  L  V  K  A  A  K  H  L  N  L  N  A  E  A  V  R  A

1711    GATATGGATGCTTTGACAGCCTCACAATTGCCATTACCAGTCATTGTTCATGTATTCAAGAAAAATAAGTTACCACACTACTATGTTGTC
         D  M  D  A  L  T  A  S  Q  L  P  L  P  V  I  V  H  V  F  K  K  N  K  L  P  H  Y  Y  V  V

1801    TATCAGGTAACTGAAAACGATTTAATTATTGGTGATCCTGATCCAACCGTTAAAACCACTAAAATATCGAAATCACAATTTGCTAAAGAA
        Y  Q  V  T  E  N  D  L  I  I  G  D  P  D  P  T  V  K  T  T  K  I  S  K  S  Q  F  A  K  E

1891    TGGACCCAGATTGCAATTATCATAGCCCCAACAGTTAAATATAAACCCATAAAAGAATCACGGCACACATTAATTGATCTAGTGCCTTTA
         W  T  Q  I  A  I  I  I  A  P  T  V  K  Y  K  P  I  K  E  S  R  H  T  L  I  D  L  V  P  L

1981    TTGATTAAACAAAAAAGATTAATTGGACTAATTATTACCGCAGCAGCTATAACAACATTAATCAGTATTGCTGGTGCATATTTCTTTCAG
        L  I  K  Q  K  R  L  I  G  L  I  I  T  A  A  A  I  T  T  L  I  S  I  A  G  A  Y  F  F  Q

2071    TTAATTATCGATACTTATTTGCCGCACTTGATGACTAATAGGCTTTCACTAGTTGCCATTGGTCTGATTGTAGCTTATGCTTTCCAAGCA
        L  I  I  D  T  Y  L  P  H  L  M  T  N  R  L  S  L  V  A  I  G  L  I  V  A  Y  A  F  Q  A

2161    ATTATCAACTATATACAAAGTTTTTTTACGATTGTATTAGGACAACGTCTCATGATCGACATCGTTTTAAAATACGTTCACCATCTTTTT
        I  I  N  Y  I  Q  S  F  F  T  I  V  L  G  Q  R  L  M  I  D  I  V  L  K  Y  V  H  H  L  F  D

2251    GATTTACCAATGAATTTTTTTACTACCCGTCATGTCGGTGAAATGACCTCACGCTTTTCTGATGCAAGCAAAATTATTGATGCACTTGGA
         L  P  M  N  F  F  T  T  R  H  V  G  E  M  T  S  R  F  S  D  A  S  K  I  I  D  A  L  G

2341    AGTACAACGCTCACCCTTTTTTTAGACATGTGGATTTTATTAGCAGTAGGGTTATTTTTGGCCTATCAAAACATCAATTTATTTTTATGC
        S  T  T  L  T  L  F  L  D  M  W  I  L  L  A  V  G  L  F  L  A  Y  Q  N  I  N  L  F  L  C

2431    TCGTTAGTTGTGGTTCCAATTTACATCTCGATTGTTTGGCTATTTAAAAAAACTTTTAATCGTTTAAATCAAGATACAATGGAAAGCAAT
        S  L  V  V  V  P  I  Y  I  S  I  V  W  L  F  K  K  T  F  N  R  L  N  Q  D  T  M  E  S  N

2521    GCAGTTCTTAATTCTGCTATTATTGAAAGTCTCAGTGGCATAGAAACCATTAAATCACTAACTGGTGAAGCAACTACAAAAAAAAAGATT
        A  V  L  N  S  A  I  I  E  S  L  S  G  I  E  T  I  K  S  L  T  G  E  A  T  T  K  K  K  I

2611    GACACACTATTTTCTGACTTATTGCATAAAAACTTGGCTTATCAAAAAGCTGATCAAGGACAACAAGCTATCAAAGCAGCTACTAAATTA
        D  T  L  F  S  D  L  L  H  K  N  L  A  Y  Q  K  A  D  Q  G  Q  Q  A  I  K  A  A  T  K  L

2701    ATCCTAACTATTGTTATCCTTTGGTGGGGTACTTTTTTTGTTATGCGACACCAACTGTCTTTAGGTCAGCTGTTAACTTATAATGCTTTG
        I  L  T  I  V  I  L  W  W  G  T  F  F  V  M  R  H  Q  L  S  L  G  Q  L  L  T  Y  N  A  L

2791    CTCGCTTACTTCTTGACCCCATTAGAAAATATTATTAATTTACAGCCTAAACTACAAGCTGCCAGAGTGGCTAATAATCGATTAAATGAG
        L  A  Y  F  L  T  P  L  E  N  I  I  N  L  Q  P  K  L  Q  A  A  R  V  A  N  N  R  L  N  E

2881    GTTTATCTAGTAGAGTCTGAATTTTCTAAATCTAGGGAAATAACTGCTCTAGAGCAACTAAATGGTGATATTGAGGTTAATCATGTTAGT
        V  Y  L  V  E  S  E  F  S  K  S  R  E  I  T  A  L  E  Q  L  N  G  D  I  E  V  N  H  V  S

2971    TTTAACTATGGCTATTGTTCTAATATACTTGAGGATGTTTCTCTAACAATTCCACATCATCAGAAGATTACTATTGTAGGCATGAGTGGT
        F  N  Y  G  Y  C  S  N  I  L  E  D  V  S  L  T  I  P  H  H  Q  K  I  T  I  V  G  M  S  G

3061    TCGGGGAAAACGACCCTAGCCAAGTTGCTAGTTGGTTTTTTTGAGCCTCAAGAACAGCACGGTGAAATTCAGATTAATCATCACAATATA
        S  G  K  T  T  L  A  K  L  L  V  G  F  F  E  P  Q  E  Q  H  G  E  I  Q  I  N  H  H  N  I

3151    TCTGATATTAGTCGCACAATTTTACGCCAATATATTAATTATGTTCCTCAAGAACCTTTCATTTTTTCGGGCTCTGTATTAGAAAATTTA
        S  D  I  S  R  T  I  L  R  Q  Y  I  N  Y  V  P  Q  E  P  F  I  F  S  G  S  V  L  E  N  L
```

Fig. 3 (continued)

```
3241    TTGTTAGGTAGCCGTCCTGGAGTAACTCAACAAATGATTGATCAAGCTTGTTCCTTTGCTGAAATCAAAACTGATATAGAAAATTTGCCT
        L  L  G  S  R  P  G  V  T  Q  Q  M  I  D  Q  A  C  S  F  A  E  I  K  T  D  I  E  N  L  P

3331    CAAGGTTATCATACTAGATTAAGTGAAAGTGGATTCAACTTATCTGGTGGGCAAAAACAGCGGTTATCAATAGCTAGAGCATTATTGTCT
         Q  G  Y  H  T  R  L  S  E  S  G  F  N  L  S  G  G  Q  K  Q  R  L  S  I  A  R  A  L  L  S

3421    CCGGCACAATGTTTCATTTTTGACGAATCAACCAGTAATTTAGACACCATTACTGAACATAAAATAGTCTCTAAGCTATTATTCATGAAA
        P  A  Q  C  F  I  F  D  E  S  T  S  N  L  D  T  I  T  E  H  K  I  V  S  K  L  L  F  M  K

3511    GACAAAACGATAATTTTTGTAGCACATCGTCTCAATATTGCGTCTCAAACCGATAAAGTTGTCGTTCTTGATCATGGAAAGATTGTTGAA
        D  K  T  I  I  F  V  A  H  R  L  N  I  A  S  Q  T  D  K  V  V  V  L  D  H  G  K  I  V  E

3601    CAGGGATCACATCGACAATTGTTAAATTATAATGGGTATTATGCACGGTTAATTCATAATCAAGAATAACCTATCAAGAACCAGTCTGCT
        Q  G  S  H  R  Q  L  L  N  Y  N  G  Y  Y  A  R  L  I  H  N  Q  E  *

3691    ATTGATAGACTATTCTTGTCCGTAAAATCCTCGCGTATTCCCGTGAGGATCATAGTATATTTAGCGCTCTTCTTAAAATTTTAAGTATAT

3781    TGATTCATATGTTTATCCMCCTAAGTTTGAAGACAAACCGGTNCATGTTATAATACTTCTACCGGCTTGTCCGGTGTTNGGGANCATTAC

3871    TCGAACTTTCTGTGGTAATCAAGTGAGT
```

Fig. 3 (continued)

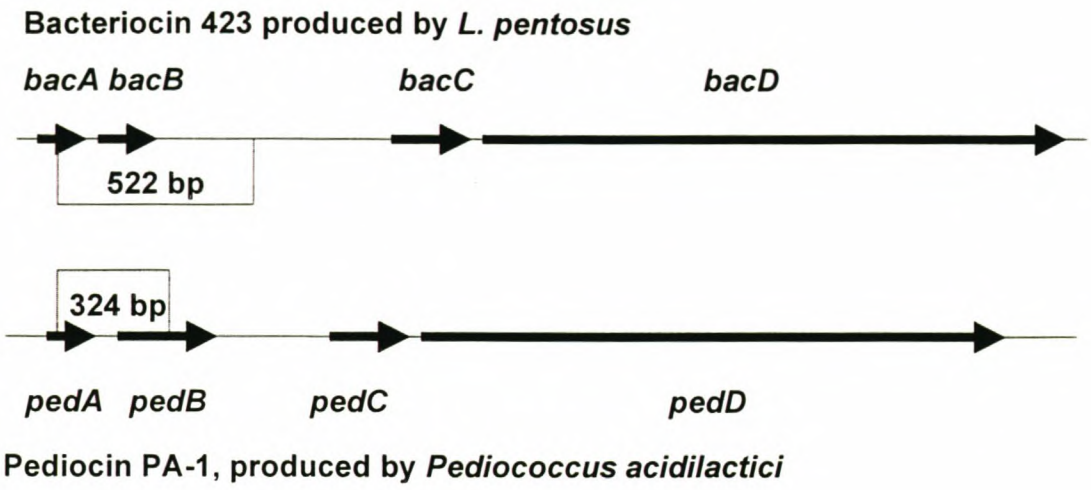


Fig. 4. Schematic overview of the similarity between the putative operon of bacteriocin 423 and the pediocin PA-1 operon. The two operons are identical except for a 522 bp fragment of DNA that occurs in the bacteriocin 423 operon, but not in the operon of pediocin PA-1. Similarly, a 324 bp fragment of DNA occurs in the operon of pediocin PA-1, but not in the bacteriocin 423 operon.

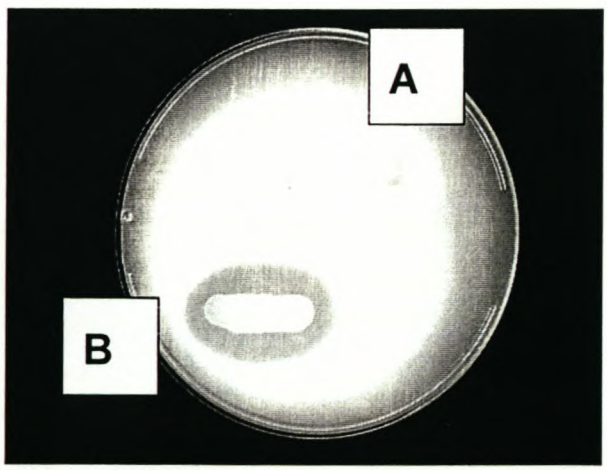


Fig. 5. Zones of inhibition produced by *Saccharomyces cerevisiae* transformants without (A) and with (B) the bacteriocin 423 gene cassettes.

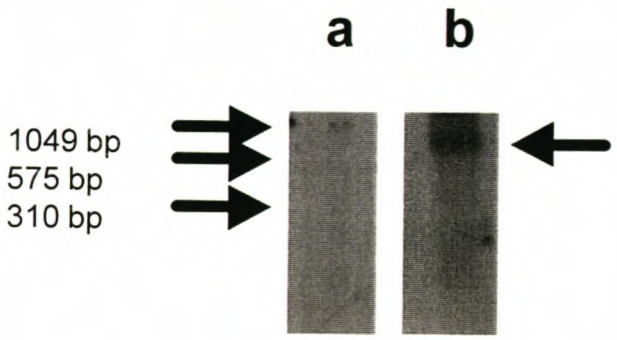


Fig. 6. Northern blot analysis of RNA prepared from a negative control yeast transformant (a) and a YEpBAc423 transformant (B). RNA ladder sizes are indicated on the left. The arrow on the right indicates the transcript.

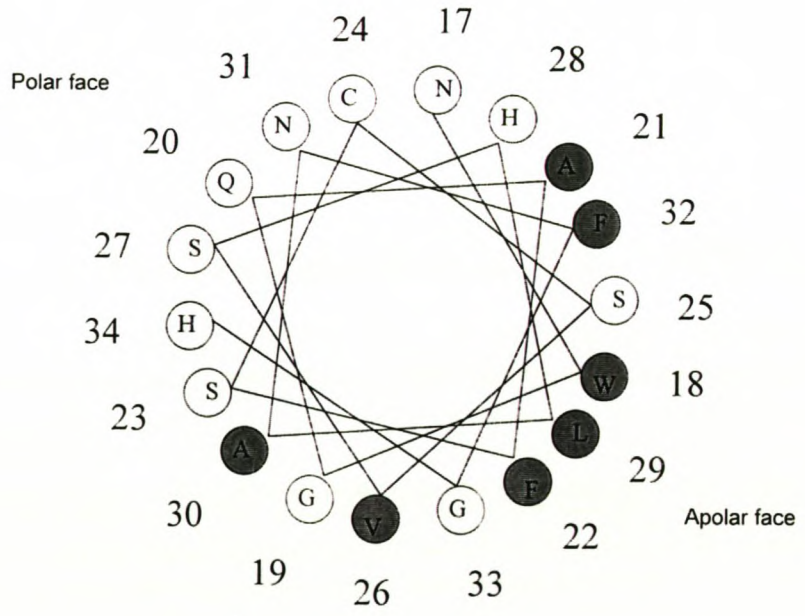


Fig. 7. Edmundson α-helical wheel representation of the amphiphatic regions of bacteriocin 423. Grey circles depict apolar (hydrophobic) amino acids.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 7

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7.1 TAXONOMY OF *Lactobacillus pentosus* 423

Lactic acid bacteria are widespread in fermentable materials. The taxonomy of these organisms is complex, and with the advent of modern molecular techniques, many of the lactic acid bacteria have been reclassified. Strain 423, a bacteriocin-producing lactic acid bacterium isolated from sorghum beer was initially identified as a Gram-positive and catalase negative rod. The lack of CO_2 production from glucose fermentation and carbohydrate fermentations using the API 50 CHL system (La Balme Les Grottes, Montalieu Vercieu, France), identified the organism as a strain of *Lactobacillus plantarum*. *Lactobacillus pentosus* is usually distinguished from *L. plantarum* by the production of acid from glycerol and/or D-xylose but not melezitose, although in many cases these characteristics were found not to be sufficient for differentiation (Bringel *et al.*, 1996). Some strains of *L. plantarum* may also ferment glycerol (Zanoni *et al.*, 1987). Isolate 423 did not ferment glycerol or D-xylose, but did ferment melezitose. Results obtained by numerical analysis of total soluble protein patterns clearly indicated that strain 423 belonged to *L. plantarum*, with some relatedness to *L. pentosus* (Van Reenen and Dicks, 1996).

Nucleic acid probes are increasingly being used for the rapid identification of economically important organisms (Hertel *et al.*, 1991). Bringel *et al.* (1996) differentiated among strains of *L. plantarum*, *L. pentosus* and *Lactobacillus paraplantarum* by using genes encoding enzymes in the pyrimidine *de novo* pathway of *L. plantarum* as a probe. Polimerase chain reactions (PCR) using random amplified polymorphic DNA (RAPD) primers have also proved useful to differentiate between several *Lactobacillus* spp. (Du Plessis and Dicks, 1995), and to distinguish *L. plantarum*, *L. pentosus* and *L. paraplantarum* (Dellaglio, personal communication). Johansson *et al.* (1995) evaluated the use of RAPD-PCR for rapid typing of known *L. plantarum* and *L. pentosus* strains. Some results showed unacceptable species designation, prompting a cautionary conclusion that one should be aware of the limitations in discrimination power between closely related strains. Numerical analysis of RAPD-PCR profiles grouped *L. pentosus* and *L. plantarum* into separate phenotypic clusters, with strain 423 related to the *L. plantarum* group. This study included several strains and type strains of *L. plantarum, Lactobacillus casei, Lactobacillus sakei* and *Lactobacillus curvatus,* and the type strain of *L. pentosus.*

16S rRNA sequence analysis identified strain 423 as *L. pentosus* (Collins, M.D.,

Department of Food Science and Technology, University of Reading, Reading RG66AP, UK, Personal communication). The discrepancy in the results of this study could be due to the selected primers used for the RAPD-PCR profiles, or to the unavailability of more reference strains of *L. pentosus,* which did not have separate species status before 1987 (Zanoni *et al.*, 1987). In an attempt to differentiate *L. plantarum* and *L. pentosus,* Berthier and Ehrlich (1998) found that the 16S rRNA sequences of these two species displayed more than 99% identity. The differentiation of the two species therefore remains difficult. Since *L. plantarum* and *L. pentosus* share such a high degree of DNA homology, it may be hypothesized that they are two subspecies within the same species. Due to this result, the bacteriocin produced by strain 423 is referred to as bacteriocin 423, rather than plantaricin 423 or pentocin 423.

7.2 PROTEIN CHARACTERIZATION OF BACTERIOCIN 423

Bacteriocin 423 inhibited various Gram-positive organisms, such as *Oenococcus oeni, Streptococcus thermophilus* and several species of the genera *Lactobacillus*, *Leuconostoc, Pediococcus, Propionibacterium, Staphylococcus*, and *Listeria.* This indicated a broad spectrum of inhibition. The bacteriocin was inactivated by the proteolytic enzymes proteinase K, pepsin, papain, α-chymotrypsin and trypsin. Heat treatments at 40°C, 60°C, 80°C and 100°C had no effect on the activity of the bacteriocin, but 50% activity was lost after 60 min at 100°C, and 75% activity was lost after autoclaving. The bacteriocin remained active at pH 1-10. A bactericidal mode of action was observed against *Oenococcus oeni* as well as *Listeria monocytogenes.*

Using tricine-SDS-PAGE, the purified bacteriocin was determined to be approximately 3.5 kDa, indicating a small, heat resistant peptide. Electrospray mass spectrometry determined the mass of the peptide to be 3930 Da. The amino acid sequence of the bacteriocin was determined as KYYGNGVTCGKHSCSVNWGQAFSCSVSHLANFGHGKC, a 37 amino acid peptide showing homology to several other Class IIa anti-listerial bacteriocins such as plantaricin C19 (Atrih *et al.*, 1993), pediocin PA-1 (Marugg *et al.*, 1992), mesentericin Y105 (Fremaux *et al.*, 1995), leucocin A (Van Belkum and Stiles, 1995), divercin V41 (Métivier *et al.*, 1998), sakacin P (Hühne *et al.*, 1996), acidocin A (Kanatani *et al.*, 1995), sakacin A (Axelsson and Hölck, 1995) and enterocin A (O'Keeffe *et al.*, 1999). The peptide contains the consensus sequence YGNGV in the N-terminal of its structure. Similar to the bacteriocins pediocin PA-1, enterocin A and divercin V41, bacteriocin has four cystein residues in its primary structure, that probably form two disulfide bonds. Most Class IIa bacteriocins only have two cystein residues.

We have also studied the fermentation optimization of bacteriocin 423 (Verellen *et al.*, 1998). The bacteriocin, produced during exponential growth, reached maximum activity at

the beginning of stationary growth, after which activity declined towards the end of fermentation. Bacteriocin activity increased if the initial pH of the MRS broth was 4.9. Meat extract, casamino acids, tryptone and peptone increased the activity of the bacteriocin. Low concentrations of manganese sulfate and magnesium sulfate stimulated the growth of *L. pentosus* 423, but only manganese sulfate further increased bacteriocin activity.

7.3 GENETIC CHARACTERIZATION OF BACTERIOCIN 423

Southern blot hybridizations, using the operon of pediocin PA-1 (Marugg *et al.*, 1992) as probe, indicated that bacteriocin 423 was plasmid-encoded. This was confirmed with curing studies using novobiocin, where loss of a plasmid of about 9 kb in size coincided with loss of bacteriocin production. The DNA sequences of the operons of bacteriocin 423 and pediocin PA-1 were virtually identical. The structural genes were identical in the N-terminal area of the peptides, but the C-terminal of the two peptides differed. The putative immunity gene of bacteriocin 423 was completely different to the immunity gene of pediocin PA-1. The putative accessory and transporter genes of bacteriocin 423 were homologous to that of pediocin PA-1. The functions of *bacB*, *bacC* and *bacD* could in future be verified by deletion analysis and overexpression of these genes as described for pediocin PA-1 by Venema *et al.* (1995).

The only major difference was observed between nucleotide 1177 and 1509 (332 bp) of pediocin PA-1 (Marugg *et al.*, 1992) and nucleotide 187 and 717 (531 bp) of bacteriocin 423 (Chapter 6). For pediocin PA-1, this section of DNA encodes the C-terminal of the structural gene and the N-terminal of the immunity gene. For bacteriocin 423, this section of DNA encodes the C-terminal of the structural gene and the complete putative immunity gene. In the operon of bacteriocin 423 the DNA encoding the C-terminal of the immunity protein of pediocin PA-1 is also present, although no function could be ascribed to this section of DNA. From this observation, one may conclude that bacteriocin 423 originated from pediocin PA-1, but evolved after DNA exchange took place at some stage. The lack of inverted repeats indicating an insertion element suggests a different mode of horizontal gene transfer (Kolstø, 1997; Smith *et al.*, 1992; Syvanen, 1994). The high percentage of identical or virtually identical bacteriocins produced by different organisms indicates that bacteria have genomic plasticity, with genetic material continually exchanged among organisms. This could also explain why some organisms produce one or more identical bacteriocins, but with different genetic arrangements, such as lactococcins A, B and M (Morgan *et al.*, 1995; Van Belkum *et al.*, 1989).

The high homology of the bacteriocin 423 operon with that of pediocin PA-1 could be explained by their phylogenetic relatedness. Based on rRNA homology, lactobacilli can be

subdivided into three major phylogenetically related groups: the *Lactobacillus delbrueckii* group, the *Lactobacillus casei-Pediococcus* group, and the *Leuconostoc* group. *Lactobacillus pentosus*, *L.plantarum* and *Pediococcus acidilactici*, the species that produces pediocin PA-1, all fall within the *L. casei-Pediococcus* group (Vandamme *et al.*, 1996).

7.4 HETEROLOGOUS EXPRESSION OF BACTERIOCIN 423

A PCR fragment identical to the DNA sequence of the mature peptide was ligated into a yeast expression/secretion system as described by Schoeman *et al.* (1999). Expression of the bacteriocin could only be detected on agar plates inoculated with a concentrated cell solution. No inhibitory activity could be detected in the unconcentrated supernatant, indicating that bacteriocin production was dependent on cell concentration. This result is similar to that found by Schoeman *et al.* (1999), who speculated that the recombinant pediocin molecule remains bound to the cell wall, or becomes stuck within the yeast plasma membrane. Although glycosylation may inhibit the secretion of heterogenous proteins, no potential glycosylation sites (Kukuruzinska et al., 1987) occur in the mature peptide of bacteriocin 423. Northern blot analysis of yeast transformants did however indicate the presence of a transcript of approximately 700 nucleotides in size, which was absent in the negative controls.

Yeast growth became increasingly slow and after about three weeks did not produce bacteriocin, indicating that the peptide could be toxic to the yeast. Inclusion of the immunity protein in the yeast expression gene cassette may improve the longevity of the yeast.

The development of yeasts with bactericidal properties remains an exciting prospect. This biological control of spoilage organisms could lead to a reduction in the use of chemical preservatives in products such as wine, beer and bakery products. Several strains of lactic acid bacteria, such as *Leuconostoc* spp., *Lactobacillus* spp., *Pediococcus* spp. and *Oenococcus oeni* occur in wine and play an important role. Sometimes the growth of lactic acid bacteria may be beneficial, as in malolactic fermentation, where malic acid is enzymatically oxidized to form lactic acid and carbon dioxide, resulting in a reduction of total acidity. Malolactic fermentation is useful in high acid, low pH wines. In low acid, high pH wines, however, the sensory properties, and chemical and microbial stability may be adversely affected (Zoecklein *et al.*, 1995). Growth of lactic acid bacteria in wine may result in wine spoilage due to overproduction of polysaccharide or flavour compounds such as diacetyl (Colagrande *et al.*, 1994; Davis *et al.*, 1985; Wibowo *et al.*, 1985). Yeasts producing antimicrobial peptides such as bacteriocin 423 may thus be useful in the control of these organisms in wine and in other yeast fermented food

products.

Much more research is necessary to optimize production of antimicrobial peptides in yeast. Expression levels of antimicrobial peptides may be enhanced using different promoters and secretion systems. Other factors that may play a role in optimization of yeast bacteriocin production include proteolytic activity and hyperglycosylation (Schoeman *et al.*, 1999).

In conclusion, bacteriocin 423 is a Class IIa anti-listerial peptide produced by *Lactobacillus pentosus* 423. The bacteriocin is plasmid-encoded by an operon-like structure. The peptide was successfully expressed in *Saccharomyces cerevisiae*.

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APPENDIX

FERMENTATION OPTIMIZATION OF PLANTARICIN 423, A BACTERIOCIN PRODUCED BY *LACTOBACILLUS PLANTARUM* 423

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Fermentation Optimization of Plantaricin 423, a Bacteriocin Produced by *Lactobacillus plantarum* 423

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More than 15 bacteriocins of *Lactobacillus plantarum* have been described. However, little information has been published on the fermentation optimization of these peptides. Plantaricin 423, produced by *L. plantarum* 423, is produced during exponential growth and reaches a maximum activity [6000 activity units (AU)/ml] at the beginning of stationary growth (*i.e.* after 15 h). This activity level is maintained for 3 h, but declines to 2600 AU/ml towards the end of fermentation (*i.e.* after 31 h). The increase in plantaricin 423 activity coincided with a decrease in pH from 5.8 to 4.0 during the first 15 h of fermentation. However, when the number of cells are taken into account (OD_{max}-value), a higher concentration of plantaricin 423 is produced in medium with an initial pH of 4.90 (2961 AU/ml/OD_{max}) than at pH 5.80 (2368 AU/ml/OD_{max}). A much lower activity of plantaricin 423 was obtained during the same fermentation period (15h) when cells were grown in MRS broth (Merck) with an initial pH of 6.9. The production of plantaricin 423 increased from 6000 AU/ml in MRS broth (Merck) to 9600, 12,800 and 19,200 AU/ml when the medium was supplemented with 1.9% (w/v) meat extract (Oxoid), 3.4% (w/v) casaminoacids (Oxoid) or 1.9% (w/v) tryptone (Oxoid), and 1.7% (w/v) bacteriological peptone (Oxoid), respectively. The activity of plantaricin 423 was even further increased with the addition of 1% (w/v) Tween 80 to MRS broth (Merck). Low concentrations of $MnSO_4 \cdot H_2O$ (0.014%, w/v) stimulated the growth of strain 423 and increased the activity of plantaricin 423. Although the addition of $MgSO_4 \cdot 7H_2O$ had the same stimulating effect on the growth of strain 423, the activity of plantaricin 423 was not increased.

Bacteriocins are defined as proteins or protein complexes antagonistic against bacteria

genetically closely related to the producer organism (1, 2). Since the discovery of colicine V in 1925, the first bacteriocin described for *Escherichia coli,* many papers have been published on the production, isolation and characterization of bacteriocins from various species, including lactic acid bacteria (3). Bacteriocins produced by *Lactobacillus, Lactococcus* and *Pediococcus*, and to a certain extent *Streptococcus, Enterococcus* and *Leuconostoc* spp. received considerable attention during recent years, with the emphasis on genetic manipulation of the producer strain and overproduction of the bacteriocin (reviewed by 4). This is not surprising, since lactic acid bacteria have GRAS (generally recognized as safe) status and are used as starter cultures in many fermented food products (5), and more recently as probiotics for humans and farm animals (6, 7).

Lactobacillus plantarum is one of the most widely distributed lactic acid bacteria in nature and is often included in mixed starter cultures for the production of fermented meat, vegetables, silage and certain dairy products (5). Since the description of lactolin A2 (8) and plantaricin SIK-83 (9, 10), no less than 13 additional bacteriocins of *L. plantarum* have been described (Table 1), of which plantacin B (11), plantaricin A (12), plantacin BN (13) and plantaricin S (14) are the best studied. Most of these studies dealt with the characterization of the bacteriocin, its antimicrobial spectrum and mechanism of activity (reviewed by 4).

Little information is available on the fermentation optimization of the bacteriocins produced by *L. plantarum*. A few papers have been published on the growth stimulation of *L. plantarum* (15, 16) and only one paper has been published on the fermentation optimization of plantaricin T (14).

In this paper we report on the fermentation optimization of plantaricin 423, a bacteriocin produced by *L. plantarum* 423 which has been isolated from traditionally fermented sorghum beer.

MATERIALS AND METHODS

Bacterial strains and growth conditions *L. plantarum* strain 423 was grown in MRS broth (Merck, Germany) at 30°C. The indicator strains used in this study are listed in Table 2. The strains were from LMG (Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium), ATCC (American Type Culture Collection) and our own collection (Department of Microbiology, University of Stellenbosch, Stellenbosch, South Africa). The indicator strains were maintained in the growth media as recommended in the respective culture collection catalogues. *Oenococcus oeni* (previously *Leuconostoc oenos*, 17) was grown in acidic grape

broth (18).

Inhibitory activity of plantaricin 423 *L. plantarum* 423 was inoculated (1%, v/v) into MRS Broth (Merck) and incubated at 30°C until mid-logarithmic growth (OD_{600}=1.4). The culture was then centrifuged (10 min at 8000 x *g*) and 10 μl of the supernatant used to determine the inhibitory activity of plantaricin 423 against the indicator strains listed in Table 2. The antimicrobial units (AU) of activity of plantaricin 423 was determined against *Listeria monocytogenes* LM1, as described by Green *et al.* (19).

Production of plantaricin 423 Production of plantaricin 423 was followed during fermentation in 200 ml MRS broth (Merck). At specific time intervals, optical density readings (at 600 nm) and the pH of the culture were determined. The AU of plantaricin 423 were also determined, as described previously.

Resistance of plantaricin 423 to heat, pH and proteolytic enzymes These tests were conducted on crude extract samples of plantaricin 423 (10 x concentrated by lyophilization). *L. monocytogenes* LM1 was used as indicator strain. Aliquots of plantaricin 423 were exposed to heat treatments of 40°C, 60°C, 80°C and 100°C for 10, 30 and 60 min, respectively, and autoclaved (121°C for 15 min). Resistance to pH and proteolytic enzymes were determined as described by Green *et al.* (19).

Effect of initial pH on the growth of strain 423 and the production of plantaricin 423 The effect of pH on the growth of strain 423 and the production of plantaricin 423 was studied in 200 ml MRS broth (Merck). The medium was buffered with 0.25 M Na_2HPO_4 and the pH adjusted to 7.1, 6.9, 6.7, 6.2 and 5.8, respectively, by adding 10 N HCl. To obtain initial pH values of 5.4 and 4.9, the MRS broth (Merck) was buffered with 0.25 M CH_3COONa and adjusted with filter-sterilized 2 N NaOH. All pH adjustments were done after autoclaving. The medium was inoculated with 1% (v/v) of *L. plantarum* 423 (OD_{600} approx. 1.4). Fermentations were conducted at 30°C for 20 h. At specific time intervals the pH of the fermented medium, optical cell density (absorbance at 600 nm) and activity (AU per ml) of plantaricin 423 was determined. *L. monocytogenes* LM1 was used as indicator strain. The experiment was done in duplicate.

Influence of medium compounds on the production of plantaricin 423

Nitrogen The influence of different nitrogen sources were tested in MRS-PM broth [MRS broth of de Man, Rogosa and Sharpe (20), but without bacteriological peptone and meat extract], supplemented with the following: 6.7% (w/v) corn steep liquor (Cerestar), 1.9% (w/v) meat extract (Oxoid), 1.9% (w/v) tryptone (Oxoid), 2.5% (w/v) yeast extract (Oxoid), 1.7% (w/v) bacteriological peptone (Oxoid), and 3.4% (w/v) casaminoacids (Oxoid), respectively.

The fermentations were conducted in 3 l fermentors (Applikon®) at 35°C. The pH was automatically controlled at 5.7 by adding 10 N NaOH with a BioController (Applikon®).

Manganese The effect of manganese on the production of plantaricin 423 was determined by using MRS-PM medium, supplemented with 2% (w/v) tryptone (Oxoid) and various concentrations of $MnSO_4 \cdot H_2O$ (0.004-0.044%, w/v). The initial pH of the medium was adjusted to 6.0 with 10 N HCl before autoclaving. The medium was inoculated with 0.4% (v/v) of an active growing culture of *L. plantarum* 423. The fermentations were conducted in 200 ml medium at 30°C for 19 h. The pH, optical density ($OD_{600\ nm}$) of the culture and activity (AU/ml) of plantaricin 423 were determined after 15.5 and 19 h, respectively.

Magnesium The influence of $MgSO_4 \cdot 7H_2O$ on the production of plantaricin 423 was studied by using the same medium, growth conditions and inoculum size as described for the manganese experiments, except for adjusting the $MnSO_4 \cdot H_2O$ level to 0.0045% (w/v). Only two experiments were conducted; one without any $MgSO_4 \cdot 7H_2O$ and the other with 0.041% (w/v) $MgSO_4 \cdot 7H_2O$ added to the medium. The pH, optical density ($OD_{600\ nm}$) of the culture and activity (AU/ml) of plantaricin 423 were determined after 15 and 19 h, respectively.

Tween 80 The effect of Tween 80 on the production of plantaricin 423 was studied in 200 ml MRS-PM medium with tryptone (2%, w/v) as sole nitrogen source. Concentrations of 0-2.0% (w/v) Tween 80 were used.

RESULTS AND DISCUSSION

Plantaricin 423 was active against *Bacillus cereus, Clostridium sporogenes, Enterococcus faecalis*, several *Lactobacillus* spp. (including *L. plantarum*), *O. Oeni*, *Listeria innocua, L. monocytogenes, Pediococcus acidilactici, Pediococcus pentosaceus, Staphylococcus carnosum* and *Streptococcus thermophilus* (Table 2). This spectrum of antimicrobial activity is similar to that reported for other plantaricins, e.g. plantaricin A (12), plantaricin C19 (21), plantaricin S (14), plantaricin 149 (22) and plantaricin SA6 (23). However, the strong bactericidal activity obtained against *L. innocua* and *L. monocytogenes* distinguishes plantaricin 423 from the other bacteriocins produced by *L. plantarum*. According to the classification system proposed by Klaenhammer (24), plantaricin 423 belongs to the group IIa (anti-Listeria) bacteriocins.

Plantaricin 423 is heat stable for up to 30 min at 100°C and 60 min at 80°C, but looses 50% of its activity after 60 min at 100°C and 75% of its activity after autoclaving (15 min at 121°C) (Table 3). The heat stability (up to 30 min at 100°C) is similar to that reported for

other plantaricins (12, 14, 21-23).

After a short lag phase of approximately 4 h the cells of *L. plantarum* increased to a maximum $OD_{(600\ nm)}$ of 1.9 (Fig. 1). During this time (27 h) the pH decreased from 5.80 to 3.75. The stationary phase was reached after 15 h, at which point the highest concentration of plantaricin 423 was recorded (6000 AU/ml). This level of plantaricin activity was retained for 3 h, but declined to 4000 AU/ml during the following 3 h, after which it remained stable for approximately 6 h and then declined to 2600 AU/ml over the remaining 4 h of fermentation.

The highest plantaricin activity was recorded at a pH of 4.0 (Fig. 1). This increase in plantaricin 423 activity as fermentation commences could be ascribed to less stringent binding of the peptide to the cell wall of *L. plantarum* as fermentation continued and the pH decreased. This phenomenon has been described for nisin, produced by *Lactococcus lactis* subsp. *lactis* (25). More than 80% of nisin remained adsorbed to the producer cell at a pH of 6.8. However, at a pH below 6.0, more than 80% of the lantibiotic was present in the culture supernatant (25). Similar results were recorded for pediocin AcH, *i.e.* maximum adsorption to the producer cells at pH 6.0-5.5 and an almost complete release at pH 1.5 (26). In the case of pediocin AcH, pre-pediocin modifying enzymes are activated at a pH below 5.0, which converts the inactive pre-pediocin AcH into its active form (27).

To determine if pre-plantaricin enzymes are involved in the activation of plantaricin 423, fermentation studies were conducted in MRS broth (Merck) at different initial pH values. The results are shown in Table 4. The best growth was obtained with an initial pH of 5.8 (OD_{max} = 1.52), followed by pH 6.2, 6.7, 5.4, 6.9, 7.1 and 4.9. The changes in pH that were recorded during growth (ΔpH) coincided with the OD_{max} - values recorded for each of the fermentations, *i.e.* the culture with the highest cell density produced more acid, which in turn resulted in a lower end pH (Table 4). The lowest final pH (pH 4.4) was obtained for cells which started at an initial growth pH of 5.8 (Table 4). The highest activity of plantaricin 423 was obtained in a medium with an initial pH of 5.8 (3600 AU/ml) and the lowest activity at pH 6.9 (300 AU/ml). According to these results, plantaricin 423 is maximally produced at the optimum growth of the strain, suggesting that primary metabolite kinetics is followed, similar to what has been reported for nisin (28). However, when the number of cells are taken into account (OD_{max} - value), a higher concentration of plantaricin 423 is produced in medium with an initial pH of 4.90 (2 961 AU/ml/OD_{max}) than at pH 5.80 (2 368 AU/ml/OD_{max}), as shown in Table 4. This phenomenon is further accentuated by the fact that the pH decreased with only 0.05 unit from 4.90 to 4.85, compared to the more drastic decrease in pH (1.40 units) from 5.80 to 4.40 (Table 4). Concluded from these results and that obtained in Fig. 1, a pre-plantaricin might be

involved in the activation of plantaricin 423 between pH 4.90 and 4.85. Increased bacteriocin activity at pH values lower than the optimal growth pH of the producer cells has been reported for other bacteriocins, *viz.* plantaricin S produced by *L. plantarum* LPCO10 (14), lacticin 481 produced by *L. lactis* subsp. *lactis* (29), leuconocin Lcm1 produced by *Leuconostoc carnosum* and sakacin A produced by *Lactobacillus sake* (30).

The effect of tryptone, bacteriological peptone, yeast extract, meat extract and corn steep liquor on the production of plantaricin 423 is shown in Fig. 2A and B. The best growth was obtained in MRS supplemented with corn steep liquor ($OD_{m.ax}$ = 2.3; Fig. 2A), but this fermentation yielded the lowest activity of plantaricin 423 (6400 AU/ml; Fig. 2B). The most active plantaricin 423 production was obtained in MRS broth (Merck) supplemented with bacteriological peptone (19,200 AU/ml), followed by casaminoacids and tryptone (both 12 800 AU/ml), and meat extract (9600 AU/ml), as shown in Fig. 2B. Corn steep liquor, bacteriological peptone and casaminoacids supported the optimum production of plantaricin 423 for a period of 6 h, which is much longer than what has been obtained with meat extract, tryptone and yeast extract, respectively (Fig. 2B). Although cells grown in the presence of meat extract, tryptone and yeast extract did not produce the highest levels (AU/ml) plantaricin 423 and the activity levels of the bacteriocin decreased after a few hours, production was maintained over the remaining time of fermentation (Fig. 2B).

The effect of $MnSO_4 \cdot H_2O$ on the growth of strain 423 and the specific activity of plantaricin 423 (AU/ml/OD_{max}) is shown in Fig. 3. Tryptone was selected as nitrogen source, since it is one of the compounds which supported the stable production of plantaricin 423, especially during the latter half (12 h) of fermentation (Fig. 2B). A concentration of 0.004% $MnSO_4 \cdot H_2O$ yielded a specific plantaricin 423 activity of approximately 36,000 AU/ml/OD_{max} (OD=1.32) after 15.5 h of fermentation, whereas a level of 0.014% $MnSO_4 \cdot H_2O$ produced approximately 48,000 AU/ml/OD_{max} plantaricin 423 (OD = 1.34) during the same period (Fig. 3). Higher concentrations of $MnSO_4 \cdot H_2O$ did not result in a further increase in the specific activity of plantaricin 423 after 15.5 h of fermentation (Fig. 3), despite the increase in cell density (OD=1.38 and 1.43 at 0.024 and 0.044% $MnSO_4 \cdot H_2O$, respectively). At each of the $MnSO_4 \cdot H_2O$ concentrations tested, lower specific activity values were recorded after 19 h of fermentation, despite better growth of the culture, as reflected in the higher optical density values recorded (OD=1.44, 1.56, 1.59 and 1.60 at 0.004, 0.014, 0.024 and 0.044% $MnSO_4 \cdot H_2O$, rspectively). The specific activity values of plantaricin 423 recorded in this study were determined at two specific time intervals. The production of plantaricin 423 is higher

during the first half (approx. 12 h) of fermentation (Fig. 2B), which may lead to higher specific activity readings. The addition of $MgSO_4 \cdot 7H_2O$ (0.041%, w/v) resulted in a growth increase of strain 423 which was similar to that recorded with $MnSO_4 \cdot H_2O$, but it did not increase the production of plantaricin 423 (data not shown). The role which manganese plays in the stimulation of bacteriocin production is not yet clear and has to be resolved.

The specific activity of plantaricin 423 could even be increased further (up to 63,200 $AU/ml/OD_{max}$) with the addition of 1% (w/v) Tween 80 to MRS broth (Fig. 4). A slightly lower specific activity value (58 400 $AU/ml/OD_{max}$) was recorded in the presence of 2% (w/v) Tween 80 (Fig. 4), despite the increase in cell density as reflected in the OD readings of 1.38 (in the presence of 2%, w/v, Tween 80) versus 1.28 (in the presence of 1%, w/v, Tween 80). In all the experiments, except with 0.5% (w/v) Tween 80, lower activities were recorded after 19 h of fermentation, despite the recorded increase in cell density readings (not shown). Based on the results obtained in the present study, a higher specific concentration of plantaricin 423 is produced after 15.5 h of fermentation than after 19 h. The production of plantaricin 423 is, however, higher during the first half (approx. 12 h) of fermentation (Fig. 2B), which may lead to higher specific activity readings. Equivalent concentrations of Tween 80, dissolved in sterile distilled water, had no inhibitory effect on the growth of *L. monocytogenes*. Stimulation of bacteriocin production by Tween 80 has been reported for pediocin AcH (31) and lactocin 705 (32).

Results obtained in this study have clearly shown that the amount of plantaricin 423 produced (measured as activity units) is highly dependent on the composition of the growth medium. We have also shown that certain growth stimulating medium compounds do not increase the production of plantaricin 423. Further studies will have to be conducted to determine the effect of other ions on the production of plantaricin 423.

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TABLE 1. Bacteriocins produced by *Lactobacillus plantarum*

Bacteriocin	Strain	Reference
lactolin	A2	8
plantaricin SIK 83	SIK 83	9
plantacin B	NDCO 1193	11
plantaricin A	C-11	12
plantaricin S	LPCO-10	14
plantaricin T	LPCO-10	14
plantacin BN	BN	13
plantaricin 406	MI406	33
plantaricin SA6	SA6	34
plantaricin C 19	C 19	21
plantaricin 149	NRIC149	22
plantaricin C	LL441	35
plantaricin F	BF001	36
plantaricin LC74	LC74	37

TABLE 2. Spectrum of antimicrobial activity of plantaricin 423

Organism	Strain	Result[a]
Bacillus cereus	LMG 13569	+
Clostridium sporogenes	LMG 13570	+
Enterococcus faecalis	LMG 13566	+
Lactobacillus brevis	ATCC 14869	+
Lactobacillus buchnerii	ATCC 4005^T	+
Lactobacillus bulgaricus	ATCC 11482^T	+
Lactobacillus casei	ATCC 334	-
Lactobacillus curvatus	LMG 13553	+
Lactobacillus plantarum	ATCC 14917	+
Lactobacillus sake	LMG 13558	+
Leuconostoc mesenteroides subsp. *cremoris*	LMG 13562	-
Listeria innocua	LMG 13568	+
Listeria monocytogenes	LM1	+
Oenococcus oeni (previously *Leuconostoc oenos*)	ML 34	+
Pediococcus acidilactici	ATCC 12697	+
Pediococcus pentosaceus	LMG 13560	+
	LMG 13561	-
Staphylococcus carnosum	LMG 13567	+
Streptococcus thermophilus	LMG 13564	-
	LMG 13565	+

[a] +, sensitive to plantaricin 423; -, resistant to plantaricin 423.

TABLE 3. The effect of heat, pH and proteolytic enzymes on the activity of plantaricin 423[a]

Treatment	Activity[b]
Heat:	
40°C, 60°C, 80°C and 100°C for 30 min	+
40°C, 60°C, 80°C for 60 min	+
100°C for 60 min	(+)
121°C for 15 min	(+)
pH:	
pH 1-10	+
Proteolytic enzymes:	
Proteinase K	-
Pepsin	-
Papain	-
α-Chymotrypsin	-
Trypsin	-

[a] The tests were done as described by Green *et al.* (19).

[b] *L. monocytogenes* LM1 was used as indicator organism. +, Active; (+), slightly active.

TABLE 4. Effect of pH on the growth of strain 423 and the production of plantaricin 423[a]

Initial pH	7.10	6.90	6.70	6.20	5.80	5.40	4.90
Final pH	6.55	6.30	6.00	5.10	4.40	4.80	4.85
Δ pH	0.55	0.60	0.70	1.10	1.40	0.60	0.05
OD_{max}	0.84	1.16	1.35	1.40	1.52	1.28	0.38
Maximum bacteriocin production (AU/ml)	500	300	900	2700	3600	3375	1125
Specific bacteriocin production (AU/ml/OD_{max})	595	259	667	1929	2368	2220	2961

[a] The values represent the average of two experiments.

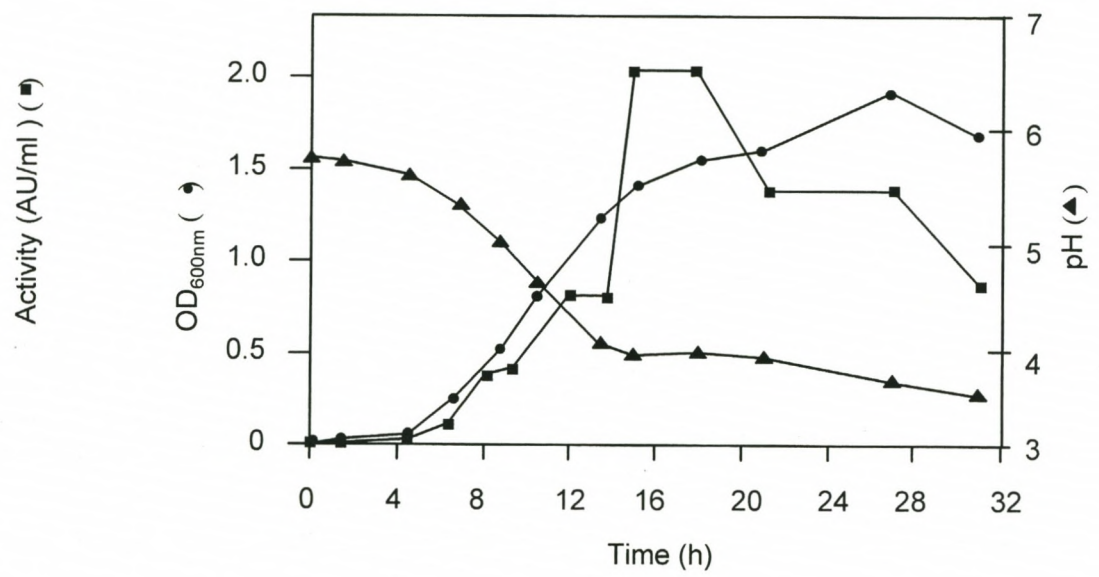

FIG. 1. Production of plantaricin 423 during growth of *L. plantarum* 423. Symbols: ●, optical density at 600nm; ■, plantaricin 423 activity in AU/ml; ▲, changes in pH.

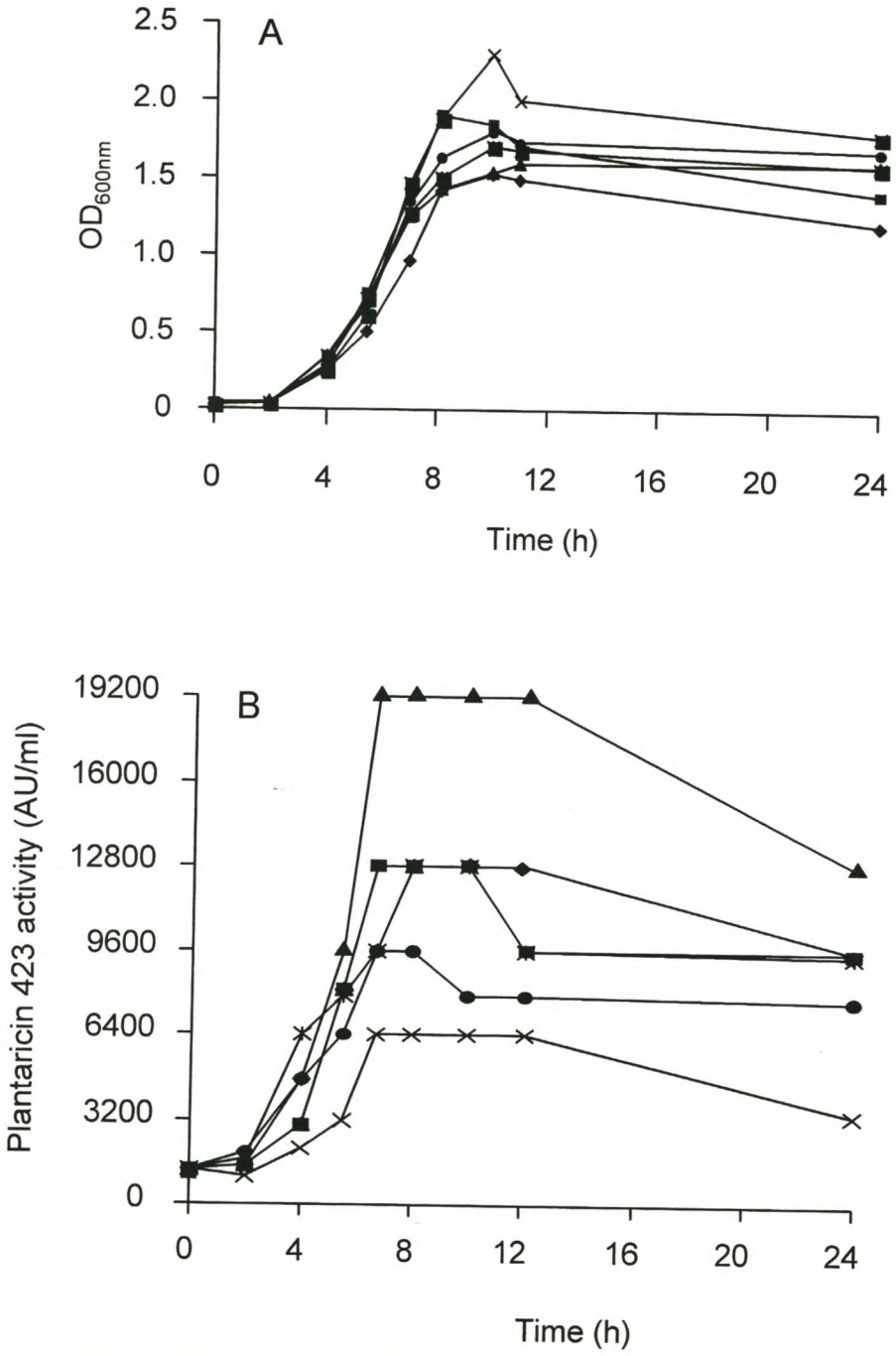

FIG. 2. (A) The effect of corn steep liquor, meat extract, tryptone, yeast extract, bacteriological peptone and casaminoacids on the growth of *L. plantarum* 423. (B) The effect of corn steep liquor, meat extract, tryptone, yeast extract, bacteriological peptone and casaminoacidson the production of plantaricin 423. Symbols: (A, B) x, corn steep liquor; ●, meat extract; ∗, tryptone; ■, yeast extract; ▲, bacteriological peptone; ◆, casaminoacids.

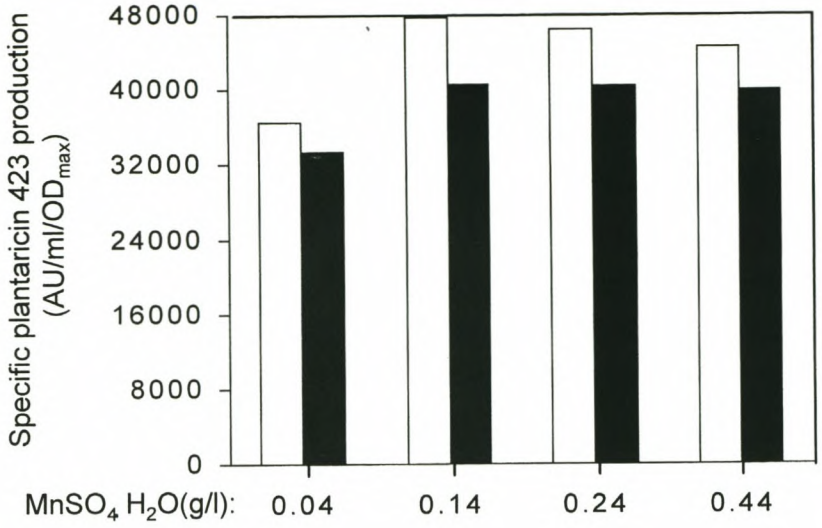

FIG. 3. The effect of $MnSO_4.H_2O$ on the production of plantaricin 423 (AU/ml/OD_{max}). Symbols: □, Activity at 15.5 h; ■, activity at 19 h.

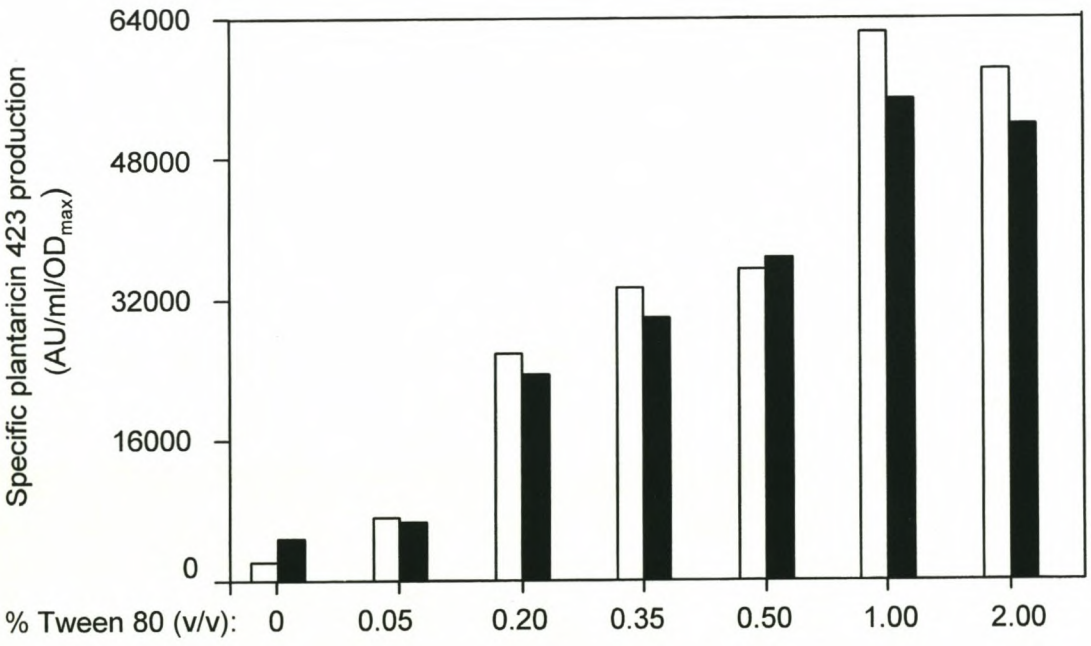

FIG. 4. The effect of Tween 80 on the production of plantaricin 423 (AU/ml/OD_{max}). Symbols: □, Activity at 15.5 h; ■, activity at 19 h.