Strategies for the Control of Malolactic Fermentation: Characterisation of Pediocin PD-1 and the Gene for the Malolactic Enzyme from *Pediococcus damnosus* NCFB 1832

by

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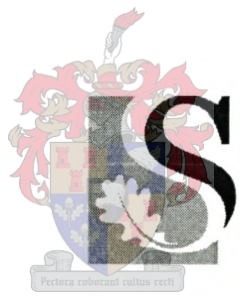

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Prof. L.M.T. Dicks

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

Malolactic fermentation (MLF) is conducted by lactic acid bacteria (LAB) and entails the decarboxylation of L-malate to L-lactate through a reaction catalysed by the malolactic enzyme (MLE). The consequence of this conversion is a decrease in total acidity. MLF plays a part in microbial stabilisation and due to the metabolic activity of the bacteria the organoleptic profile of the wine is modified. In some wines MLF is considered as spoilage, especially in warm viticultural regions with grapes containing less malic acid. In addition to undesirable organoleptic changes, MLF can alter wine colour, and biogenic amines may be produced. To induce MLF we provided *S. cerevisiae* with the enzymatic activities required for MLF, which is then conducted by the yeast during alcoholic fermentation. The malolactic enzyme-encoding gene (*mle*D) was cloned from *Pediococcus damnosus* NCFB 1832, characterised and expressed in *S. cerevisiae*. The activity of this enzyme was compared to two other malolactic genes, *mle*S from *Lactococcus lactis* MG1363 and *mle*A from *Oenococcus oeni* Lal1, expressed in the same yeast strain. All three recombinant strains of *S. cerevisiae* converted L-malate to L-lactate in synthetic grape must, reaching L-malate concentrations of below 0.3 g/L within 3 days. However, a lower conversion rate and a significant lower final L-lactate level were observed with the yeast expressing *mle*D. In order to inhibit MLF, we show that the growth of *O. oeni*, the main organism responsible for MLF, could be safely repressed with a ribosomaly synthesised antimicrobial peptide, pediocin PD-1, produced by *P. damnosus* NCFB 1832, without effecting yeast growth. Pediocin PD-1 is stable in wine at 4°C–100°C, and ethanol or SO_2 does not affect its activity. The peptide was purified to homogeneity and sequence analysis suggests that the peptide is a member of the lantibiotic family of bacteriocins. The molecular mass was estimated by mass spectroscopy to be 2866.7 ± 0.4 Da. Pediocin PD-1 forms pores in sensitive cells, as indicated by the efflux of K^+ from *O. oeni*, combined with inhibition of cell wall biosynthesis, leading to cell lysis. Loss of cell K^+ was reduced at low temperatures, presumably as a result of the increased ordering of the lipid hydrocarbon chains in the cytoplasmic membrane. Although pediocin PD-1 is active over a broad pH range, optimal activity was recorded at pH 5.0. The petide is, however, more stable between pH 2.0 and 5.0, with the best stability observed between pH 3.0 and 4.0. Pediocin PD-1 provides a safer biological alternative than chemical preservatives such as SO_2.

OPSOMMING

Appelmelksuurgisting (AMG) word deur sekere melksuurbakterieë (MSB) uitgevoer en verwys na die dekarboksilering van L-malaat na L-laktaat, 'n reaksie gekataliseer deur die appelmelksuurensiem (AME). AMG verlaag die suurvlakke in wyn, speel 'n rol in mikrobiologiese stabiliteit, en verander die organoleptiese profiel van die wyn. In sommige wyne word AMG beskou as bederf, veral in warm wynbou streke met minder malaat in druiwe. AMG kan ongewenste organoleptiese veranderinge teweeg bring, die wyn se kleur beinvloed, en tot die produksie van biogene amiene lei. Vir die bevordering van AMG het ons *S. cerevisiae* met die ensiematiese aktiwiteit benodig vir AMG voorsien wat dan veilig deur die gis tydens alkoholiese fermentasie uitgevoer word. 'n AME-koderende geen (*mle*D) is uit *Pediococcus damnosus* NCFB 1832 gekloneer, gekarakteriseer en in *S. Cerevisiae* uitgedruk. Die aktiwiteit van die ensiem is vervolgens vergelyk met die aktiwitet van twee ander AME gene, *mle*S van *Lactococcus lactis* MG1363 en *mle*A van *Oenococcus oeni* Lal1, uitgedruk in dieselfde gisras. Al drie rekombinante gisrasse het L-malaat binne die bestek van drie dae na L-laktaat omgeskakel en die finale L-malaat vlakke was minder as 0.3 g/L. Die tempo van omkakeling was egter laer in die gis wat die *mle*D geen uitdruk en die finale L-laktaat vlakke was veel laer. Om AMG te inhibeer is die groei van *O. oeni*, die organisme hoofsaaklik verantwoordelik vir AMG, onderdruk deur die byvoeging van 'n ribosomaal gesintetiseerde antimikrobiese peptied, pediocin PD-1, geproduseer deur *P. damnosus* NCFB 1832. Gisgroei is nie geaffekteer nie. Pediocin PD-1 is stabiel in wyn by temperature wat wissel tussen 4°C en 100°C, en die aktiwiteit van die peptied word nie geaffekteer deur ethanol of SO_2 nie. Die peptied is gesuiwer volgens 'n eenvoudige metode wat amoniumsulfaat-presipitasie en katioon uitruilings-chromatografie insluit. Aminosuur volgorde bepaling van gesuiwerde peptied dui daarop dat pediocin PD-1 tot die lantibiotiese familie van bakteriosiene behoort. Die molekulêre massa van die peptied, soos bepaal deur massa spektroskopie, is 2866.7 ± 0.4 Da. Pediocin PD-1 vorm porieë in selmembrane van sensitiewe selle soos aangedui deur die uitvloei van K^+ vanuit *O. oeni* selle. Die peptied kombineer hierdie aksie met die inhibisie van selwand biosintese wat lei tot sel lise. Verlies van sellulêre K^+ verminder by laer temperature, waarskynlik as gevolg van verandering in die lipied- en protein inhoud van die sitoplasmiese membraan. Alhoewel die peptied aktief is oor 'n breë pH grens, is die antimikrobiese aksie optimaal by pH 5.0. Die peptied is meer stabiel tussen pH 2.0 en 5.0 en toon die beste stabiliteit tussen pH 3.0 en 4.0. Peiocin PD-1 is 'n veilige biologiese alternatief vir chemiese preserveermiddels soos SO_2.

BIOGRAPHICAL SKETCH

Rolene Bauer (née Kritzinger) was born in George, South Africa, on 24 August 1973. She obtained a BScAgric degree, majoring in Microbiology, Biochemistry and Genetics, at Stellenbosch University. All postgraduate studies were conducted in the Department of Microbiology, Stellenbosch University, with Prof. Leon M.T. Dicks as supervisor. During this period she was employed as a part-time technical assistant and laboratory manager. She completed a degree course in Oenology at Stellenbosch University and is a qualified wine taster. Rolene is married to Prof. Florian F. Bauer, Institute of Wine Biotechnology, Department of Viticulture and Oenology, University of Stellenbosch. They have one son and reside in Stellenbosch.

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Prof. L.M.T. Dicks, as my supervisor for his guidance and support

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PREFACE

This dissertation is presented as a compilation of manuscripts and each chapter is introduced separately. Chapters 2 and 5 are literature reviews and are written according to the style of "American Journal of Enology and Viticulture" and "International Journal of Food Microbiology" respectively. Chapters 3, 4 and 6 were either published or submitted for publication in international peer reviewed journals, and are written according to the style of the respective journal. The Appendix is added for additional information pertaining to this research.

Chapter 2

Bauer, R., and L.M.T. Dicks. Control of malolactic fermentation in wine: A review.

Chapter 3

Bauer R, H. Volschenk and L.M.T. Dicks. 2003. Cloning and expression of the malolactic gene of *Pediococcus damnosus* NCFB 1832 in *Saccharomyces cerevisiae.* Submitted for publication: American Journal of Enology and Viticulture.

Chapter 4

Bauer, R., H. A. Nel, and L.M.T. Dicks. 2003. Pediocin PD-1 as a method to control growth of *Oenococcus oeni* in wine. American Journal of Enology and Viticulture 54:86-91.

Chapter 5

Bauer, R., and L.M.T. Dicks. Mode of action of lipid II mediated lantibiotics: A review.

Chapter 6

Bauer, R., M.L. Chikindas, and L.M.T. Dicks. 2003. Purification, partial amino acid sequence and mode of action of pediocin PD-1, a bacteriocin produced by *Pediococcus damnosus* NCFB 1832. Submitted for publication: Applied and Environmental Microbiology.

Appendix I

Nel, H.A., R. Bauer, E.J. Vandamme, and L.M.T. Dicks. 2001. Growth optimization of *Pediococcus damnosus* NCFB 1832 and the influence of pH and nutrients on the production of pediocin PD-1. Journal of Applied Microbiology 91:1131-1138.

Appendix II

Nel, H.A., R. Bauer, G.M. Wolfaardt, and L.M.T. Dicks. 2002. The effect of bacteriocins pediocin PD-1, plantaricin 423 and nisin on biofilms of *Oenococcus oeni* on a stainless steel surface. American Journal of Enology and Viticulture 53:191-196. **Best Paper Award**.

CONTENTS

CHAPTER 1

General introduction and project aims

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Winemaking normally involves two fermentation processes, an alcoholic fermentation conducted by *Saccharomyces cerevisiae*, followed by a secondary malolactic fermentation (MLF). MLF involves the decarboxylation of L-malic acid to L-lactic acid (Henick-Kling, 1995). The removal of L-malic acid, one of the main organic acids of grape must, is essential for the deacidification and stabilisation of wine produced in cool regions. L-malate is metabolised by LAB by one of three enzymatic pathways (Radler, 1986). The LAB identified in wine contains a malolactic enzyme (MLE), which decarboxylates L-malate directly to L-lactate without free intermediates. The malic enzyme, on the other hand, converts L-malate to pyruvate, which is then in part reduced to L-lactic acid. The third pathway has been described for *L. fermentum*, where L-malate is reduced by malate dehydrogenase to oxaloacetate, followed by decarboxylation to pyruvate.

The strains of LAB isolated from wine belong to the genera *Lactobacillus, Leuconostoc, Oenococcus* and *Pediococcus*, with *Oenococcus oeni* (previously *Leuconostoc oenos*, Dicks et al. 1995) being the most dominant due to its acidophilic nature and resistance to the alcohol and SO_2 levels in wine (Van Vuuren and Dicks, 1993; Lonvaud-Funel, 1999). Spontaneous MLF is in most cases driven by *O. oeni,* which is also the preferred organism to conduct the secondary fermentation. MLF plays an important role in determining the final quality of most red and some white wines (determined strictly by the wine style) and classic sparkling wines. The direct consequence of the conversion is a decrease in total acidity, but MLF also plays a part in microbial stabilisation, while the metabolic activity of the bacteria modifies the organoleptic profile of the wine. Spontaneous MLF is unpredictable, since it may occur any time during, or many months after, the completion of alcoholic fermentation, and the bacteria may become infected by phage during such extended fermentation (Henick-Kling, 1995). Even with the use of bacterial starter cultures, stuck or sluggish MLF often causes delays in cellar operations. Furthermore, reduced sulphite levels in such wine may lead to proliferation of spoilage organisms, producing off-odours and biogenic amines (Lonvaud-Funel, 1999). MLF may also result in undesirable changes in the wine sensory properties and it can alter wine colour.

Wild type strains of *S. cerevisiae* metabolise insignificant amounts of L-malate during alcoholic fermentation due to the absence of an active transport system for malate (Van Vuuren et al., 1995) and the low substrate affinity of its malic enzyme (Fuck et al., 1973). On the other hand, efficient malo-ethanolic fermentation by *Schizosaccharomyces pombe* is accomplished under anaerobic conditions (Osothsilp et al., 1986) through the constitutive synthesis of malate permease, encoded by the

mae1 gene (Grobler et al., 1995), and the malic enzyme, encoded by the *mae2* gene (Viljoen et al., 1994). Strains of *S. pombe* are however, unsuitable for vinification due to the production of off-flavours and the high temperature required for fermentation. The ability of engineered *S. cerevisiae* strains that degrade malate efficiently during alcoholic fermentation should prevent problems experienced with bacterial strains. Volschenk et al. (2001) constructed a *S. cerevisiae* strain with the *S. pombe mae1* and *mae2* genes integrated in the genome, which efficiently degraded L-malate. The ability of engineered yeast strains to perform both alcoholic and malolactic fermentation to achieve better control over MLF has also been studied (Denayrolles et al., 1995; Ansanay et al., 1996; Bony et al., 1997; Volschenk et al., 1997a and b). The malolactic *S. cerevisisae* strain constructed by Volschenk et al., (1997a), co-expressing the *mae1* gene and the *Lactococcus lactis* malolactic gene (*mle*S), showed rapid growth at very low pH values, where even the acid tolerant *Oenococcus oeni* is unable to grow (Kunkee, 1997). Whether this new yeast can replace MLF in all cases is doubtful, but it opened a field of research with exiting future prospects.

Malolactic fermentation is discouraged and can be considered a spoilage factor in certain wines (Kunkee, 1997). In warm regions, white grapes may contain too little malic acid to begin with. It is also discouraged in aromatic wines, such as Sauvignon blanc, Riesling or Gewürztraminer, where varietal aromas may be changed or masked. Although MLF is occasionally difficult to induce, prevention of the development of lactic acid bacteria (LAB) is likewise difficult. Several methods have been implemented with varying degrees of success. Fumaric acid inhibits malolactic fermentation but is metabolised by yeast and lactic acid bacteria, rendering it unstable (Ough and Kunkee, 1974). Dimethyldicarbonate (DMDC) is lethal against yeast and bacteria, and can be used to sterilise wine (Terrell et al., 1993). Since DMDC is hydrolysed to CO_2 and methanol, low concentrations may be left to protect the bottled wine. The winemaking process relies on the use of SO_2 to inhibit microbial growth. However, its use is strictly regulated due to associated health risks and organoleptic changes. Mounting consumer demands for safe alternatives to chemical preservatives has led to an increased interest in different solutions. For this reason, natural antimicrobial compounds from plants (e.g., phenolics), animals (e.g., enzymes such as lysozyme) and micro-organisms (e.g., bacteriocins) have been the focus of research in recent years (Abee et al., 1995). Of these, lysozyme and bacteriocins appear to be the most promising candidates for wine preservation.

The bacteriolytic enzyme lysozyme is active against most Gram-positive bacteria (Gould et al., 1996). Lysozyme has no effect on yeast, is not affected by alcohol and remains active in the pH range of the winemaking process (Fugelsang, 1997). The Office International de la Vigne et du Vin (OIV) has recently approved the addition of lysozyme to the winemaking process, but the high cost of using lysozyme is still a limiting factor. Bacteriocins, ribosomal synthesised antimicrobial peptides or proteins, are odourless, colourless, and non-toxic (Hansen, 1994). They have been

grouped into three classes based on their structure (Klaenhammer, 1993). The lantibiotics (class I) are small heat-resistant peptides that undergo post-translational modifications, which leads to the formation of rings through reaction between dehydrated serine and threonine residues with the sulfhydryl group of cysteine (Jung et al., 1991). The lantibiotics are further divided into two sub-groups, namely the elongated and amphipathic pore-forming type A peptides (e.g. nisin), and the globular peptides of the type B category, e.g. mersacidin and actagardine (Brötz et al., 1998a and b; McAucliffe et al., 2001). Class II consists of small (<15 kDa), heat-stable, membrane-active, unmodified peptides (Sablon et al., 2000). Included in this class are the pediocin-like peptides, named after pediocin PA-1 produced by *Pediococcus acidilactici*. All pediocin-like bacteriocins have a consensus amino acid sequence motif in the N-terminal part of the mature peptide. Class III is represented by heat-labile proteins with sizes in excess of 15 kDa.

Contrary to lysozyme and antibiotics, bacteriocins are very specific and only affect a small group of microorganisms. The mode of action of bacteriocins is not yet fully understood. It is generally assumed that the type A subgroup of lantibiotics and the Class II bacteriocins kill microbes by disturbing the integrity of the cell membrane which leads to rapid efflux of ions or cytoplasmic solutes (Sablon et al., 2000). Models for nisin-membrane interactions suggest that the peptides form wedge-like poration complexes in the membrane (Breukink and de Kruijff, 1999). The positively charged C terminus of nisin bind via electrostatic interactions with the anionic lipids, provided the cell membrane contains at least 50% negatively charged phospholipids (Wiedemann et al., 2001). Nisin can also permeabilise membranes via a targeted mechanism by using lipid II, the bactoprenol-bound precursor of the bacterial cell wall, as a docking station (Brötz et al., 1998b). Type-B lantibiotics exert their activity by binding to specific membrane lipids and through interfering with enzyme activities by blocking the respective substrate (Brötz et al., 1998b; Sahl and Bierbaum, 1998). Within this group, the mersacidine subtype was also shown to form a complex with lipid II, but binding only blocks the incorporation of lipid II into peptidoglycan, resulting in slow cell lysis rather than pore formation (Breukink et al., 1999). The mechanism of action of the class III bacteriocins remains unclear (Klaenhammer, 1993).

Bacteriocins are introduced into food by either direct addition of the peptide (usually in a purified form), adding of the culture supernatant (a crude extract of the peptide), or by using a bacteriocin-producing starter culture in fermented foods. To date, nisin is the only purified bacteriocin (lantibiotic) allowed in food (Van Kraaij et al., 1999). The effectiveness of nisin in preventing the growth of lactic acid bacteria in beer (Ogden, 1986) and wine (Radler, 1990 a and b) has been demonstrated. Another bacteriocin showing potential in preventing the growth of LAB, such as *O. oeni*, in wine is pediocin PD-1. This peptide was first described by Green et al. (1997) and is produced by *P. damnosus* NCFB 1832, a strain isolated from beer. We have determined the fermentation conditions required for optimal production of this peptide (Nel et al., 2001). It is active against a number of LAB, including malolactic

strains of *Lactobacillus, Leuconostoc* and *Oenococcus* spp. In one of our previous studies (Nel et al., 2002), when compared with nisin and plantaricin 423, we have shown that pediocin PD-1 is the most effective in removal of an established biofilm of *O. oeni* from stainless steel surfaces in Chardonnay must.

Bacteriocins have not yet been approved for use in the wine industry. Cost considerations will play a major role in the acceptance of peptide-based wine preservation strategies. Problems such as low production levels and instability of bacteriocins need to be addressed. It is furthermore important to understand the mode of action of the applied bacteriocin and to examine the antimicrobial effect of the peptide under a range of growth phases and conditions before drawing generalised conclusions about its efficiency. Considering the advantages of a biological system over classical chemical control methods, however, there is little doubt that once these systems have been developed sufficiently, they will impose themselves as healthier and more ecologically friendly alternatives.

1.2 PROJECT AIMS

1. The first aim of this study was to construct genetically engineered strains of *S. cerevisiae* that differ in their ability to degrade L-malate via the malolactic pathway. The specific approaches of this project were as follows:
   i) Cloning, sequencing and heterologous expression of the *mle* gene (*mle*D) from *Pediococcus damnosus* NCFB 1832 in a laboratory strain of *S. cerevisiae*.
   ii) Assessing the ability of the *mle*D gene product to convert L-malate into L-lactate when expressed in *S. cerevisiae*, and comparing the efficiency of the conversion with that of the *mle* gene products from *O. oeni* and *L. lactis* subsp. *lactis* expressed in the same yeast.
2. The second aim of this study was to determine if the antimicrobial peptide, pediocin PD-1, can be used to control the growth of *O. oeni* in wine. The specific approaches of this project were as follows:
   i) Determination of the levels of antimicrobial activity needed to effectively control the growth of *O. oeni* in must and wine.
   ii) Study of the stability of the peptide in must and wine.
   iii) Determination of the conditions required for the production of pediocin PD-1 by *P. damnosus* NCFB 1832 in must to assess whether pediocin PD-1 production in wine may be responsible for some of the antagonistic effects observed amongst LAB during vinification.
3. Lastly, we focused on the characteristics of pediocin PD-1. This study included the following:
   i) Establishment of a quick method for purification of the peptide.
   ii) Determination of the amino acid sequence of the mature peptide.

iii) Study the mode of action of pediocin PD-1.

iv) Influence of environmental parameters, such as pH and temperature, and the growth phase of *O. oeni* on the activity of the antimicrobial peptide.

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CHAPTER 2

LITERATURE REVIEW

Control of malolactic fermentation in wine

LITERATURE REVIEW

Control of malolactic fermentation in wine

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ABSTRACT

Malolactic fermentation (MLF) is conducted by lactic acid bacteria (LAB) and refers to the decarboxylation of L-malate to L-lactate. This secondary fermentation is difficult to control and is mainly driven by *Oenococcus oeni*. Uncontrolled MLF, especially in wines with a high pH, which is typical to warmer viticultural regions, may render the wine unpalatable or even cause spoilage. In this review we focus on wine compounds and stress factors that affect the growth of *O. oeni* and MLF, and discuss practical applications. We also explore alternative technologies that may enable better control over MLF.

2.1 INTRODUCTION

Winemaking normally involves two fermentation processes, i.e. an alcoholic fermentation conducted by yeast and malolactic fermentation (MLF) performed by lactic acid bacteria (LAB) containing a malolactic enzyme (MLE). MLF plays an important role in determining the final quality of most red wines, but also certain white wines and classic sparkling wines. Apart from an increase in pH, additional sugars are fermented, and aromatic compounds are produced which change the organoleptic profile of the wine. The cells gain energy from the uniport of monoanionic L-malate through the generation of a proton gradient across the cell membrane (Salema et al. 1996). Only strains of *Lactobacillus, Leuconostoc, Oenococcus* and *Pediococcus* resistant to low pH and high SO_2 and ethanol levels, survive in wine (Van Vuuren and Dicks 1993; Lonvaud-Funel 1999). *Pediococcus damnosus*, *Leuconostoc mesenteroides* and *Oenococcus oeni* predominate during alcoholic fermentation (Lonvaud-Funel 1999). However, towards the end of alcoholic fermentation spontaneous MLF is mainly driven by *O. oeni* (Van Vuuren and Dicks 1993), a species formerly known as *Leuconostoc oenos* (Dicks et al. 1995).

MLF is encouraged in cool viticultural regions where grapes may have high levels of malic acid, in wine aging in oak barrels, when long-time maturation in bottles are part of the process (e.g. Champagne), or when a specific organoleptic profile is required, as in Chardonnay, Burgundy white wines and Bordeaux red wines. In some

wines, MLF is considered spoilage, especially in warm viticultural regions with grapes containing less malic acid. In addition to undesirable organoleptic changes, the color of red wine may be reduced by as much as 30% (Van Vuuren and Dicks 1993), and biogenic amines may be produced (Lonvaud-Funel and Joyeux 1994).

Spontaneous MLF is unpredictable, since it may occur any time during or several months after the completion of alcoholic fermentation. The wine may also become infected by bacteriophages, especially during extended fermentation (Henick-Kling 1995). The use of starter cultures to induce MLF is often unsuccessful because of the rapid loss of cell viability after inoculation. Hence, studies on factors affecting the growth and survival of *O. oeni* in wine are important and methods to control MLF remain a priority.

2.2 MALOLACTIC FERMENTATION AND THE MALOLACTIC ENZYME

LAB are strictly fermentative and, with the exception of a few streptococci, lack electron transfer chains (Salema et al. 1996a). Therefore, generation of a proton motive force (PMF) can only be achieved by proton translocation via the membrane-bound F_0F_1 H^+-ATPase driven by the hydrolysis of ATP, or by some other chemiosmotic processes. Three chemiosmotic mechanisms for PMF generation have been described for LAB: (i) carrier mediated excretion of fermentation end products in symport with protons (Ten Brink et al. 1985), (ii) electrogenic precursor-product exchange (Poolman 1990) and (iii) electrogenic uniport (Salema et al. 1994) in combination with metabolic breakdown of the substrate in the cell. MLF (Salema et al. 1994) and citrate metabolism (Ramos et al. 1995a) are examples of the anion uniport mechanism in *O. oeni*. MLF is a PMF-generating process conducted by some LAB and, as a consequence, metabolic energy is conserved (Cox and Henick-Kling 1989 and 1990). The metabolic pathway is based on the electrogenic uptake of L-malate, its intracellular conversion to L-lactate plus CO_2, and the excretion of the end products (Salema et al. 1994). The mechanism of metabolic energy generation by MLF in *O. oeni* was inferred from transport studies with membrane vesicles (Salema et al. 1994). Monoprotonated L-malate (L-malate$^-$) is taken up by electrogenic uniport with a net negative charge being moved inwards, thereby creating an electrical potential, $\Delta\psi$ (inside negative relative to outside). Once inside the cell, L-malate is decarboxylated to L-lactate and carbon dioxide in a reaction that requires one proton. This alkalization of the cytoplasm results in the creation of a pH gradient (ΔpH) that, together with the $\Delta\psi$, forms the proton motive force (expressed in Δp) across the cytoplasmic membrane. The PMF generated under such conditions is sufficient to drive ATP synthesis via the membrane-bound F_0F_1 ATPases (Olsen et al. 1991; Poolman et al. 1991). L-lactate and CO_2 appear to leave the cell as neutral species (Salema et al. 1994). The latter mechanism of PMF generation was confirmed by *in vitro* reconstitution of the MLF pathway of *O. oeni* (Salema et al. 1996b).

Decarboxylation of L-malate to L-lactate is catalyzed by the malolactic enzyme (MLE) with the requirement of NAD^+ and Mn^{2+}, and does not generate intermediate nor cofactor reduction, which is different from the malic enzyme leading to pyruvate. MLE, the only enzyme involved in MLF, has been purified from several LAB (Caspritz and Radler 1983; Lonvaud-Funel and Strasser de Saad 1982; Naouri et al. 1990; Spettoli et al. 1984). The active form is composed of two or four identical subunits of 60-70 kDa and the protein is strongly homologous to malic enzymes from different organisms. Malic and malolactic enzymes are, however, distinct at the phylogenetic level, except for malic enzymes of yeast and *E. coli*, which are closer to malolactic enzymes than other malic enzymes (Groisilliers and Lonvaud-Funel 1999). In the presence of NAD and Mn^{2+}, the activity of MLE is similar to the malic enzyme combined with lactate dehydrogenase, but without the release of intermediate products. The complete nucleic acid sequence of the *mle* gene has been determined for *Lactococcus lactis* (Denayrolles et al. 1994), *O. oeni* (Labarre et al. 1996) and *P. damnosus* (Bauer et al. 2003a).

2.3 INFLUENCE OF PHYSICAL AND CHEMICAL FACTORS ON MLF

2.3.1 TEMPERATURE

Temperature affects the growth rate and length of the lag phase of LAB. The optimal growth rate of strains of *O. oeni* is close to 25°C (Henick-Kling 1993). Survival of *O. oeni* in wine and its ability to perform MLF was, however, improved by pre-incubation at 42°C (Guzzo et al. 1994). The latter temperature induces synthesis of stress proteins in *O. oeni* (Guzzo et al. 1997). Many of these proteins may function as molecular chaperones or proteases that participate in the refolding of proteins or the degradation of denatured cellular proteins (Craig et al. 1993). At low growth temperatures, *O. oeni* became more resistant to pore forming antimicrobial peptides, such as pediocin PD-1, (Bauer et al. 2004c).

Tourdot-Maréchal et al. (2000) compared the kinetics of membrane fluidity variation of instantaneously stressed *O. oeni* cells with cells adapted to the stress factor by a pre-incubation in inhibitory growth conditions. Membrane fluidity of heat adapted cells increased only slightly when exposed to 42°C and the rate of membrane fluidisation was five-fold lower than with non-adapted cells. To maintain optimal fluidity under various growth conditions, cells regulate the lipid composition in their cell membranes (Lehninger et al. 1993). An increase in growth temperature induces an increase in the incorporation of saturated fatty acids, while unsaturated fatty acids decrease. A decrease in temperature has the opposite effect. The higher the proportion of saturated fatty acids, the higher the solid-to-fluid transition temperature of the cell membrane. According to Tourdot-Maréchal et al. (2000), the ability of *O. oeni* to regulate its membrane fluidity, as described here, represents a stress tolerance mechanism. The decrease in pediocin PD-1-induced K^+ efflux

observed at lower temperatures (Bauer et al. 2003a) may thus be due to changes in the lipid and protein content in the cell membrane of *O. oeni*.

2.3.2 ETHANOL

Ethanol strongly interferes with the growth and metabolic activity of lactic acid bacteria. High ethanol concentrations decrease the optimal growth temperature of LAB and ethanol tolerance is decreased at elevated temperatures (Henick-Kling 1993). Although ethanol is not inhibitory towards malolactic activity (Capucho and San Romão 1994), the growth rate of *O. oeni* decreases linearly with increasing ethanol concentrations, with 14% (v/v) being the upper limit tolerated by most strains (Henick-Kling 1993). Growth is completely inhibited at 25°C and above in the presence of 10 to 14% (v/v) ethanol. Optimum growth (shortest lag time, fastest growth rate, and highest cell yield) at these alcohol concentrations occurs between 18 and 20°C compared to 30°C at 0 to 4% (v/v) ethanol (Henick-Kling 1993). Cell yield is less affected by ethanol and temperature than growth rate with maximum cell yield in media containing 0 to 8% (v/v) ethanol at approx. 22°C. The degree of ethanol tolerance is, however, strain dependant and also depends on the pH and nitrogen status of the culture medium (Britz and Tracey 1990).

The cell membrane is likely to be the primary site for the expression of an adaptive response to ethanol, with lipids being the main target (Jones 1989). Changes in the membrane lipid composition induced by ethanol have been described for *Bacillus subtilis* (Rigomier et al. 1980), *Escherichia coli* (Dombeck and Ingram 1984), *Lactobacillus hilgardii* (Couto et al. 1996), and *O. oeni* (Garbay et al. 1995; Tracey and Britz 1989a). The adaptive response to the presence of high concentrations of ethanol is aimed at maintaining the fluidity and integrity of the cell membrane (Couto et al. 1996). Ethanol-induced changes in the fatty acid profile of *Bacillus subtilis* cell membranes coincided with a decrease in membrane fluidity (Rigomier et al. 1980). The model proposed for *E. coli* (Dombeck and Ingram 1984) also predicts a decrease of membrane fluidity in cells grown in the presence of ethanol. On the other hand, the membrane fluidity of cells of *L. hilgardii* and *O. oeni* was increased in the presence of ethanol (Couto et al. 1996, Teixeira et al. 2002; Tourdot-Marcéchal et al. 2000).

Tourdot-Maréchal et al. (2000) showed that the rate of membrane fluidization after an ethanol shock was threefold lower with cells pre-incubated in ethanol than with non-adapted cells. The positive effect of adaptation was time-limited, since membrane fluidity was similar at the end of the treatment. Incubation in the presence of ethanol induced a rapid increase in membrane rigidity. Based on the hypothesis of 'homeoviscous adaptation' (Sinensky 1974) the production of a more fluid membrane is a compensation for the increase in rigidity generated by ethanol stress.

Teixeira et al. (2002) studied the lipid and protein composition of the membrane of *O. oeni* in the presence of different ethanol concentrations. The percentage of membrane lactobacillic acid increased at the expense of *cis*-vaccenic acid when cells

were grown in the presence of ethanol higher than 8% (v/v). Lactobacillic acid is a ring-containing fatty acid produced during late exponential to stationary phase growth and is formed by conversion of the unsaturated position of *cis*-vaccenic acid to a cyclopropane ring. Other than this, the membrane fatty acid profile was similar along the cell growth cycle for all the ethanol concentrations assayed. The increase of lactobacillic acid in the membrane of *O. oeni* appears to provide protection against the toxic effect of ethanol, balancing the increase of membrane fluidity normally attributed to ethanol. By cyclizing the unsaturated fatty acids, bacteria may stabilize their plasma membrane, particularly at stationary phase. This could explain why bacteriocin-induced cell lysis of *O. oeni* was least prominent in stationary phase cells (Bauer et al. 2003c).

Ethanol at concentrations up to 8% (v/v) induced an increase in membrane permeability in resting cells of *O. oeni*, but not in cells grown in the presence of 8% (v/v) ethanol (Teixeira et al, 2002). The total membrane protein content of cells grown in the presence of 8% (v/v) or higher ethanol decreased (Teixeira et al. 2002). However, the synthesis of low molecular weight stress proteins was induced and may be involved in cell adaptation (Guzzo et al. 1997; Guzzo et al. 2000; Teixeira et al. 2002; Tourdot-Maréchal et al. 2000). In conclusion, the development of ethanol resistance in *O. oeni* is a complex and multi-layered phenomenon, which depends on the severity and duration of the shock and on culture conditions such as medium composition, pH, and temperature.

2.3.3 PH

Wine pH plays an important role in determining which LAB species will survive and develop as well as the growth rate of the bacteria. In terms of initiation and completion of MLF, wines of pH 3.3 and above generally exhibit little problems, whereas at lower pH, difficulties may be experienced (Kunkee 1967). *O. oeni* usually represents the dominant species in wine below pH 3.5. At higher pH *Lactobacillus* and *Pediococcus* spp. may survive and grow. The pH strongly affects malolactic activity of the cell (Henick-Kling 1993). Although sugar utilization and growth of *O. oeni* are inhibited by low pH, malolactic activity is the highest at pH 3.5 to 4.0. Also, malate transport activity in *L. plantarum* is higher in cells grown at pH 3.5 compared to cells grown at pH 6.0 (Olsen et al. 1991).

Survival of *O oeni* in wine improved when cells were subjected to an acid shock before inoculation, presumably due to the synthesis of specific stress proteins (Guzzo et al. 1994, Guzzo et al. 1997 and 1998, Guzzo et al. 2000). However, physiological studies concerning acid tolerance have mainly been focused on MLF. The energy-yielding MLF pathway explains the physiological benefits of MLF, particularly under very acid conditions. The fermentation of L-malate generates both a transmembrane pH gradient and an electrical potential gradient. Proton consumption during the decarboxylation of L-malate participates in the regulation of intracellular pH, while the PMF generated by MLF is used for additional ATP synthesis (Henick-Kling 1995).

A mechanism that seems to be strictly linked to acid tolerance in LAB is ATP hydrolysis and proton extrusion by the membrane bound H^+-ATPases (Tourdot-Maréchal et al. 1999). Since bacteria extrude H^+ at acidic pH, this process plays an important role in PMF maintenance and pH homeostasis. In the case of anaerobic enterococci the only function of the membrane H^+-ATPase is to regulate the intracellular pH (pH_{in}) and maintain a ΔpH across the membrane (Shibata et al. 1992). When the pH_{in} was lowered below a certain threshold, the activity and synthesis of the H^+-ATPase increased. A study on the H^+-ATPase of *Enterococcus hirae* revealed a sub-unit composition identical to other bacterial F_0F_1 ATPases. Unfortunately, little is known about H^+ATPases and their role in pH homeostasis for other LAB. Drici-Cachon et al. (1996) have shown that the ATPase activity of an acidophilic *O. oeni* mutant significantly increases when grown at pH 2.6, which is usually lethal for the wild-type strain. The survival of LAB under acid conditions, therefore, depends on the activation of membrane-bound H^+-ATPase.

Tourdot-Maréchal et al. (1999) isolated *O. oeni* neomycin-resistant mutants as H^+-ATPase-deficient strains. The acid sensitivity of these mutants supported the hypothesis that the major role of H^+-ATPase is maintenance of intracellular pH. Surprisingly, all the mutants were devoid of malolactic activities. Since the growth rates of the mutant strains were also impaired when cultured under optimum conditions, acid sensitivity could not be the primary consequence of the lack of L-malate metabolism in energy production and intracellular pH homeostasis. The results suggested that the ATPase and malolactic activities of *O. oeni* are linked and play a crucial role in resistance to acid stress.

Another surprising observation was that no significant increase of ATPase activity was detected in wild-type *O. oeni* cells incubated at low pH. This absence of induction could be explained by the existence of several cation transport ATPase systems of which maximal activities depend on the pH of the media. Using inhibitors specific for different types of ATPases, Guzzo et al. (2000) demonstrated the existence of H^+-ATPase and K^+-translocating ATPase, which is also referred to as the P-type ATPase.

2.3.4 SULFUR DIOXIDE

It is common practice to add SO_2 (50 to 100 mg/L) to must at the beginning of the vinification process to restrict the growth of indigenous yeast and bacteria, mainly acetic acid bacteria (Fleat and Heard 1993). Some yeast strains also produce relatively large quantities of SO_2 (King and Beelman 1986). At low pH such as in wine (pH of 3 to 4), sulfite predominates as free SO_2 (Usseglio-Tomasset 1992), consisting mainly of bisulfate anion (HSO_3^{-1}) and a small proportion of molecular SO_2 ($SO_2 \cdot H_20$) and sulfite anion (SO_3^{-2}). Molecular SO_2, the only form of SO_2 that can cross cell walls of yeast and bacteria, enters the cell by diffusion and is converted to HSO_3^{-1}. In the cell, sulfite may react with proteins, nucleic acids and cofactors, affecting the growth of LAB (Carreté et al. 2002) and yeast (Constantí et. al., 1998).

The majority of *O. oeni* cells died within 3 hrs in the presence of 15 mg/L free sulfite (Guzzo et al. 1998). Levels of 5 mg/L free SO_2 resulted in complete MLF lasting longer than 40 days (Carreté et al. 2002). The F_0F_1 ATPase activity of *O. oeni* cells was more than 50% inhibited in the presence of 20 mg/L free SO_2 (Carreté et al. 2002). Malolactic activity is also influenced by SO_2 (Henick-Kling 1993). Bound SO_2 at 20 mg/L reduces L-malate degradation by 13%, 50 mg/L reduces it by 50%, and 100 mg/L inhibits malolactic activity completely.

A number of carbonyl compounds (mainly acetaldehyde, α-ketoglutaric acid and pyruvic acid) binds with free SO_2 (especially HSO_3^{-1}) to form a complex compound (bound SO_2) which has only weak antimicrobial properties. Bound SO_2 at 30 mg/L delays the growth of LAB whereas bound SO_2 at more than 50 mg/L may completely inhibit growth (Henick-Kling 1993). Furthermore, free SO_2 released upon microbial metabolism of bound acetaldehyde may cause microbial inhibition resulting in stuck or sluggish MLF (Osborne et al. 2000). Other SO_2-binding compounds, such as α-ketoglutaric acid and pyruvic acid are also substantially reduced during MLF and may therefore lead to similar results (Nielsen and Riechelieu 1999).

O. oeni developed a tolerance to sulfite as high as 30 mg/L and cells adapted to low pH survived better than non-adapted cells (Guzzo et al. 1998). Addition of a sub-lethal concentration of sulfite (15 mg/L) during the adaptation step in acidic medium (pH 3.5) increased sulfite tolerance. Higher concentrations of sulfite (60 mg/L) induced the synthesis of Lo18, a small heat shock protein. It appears, therefore, that several adaptation mechanisms, including pH homeostasis and stress protein synthesis, could be involved in the induction of sulfite resistance in *O. oeni*.

2.3.5 CARBOHYDRATES

The major residual sugars in wine after completion of alcoholic fermentation are glucose and fructose, which may vary from 10 g/L to less than 0.5 g/L, depending on the style of wine. Fructose is always found in higher concentrations than glucose. Although glucose is preferred by *O. oeni*, fructose is the most efficiently metabolized sugar, leading to maximum biomass levels during co-metabolism with glucose (Maicas et al. 1999a). Fructose is not only metabolized via the heterofermentative pathway, but is also reduced to mannitol by mannitol dehydrogenase (Figure 1). Sugars other than glucose and fructose may be present in wine at concentrations as high as 1.3 g/L (Henick-Kling 1995). The ability of these sugars to support growth of *O. oeni* is strain specific.

MLF is reduced by 50% in the presence of 2 mM glucose (Miranda et al 1997). At 5 mM or higher approx. 70% inhibition was observed. The activity of acetaldehyde dehydrogenase is very low compared to the activity of NAD(P)H-forming enzymes in the early steps of glucose metabolism (Veiga-da-Cunha et al. 1993). This prevents efficient NAD(P)H disposal during glycolysis, leading to a high intracellular concentration of NAD(P)H. Consequently, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are inhibited, which results in the accumulation

of glucose-6-phosphate and 6-phosphogluconate, respectively (Figure 1). Nuclear magnetic resonance (NMR) spectroscopic analysis revealed the accumulation of phosphorylated intermediates during glucose-malate co-metabolism (Miranda et al. 1997). The data showed that NADH, which is expected to accumulate during glucose catabolism as a result of inefficient NAD(P)H disposal, causes glucose-induced inhibition of malolactic activity. NADH at a concentration of 25 μM resulted in 50% inhibition of the malolactic enzyme purified from *O. oeni*, whereas NADPH had no inhibitory effect. Although slightly lower than glucose, galactose, trehalose, maltose, and mannose inhibited the malolactic activity in whole cells in a manner similar to that observed for glucose.

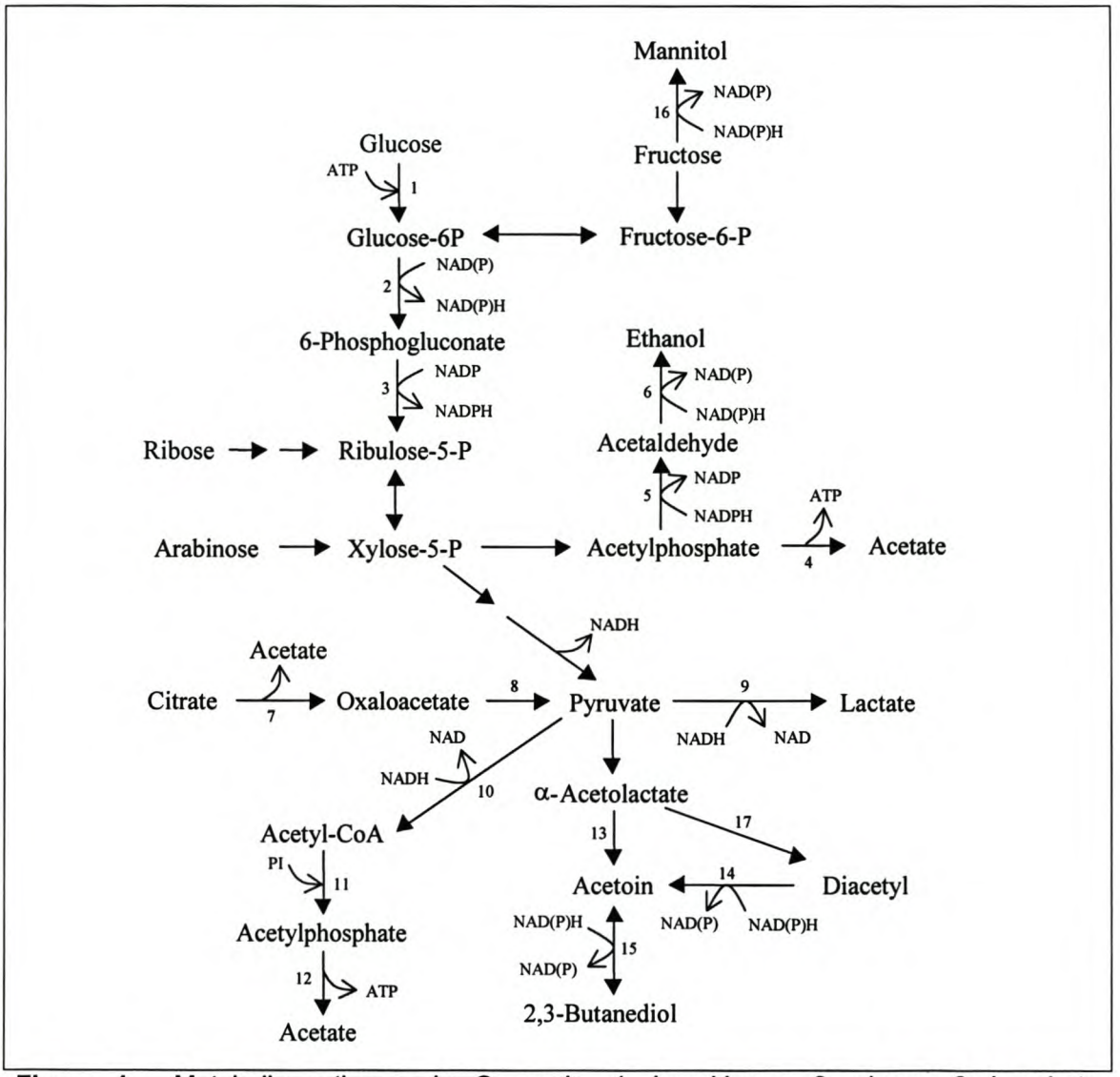

Figure 1. Metabolic pathways in *O. oeni*. 1, hexokinase; 2, glucose-6-phosphate dehydrogenase; 3, 6-phosphogluconate dehydrogenase; 4, acetate kinase; 5, acetaldehyde dehydrogenase; 6, alcohol dehydrogenase; 7, citrate lyase; 8, oxaloacetate decarboxylase; 9, lactate dehydrogenase; 10, pyruvate dehydrogenase complex; 11, phosphotransacetylase; 12, acetate kinase; 13, α-acetolactate decarboxylase; 14, diacetyl reductase; 15, acetoin reductase; 16, mannitol dehydrogenase; 17, nonenzymatic decarboxylative oxidation of α-acetolactate.

Ribose did not affect the rate of malolactic activity (Miranda et al. 1997). This observation was explained by the fact that ribose does not undergo oxidative-decarboxylation, since it enters the heterofermentative pathway at the level of xylose-5-phosphate (Figure 1). Fructose is partially converted to mannitol via mannitol dehydrogenase, thus providing an extra route for the reoxidation of NAD(P)H (Salou et al.1994). This provides cells with additional oxidized redox power compared to that obtained from glucose alone, hence the increase in biomass production when both sugars are present (Maicas et al. 1999a). Moreover, the addition of fructose completely relieved glucose-induced inhibition of MLE (Miranda et al. 1997). The same was observed in the presence of citrate (see section on citrate metabolism). The intracellular pool of NAD(P)H decreases during the co-metabolism of citrate and glucose, due to pyruvate being increasingly converted to lactate and 2,3-butanediol, with a concomitant regeneration of $NAD(P)^+$ (Ramos and Santos 1996).

2.3.6 L-MALATE

Grape juice contains between 1 and 8 g/L malate (Henick-Kling 1993). The concentration of malate decreases during grape maturation. In cool viticultural regions final concentrations in grape must are typically 2-5 g/L, while the malate content is much lower in warm climates (typically <2 g/L). LAB metabolize L-malate by one of three different enzymatic pathways, converting it to L-lactate and CO_2 (Radler 1986). Some LAB possess an active MLE, which decarboxylates L-malate directly to L-lactate without free intermediates. *L. casei* and *Enterococcus faecalis* possess a malic enzyme that converts L-malate to pyruvate which is in part reduced to L-lactate, and enables growth on malate as carbon source. A third pathway has been described for *L. fermentum*, where L-malate is reduced by malate dehydrogenase to oxaloacetate, followed by decarboxylation to pyruvate.

Several studies have shown that L-malate stimulates the growth and biomass production of *O. oeni* (Champagne et al. 1989; Firme et al. 1994; Tracey and van Rooyen, 1988). At low pH, L-malate is metabolized at a high rate, whereas carbohydrate metabolism proceeds very slowly. The resulting increase in pH allows an increase in carbohydrate utilization, which explains malate-induced growth (Miranda et al. 1997). L-malate degradation also stimulates growth in a pH-independent fashion (Pilone and Kunkee 1976) by generating a PMF that drives ATP synthesis (Cox and Henick-Kling 1989 and 1990).

2.3.7 L-LACTATE

Lactate (0.1 to 7 g/L in wine) can only be metabolized aerobically by LAB and will result in wine spoilage (Henick-Kling 1993). L-lacate at 0.5 g/L reduced the growth of *O. oeni* in synthetic medium (pH 3.5) and at 3 g/L growth was completely inhibited (Henick-Kling 1995). High lactate concentrations in wine may also limit the level of energy obtained from MLF by slowing the export of lactate from the cell.

2.3.8 CITRATE

Citrate (0.1 to 0.7 g/L) is a major component in must and wine (Henick-Kling 1993). During MLF *O. oeni* metabolizes citrate (1 to 5 mM) and the residual carbohydrates present after alcoholic fermentation (Ramos and Santos 1996). *O. oeni* is not able to grow on citrate as sole energy source (Salou et al. 1994, Ramos and Santos 1996). However, in the presence of glucose the specific growth rate and biomass production yields of *O. oeni* are enhanced (Salou et al. 1994). Since citrate catabolism is also of importance in the production of flavor compounds, such as diacetyl and acetoin, several studies have dealt with the co-metabolism of citrate and sugars (Miranda et al. 1997; Ramos and Santos 1996; Salou et al. 1994).

Ramos and Santos (1996) used ^{13}C nuclear magnetic resonance spectroscopy (NMR) to distinguish between end products derived from the metabolism of citrate and glucose. In the presence of glucose, the metabolic flux from pyruvate was mainly directed towards the production of 2,3-butanediol and lactate, whereas acetoin was the main product of citrate metabolism (Figure 1). The use of additional pathways for re-oxidation of NAP(P)H, in the presence of citrate, allows for the diversion of sugar carbon to reactions in which ATP is synthesized. Not only did the intracellular NAD(P)H/$NAD(P)^+$ ratio decrease during citrate-glucose co-metabolism, but also the intracellular concentration of glucose-6-phosphate (Ramos and Santos 1996). Moreover, in the presence of citrate the rate of glucose consumption increased. This is due to the relief of inhibition of NAD(P)H on glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Veiga-da-Cunha et al. 1993).

Citrate-induced growth enhancement is in part due to the increased ATP yield from glucose during co-metabolism with citrate (Ramos and Santos 1996). ATP is formed via substrate-level phosphorilation in the reaction catalyzed by acetate kinase, with consequent formation of acetate at the expense of ethanol. Although acetate formation via acetate kinase is negligible in the absence of glucose and at low pH (Ramos et al. 1995b), more ATP is derived from citrate metabolism than from glucose metabolism (Ramos and Santos 1996). Uniport of the monoanionic species of citrate and further metabolism generate a PMF composed of a transmembrane electrical potential and a pH gradient (Ramos et al. 1994). The generated PMF is high enough to drive ATP synthesis. During growth of *O. oeni* on citrate-glucose mixtures, the energy generated by the secondary transport of citrate supplements the energy obtained from glucose by substrate-level phosphorilation, which in turn renders competitiveness to *O. oeni* (Ramos and Santos 1996). Moreover, the addition of citrate completely relieved glucose-induced inhibition of malate utilization caused by the inhibition of MLE by NADH (Miranda et al. 1997), which is expected to accumulate during glucose catabolism as a result of inefficient NAD(P)H disposal (see section on carbohydrates). The relief of inhibition of MLF is due to the regeneration of $NAD(P)^+$ in the presence of citrate (Ramos and Santos 1996).

Production of diacetyl and acetoin by *O. oeni* is stimulated by increased citrate concentrations (Nielsen and Riechelieu 1999). Diacetyl is considered one of the most important flavors produced during MLF. When present at a concentration above the sensory threshold, which varies from 0.2 mg/L in Chardonnay wine to 2.8 mg/L in Cabernet Sauvignon wine, diacetyl gives the wine an aroma characterized as buttery or nutty (Martineau et al. 1995). An unstable compound, α-acetolactic acid (ALA) is the only source of diacetyl in wine (Hugenholtz 1993). At high redox potential and O_2 concentrations, and at low pH, ALA decarboxylates spontaneously to diacetyl (Richelieu et al. 1997). At low redox potential and O_2 concentration, ALA is converted, either chemically or by bacterial ALA decarboxylase, to acetoin. However, during MLF the degradation of citric acid is delayed compared to the degradation of L-malate (Nielsen and Riechelieu 1999). This may be due to an inhibitory action of malate on the synthesis of citrate permease, since citric acid transport is inhibited in the presence of malate (Martineau and Henick-Kling 1995). As a result, the maximum concentration of diacetyl coincides with the exhaustion of L-malate. This is then followed by degradation by *O. oeni* and yeast to acetoin and 2,3-butandiol, which in normal concentrations has no influence on wine aroma (Nielsen and Riechelieu 1999). If the buttery note from diacetyl is overpowering after depletion of L-malate, it is advisable to delay the addition of sulfite until the diacetyl concentration has been reduced to acceptable levels. SO_2 binds rather strongly with diacetyl and thereby reduces the buttery flavor. In contrast to microbial reduction, this reaction is reversible. It is therefore important to take into consideration that the diacetyl concentration will increase again as the concentration of SO_2 decreases during storage of the wine.

2.3.9 OTHER ORGANIC ACIDS

L-malate and tartaric acid (2 to 10 g/L) are the predominant organic acids in wine. Unlike malate, tartrate can only be metabolized aerobically by LAB, which means wine would have to be exposed to air (Henick-Kling 1993).

Succinate (0.2 to 2 g/L) is produced by yeast during alcoholic fermentation and is not metabolized by LAB, while acetic acid (0.05 to 0.2 g/L in dry wine) is produced during bacterial growth due to sugar and citric acid metabolism (Krieger et al. 1992). Low concentrations of gluconate (0.05 to 1.1 g/L) and pyruvate (0.03 to 0.3 g/L) are present in wines. These acids may be metabolized by LAB under winemaking conditions via the hexose monophosphate (HMP) pathway to lactate, acetate and CO_2. Since pyruvate binds SO_2, removal through growth of LAB may decrease the need to add SO_2 for protection against oxidation and microbial spoilage.

Other acids, such as fumarate and sorbate, are only present in significant amounts if added after alcoholic fermentation to prevent growth of LAB (Henick-Kling 1993). Fumarate is bactericidal against LAB at concentrations between 0.4 to 1.5 g/L, and the effect is synergistic with decreasing pH. Bacteria may overcome inhibition by converting fumarate to malate through a reaction catalysed by fumarase.

Sorbate is effective against yeast in wine at concentrations ranging from 150 to 250 mg/L and may be added to wines in the USA at concentrations up to 300 mg/L. *O. oeni* metabolizes sorbate to a geranium off-odor (Edinger and Splittstoesser 1986).

Apart from the antimicrobial action of organic acids, the pH of the wine is lowered. Although organic acids have no known effect on specific malolactic activity, malate degradation is the highest at low pH (Henick-Kling 1993).

2.3.10 FATTY ACIDS

Tween 80 (polyoxyethylene-sorbitan-mono-oleate) is often included in synthetic culture media for LAB, since it enhances bacterial growth (Johnsson et al. 1995) and may improve the production of antimicrobial peptides (Nel et al. 2002). According to Lonvaud-Funel and Desens (1990), cells of *O. oeni* grown in the presence of Tween 80 incorporate oleic acid (C18:1Δ9) into their cell membranes and form the methylated derivate, dihydrosterculic acid (C19:0cy9). Cells grown without Tween 80 lack both these acids, but contain higher levels of the cyclic lactobacillic acid (C19:0cy11). Cyclopropane acids originate from a methylation of the corresponding octadecenoic acids, explaining the inability of *O. oeni* to synthesize oleic acid.

Strains of *O. oeni* differ in their ability to assimilate oleic acid from culture medium (Bastianini et al. 2000, Guerrini et al. 2002). Strains possessing higher percentages of oleic acid and dihydrosterculic acid revealed higher cell viability and conducted complete MLF after inoculation into wine without oleic acid (Guerrini et al. 2002). In wines supplemented with Tween 80, oleic acid acted as a survival factor for strains with low capacity to assimilate oleic acid and acted as a growth factor for strains with high assimilative capacity. Survival factors are unable to affect total growth, but maintain viability of resting cells and their metabolic activities. Growth factors increase biomass without affecting population viability during the decline phase.

Since MLF depends on the ability of the malolactic starter culture to maintain high cell viability in wine, the presence of oleic acid is recommended. The success of MLF is influenced by the ability of the strain to assimilate oleic acid. If a wine lacks oleic acid, which could be due to must clarification practices, the success of MLF, unless inoculated at very high cell densities, will depend on the level of C18:1Δ9 + C19:0cyΔ9 acids present in the strain.

Antagonism between yeast and LAB during alcoholic fermentation may be, at least in part, explained by the production of medium-chain fatty acids (C_6 to C_{12}), derived from yeast metabolism (Edwards et al. 1990). Decanoic (0.6 to 14 mg/L) and dodecanoic acids are the most common fatty acids in wine (Lafon-Lafourcade et al. 1984). Decanoic acid up to 12.5 mg/L and dodecanoic acid up to 2.5 mg/L act as growth factors and stimulate malolactic activity in the presence of 4% (v/v) ethanol (Capucho and San Romão 1994). At higher concentrations these acids exerted an inhibitory effect and the toxicity increased when the pH of the media decreased from 6 to 3, indicating that the undissociated molecule is the toxic form. This form is highly

soluble in membrane phospholipids and enters the cell by passive diffusion. A fraction of these fatty acids may be incorporated into the plasma membrane and modify its composition and permeability. An increase in L-malate degradation at low concentrations of fatty acids may be due to an increase in passive transport of L-malate into the cell as a result of increased membrane permeability. In the presence of decanoic acid (20 mg/L) and dodecanoic acid (5 mg/L) the ATPase activity of *O. oeni* was reduced by approx. 5% and 42%, respectively (Carreté et al. 2002). Longer chain fatty acids are more toxic due to their higher liposolubility (Sá-Coreia 1986). The toxicity of decanoic acid, increased significantly in the presence of ethanol (Carreté et al. 2002). Although ATPase activity was only slightly inhibited by 12% (v/v) ethanol, it was reduced to approx. 65% in the presence of decanoic acid. The synergetic inhibition by ethanol and fatty acids has also been shown in yeast (Sá-Coreia 1986). The growth of certain LAB in wine could be encouraged by the presence of fungal polysaccharides produced by *Botrytis cinerea.* These polysaccharides could act by protecting LAB against the inhibitory action of some fatty acids (Henick-Kling 1993).

2.3.11 AMINO ACIDS

The efficiency of MLF is influenced by the nutrient composition of the wine and free amino acids appear to be of great significance. However, only a few studies have focused on the amino acid requirements of *O. oeni* and their effect on malolactic conversion (Fourcassie et al. 1992; Garvie 1967; Tracey and Britz 1989b). Fourcassie et al. (1992) demonstrated the absolute requirement for four amino acids (arginine, glutamic acid, tryptophan and isoleucine), while six others (valine, methionine, cysteine, leucine, aspartic acid and histidine) are required for optimum growth of *O. oeni.*

Vasserot et al. (2001) studied the effect of high concentrations of the non-essential amino acid, L-aspartic acid, on the growth of *O. oeni* and MLF. Bacterial growth in medium without L-aspartic acid was reduced by 30 to 50%, depending on the strain of *O. oeni* studied (Fourcassie et al. 1992; Vasserot et al. 2001). The favorable effect of L-aspartate on bacterial growth may be due to the ability of *O. oeni* to metabolize it to the essential amino acid L-isoleucine (Saguir and Manca De Nadra 1995). On the other hand, high concentrations of L-aspartate almost completely inhibited bacterial growth and reduced D-glucose fermentation and L-malic consumption (Vasserot et al. 2001). L-aspartate interacted with the essential amino acid L-glutamic acid and, as a result, L-glutamic acid transport is competitively inhibited. Such antagonistic interactions between amino acids could explain some of the difficulties experienced with the induction of MLF in wine.

O. oeni grows poorly under aerobic conditions with glucose as the only carbohydrate (Maicas et al. 2002). When cysteine is added, glucose consumption in aerobic conditions reaches rates similar to those found in anaerobic conditions. Cysteine acts as an electron acceptor, scavenging oxygen, and suppresses

inactivation of the ethanol-forming pathway enzymes by molecular oxygen, allowing the regeneration of NAD(P)H (see section on oxygen and carbon dioxide).

Arginine, being one of the most important amino acids in grape must and wine, represents a potential source of energy and increases the viability of *O. oeni* (Tonon and Lonvaud-Funel 2000). In wine, heterofermentative LAB may degrade arginine during MLF via the arginine deiminase (ADI) pathway, leading to the formation of ammonia, ornithine, citrulline, ATP and CO_2 (Liu et al. 1996). Arginine degradation by LAB has several enological implications. The production of ammonia increases pH and, therefore, increases the risk of growth of spoilage microorganisms (Mira de Orduña et al. 2001). Formation of ATP may give arginine-positive LAB, including spoilage LAB, an ecological advantage. Two major precursors for the formation of carcinogenic ethyl carbamate (EC) in wine are citrulline (Liu et al. 1994) and urea (Kodama et al. 1994). Ethyl carbamate is formed from a non-enzymatic and spontaneous reaction between alcohol and excreted citrulline. The reaction is favored upon wine storage in warm cellars. Urea, however, is formed by yeast arginase. Since alcoholic fermentation by yeast is traditionally conducted before MLF, control of EC formation has been focused on the reduction of arginine levels in must and wine and the selection of low-urea-producing yeast or yeast that reutilize most of the produced urea (Mira de Orduña et al. 2001).

Although most arginine is degraded by yeast during alcoholic fermentation, some wines have arginine levels as high as 2 to 5 g/L after alcoholic fermentation (Lehtonen 1996). Oenococci were able to degrade arginine at pH 3.9 and partially at pH 3.6, but not at pH 3.3 (Mira de Orduña et al. 2001). Lactobacilli degraded arginine at all pH values tested, excreting considerable amounts of citrulline. In addition to higher minimum pH requirements, arginine degradation by oenococci was delayed in comparison to L-malate degradation. In practice, this would allow the winemaker to avoid arginine degradation by carefully monitoring L-malate degradation and removing cells or inhibiting cell activity after L-malate depletion. Pure cultures of *O. oeni* and non-arginine degrading strains should be used to induce MLF.

Many LAB strains in wine are able to decarboxylate amino acids, producing high concentrations of biogenic amines (Lonvaud-Funel 2001). This reaction favors growth and survival in acidic media, since it results in an increase in pH. If biogenic amine-producing strains are present, the winemaker is encouraged to inoculate with selected malolactic starter cultures to replace the indigenous microflora.

2.3.12 OXYGEN AND CARBON DIOXIDE

LAB have a fermentative metabolism and do not usually grow well under absolutely aerobic conditions. However, some strains of *Leuconostoc* yielded higher biomass production when cultured aerobically, due to the presence of inducible NAD(P)H oxidases. These enzymes enable the cells to gain an ATP molecule from the transformation of acetyl phosphate to acetate (Lucey and Condon 1986; Plihon et al.

1995; Sakamoto and Komagata 1996). Other LAB, such as *L. plantarum* and *Lactococcus lactis*, do not benefit from O_2, but they are not inhibited by its presence (Cogan et al. 1989; Murphy and Condon 1984).

Growth of *O. oeni* is stimulated under strict anaerobic conditions (Henick-Kling 1993). Cells did not grow under aerated conditions with glucose as the only carbohydrate (Maicas et al. 2002). Oxygen inactivates the enzymes of the ethanol-forming pathway, acetaldehyde dehydrogenase and alcohol dehydrogenase (Figure 1), thus stopping the reoxidation of cofactors produced in the first steps of heterolactic sugar catabolism. Moreover, *O. oeni* lacks significant NAD(P)H-oxidase activities under aerobic conditions. These results suggest that the regeneration of cofactors is the limiting factor for aerobic metabolism of glucose.

The addition of fructose or pyruvate, which act as external electron acceptors, stimulated the growth of *O. oeni* slightly. Fructose was converted to mannitol, oxidizing two molecules of NAD(P)H, and pyruvate was transformed to lactate, enabling the regeneration of NAD^+. In the presence of cysteine, the metabolism of glucose under aerobic conditions reached similar rates to those under anaerobic conditions. Cysteine suppressed the oxygen-induced inactivation of the ethanol-forming pathway enzymes. Improved growth in the presence of added substrates that act as electron acceptors is important if high biomass levels are needed, as in the preparation of commercial starters for MLF.

2.3.13 ACETALDEHYDE

Acetaldehyde is one of the most important sensory carbonyl compounds formed during vinification, constituting more than 90% of the total aldehyde content in wine, and originates mainly from yeast metabolism (Liu and Pilone 2000). Variable levels of acetaldehyde have been described, ranging from 4 to 212 mg/L in red wine and 11 to 493 mg/L in white wine, with average values of about 30 mg/L and 80 mg/L for red and white wine, respectively. Acetaldehyde is highly volatile and has a sensory threshold value of 100 to 125 mg/L in wine. At low levels, acetaldehyde gives a pleasant fruity aroma, but results in an undesirable aroma described as green, grassy, or apple-like when present in excess (Zoecklein et al. 1995). The aroma can be masked by the addition of SO_2. Binding of SO_2 to acetaldehyde reduces its effectiveness as an antimicrobial compound and its antioxidative effect. The interaction of acetaldehyde with phenolics improves red wine color by forming stable polymeric pigments resistant to SO_2 bleaching, but it may also induce phenolic haze and eventual deposition of condensed pigments (Liu and Pilone 2000).

The impact of free acetaldehyde on wine LAB such as *O. oeni* has not been defined. Since acetaldehyde (<100 mg/L) stimulates the growth of hetero-fermentative dairy LAB (e.g. *Leuc. mesenteroides*), it has been suggested that acetaldehyde acts as an electron receptor during heterofermentation with the formation of additional energy (Liu et al. 2000). However, high levels (>100 mg/L) inhibits the growth of LAB.

The inhibitory effect of acetaldehyde-bound SO_2 on LAB growth has been well-documented (Fornachon 1963; Hood 1983). Nielsen and Riechelieu (1999) measured a decrease in the concentration of acetaldehyde in Chardonnay wine from 17 mg/L before MLF to 1.5 mg/L after MLF. Subsequently, it was shown that oenococci and lactobacilli are able to convert free and SO_2-bound acetaldehyde to mainly ethanol and acetate (Osborne et al. 2000). Free SO_2 released from the degradation of SO_2-bound acetaldehyde by SO_2-sensitive strains of *O. oeni* may cause microbial inhibition, resulting in stuck or sluggish MLF. By using efficient acetaldehyde-degrading strains to conduct MLF, the addition of SO_2 to reduce acetaldehyde aroma can be minimized.

2.3.14 PHENOLIC COMPOUNDS

Red wines contain large quantities of phenolic compounds, such as carboxylic acids (240 to 500 mg/L); anthocyanins (40 to 470 mg/L); flavonols (65 to 240 mg/L), e.g. quercetin (1 to 30 mg/L); and flavan-3-ols (25 to 560 mg/L), e.g. catechin (15 to 390 mg/L)(de Beer et al. 2002). Carboxylic/phenolic acids belong to the non-flavanoid group of phenolics in wine and are derivatives of benzoic and cinnamic acids. The most common carboxylic acids are gallic (3,4,5-trihydroxy-benzoic acid), caffeic (3,4-dihydroxy-cinnamic acid), ferulic (3-methoxy-4-hydroxy-cinnamic acid) and ρ-coumaric acid (4-hydroxy-cinnamic acid). In red cultivars of *Vitis vinifera* grapes, anthocyanins occur only as monoglucosides. Flavonols are reduced products of anthocyanins. Flavan-3-ols differ from other flavanoids, in that they do not generally occur as glycosides. Phenolic compounds may influence growth and metabolism of bacteria and the rate of MLF. The antimicrobial properties of tannins, polymers of carboxylic acids and flavanoid phenols, are well documented (Scalbert 1991). Some phenolic compounds may be involved in the release of fermentable sugars, or serve as oxygen scavengers and thereby reduce the redox potential of wine.

At high concentrations, hydroxycinnamic acids are inhibitory against growth of wine-spoilage LAB (Stead 1993) and *O. oeni* (Reguant et al. 2000). Since the pKa of these compounds is in the 5 to 7 range, a low pH would produce greater proportions of the undissociated form, which is inhibitory towards growth because of its ability to enter the cell and acidify the cytoplasm. For some *Lactobacillus* spp. a stimulatory effect on growth at low concentrations has been described (Stead 1993). These species are able to metabolize hydroxycinnamic acids by reduction to ethyl phenols, a noninhibitory form. *O. oeni* is unable to metabolize hydroxycinnamic acids (Reguant et al. 2000).

Gallic acid (3 OH in *ortho* position) is metabolized by *O. oeni* and stimulates growth (Reguant et al, 2000; Vivas, et al. 1997). Vivas et al. (1997) not only observed an increase in the rate of MLF in the presence of gallic acid, but also an increase in specific malolactic activity. Two other phenolic acids of the benzoic series, prorocatechuic acid (2 OH in *ortho* position) and vanillic acid (1 OH and

1 OCH_3 in *ortho* position), displayed no effect and a slight inhibiting effect, respectively, on MLF (Vivas et al. 1997).

Anthocyanins are metabolized by *O. oeni*, stimulating both growth and MLF (Vivas et al. 1997). The increase in the rate of MLF is, however, not due to an increase in specific malolactic activity, but rather to an increase in growth rate. The bacteria use the glucose moiety of the anthocyanins as an energy source. Both the flavonoid compounds catechin and quercetin stimulated MLF, although only catechin stimulated the growth of *O. oeni* (Reguant et al. 2000). It remains unclear how phenolic compounds such as quercetin and gallic acid increases the specific activity of the malolactic enzyme.

2.3.15 PESTICIDES

Chemical treatment against fungi, such as mildew and *Botrytis*, can lead to pesticide residues in the must and wine (Garcia-Cazorla and Xirau-Vayreda 1994). These residues not only effect yeast but also LAB in wine and delay MLF (Cabras et al. 1994). Vidal et al. (2001) examined the inhibitory effect of two commonly used pesticides, copper and dichlofluanid, on several strains of *O. oeni* and on MLF in simulated wine. Sensitivity to these pesticides varied and was enhanced by the presence of ethanol. Inhibition was due to a decrease in cell number and not to a decrease in malolactic activity. Carreté et al. (2002) recorded an approx. 25% reduction in F_0F_1 ATPase activity of *O. oeni* in the presence of 20 mg/L copper.

2.3.16 PRE-CULTURE CONDITIONS

Most LAB grown in rich and synthetic media do not survive in wine without a preculturing or a reactivation process. A limiting medium with composition close to that of wine is recommended (Nault et al. 1995). The rate of MLF in wine is directly linked to cell density and to the specific malolactic activity of the cell, with malolactic activity at its highest during the early stages of growth (Krieger et al. 1992). However, survival of a culture of *O. oeni,* and consequently malolactic activity following inoculation into wine, was the highest when the pre-culture was harvested 18-24 hrs after it entered stationary phase. Establishing an arbitrary duration of the reactivation process is not that simple and following the growth phase of bacteria under conditions in a winery is not always possible. A more practical approach to determine the best moment for starter collection would be to follow L-malate degradation. If this is the method of choice, inoculation into wine should only commence after all the L-malate of the medium is degraded (Nault et al. 1995). Furthermore, the cell numbers in the pre-culture medium should be between 10^6 and 10^7 cfu/mL to ensure that L-malate degradation follows bacterial growth. Higher cell numbers leads to high malate decarboxylation by non-proliferating cells. Survival of *O. oeni* in wine and its ability to perform MLF was also improved by pre-treating the cells at 42°C for 1h (Guzzo et al. 1994). The positive effect of a heat shock may be attributed to the synthesis of stress proteins, which are induced in stationary growth

phase (Guzzo et al. 1997). This is in agreement with the observation that stationary phase cells survive better in wine after direct inoculation (Krieger et al. 1992).

Contamination with yeast and other bacteria during reactivation and cultivation of a starter culture is difficult to avoid in a winery. Starter cultures developed for direct inoculation after simple rehydration in water will improve the management of MLF in wine. Freeze-dried cultures of *O. oeni* are commercially available (Henick-Kling 1995) and modifications of freeze-drying techniques have resulted in improved cell viability (Nielsen et al. 1996).

2.4 ALTERNATIVE TECHNOLOGIES FOR PROMOTING MLF

2.4.1 BIOREACTORS BASED ON HIGH BIOMASS OF FREE CELLS

High cell numbers of *O. oeni* have long been used to improve MLF (Gao and Fleet 1994; Maicas et al. 2000). At high cell densities (approx. 10^7 to 10^8 cfu/mL) the inhibition of MLF by low pH is diminished, as bacterial development is not essential to perform MLF. Approaches to increase productivity in high cell density fermentations by using bioreactors have been explored and recently reviewed (Maicas 2001). Cell-recycle bioreactors uses a tangential flow or hollow-fiber filter to separate the cells from the wine. Cells remain in the vessel and reach high cell densities, with the wine being constantly removed to prevent inhibition of cell growth by lactic acid production and low pH. Limitations include stress on cells entering the filtration unit, potential difficulties in up scaling due to the filtration system, and a drastic decrease in malolactic activity after only a few days. Recently, Maicas et al. (1999b) made use of free *O. oeni* cells in a continuous stirred tank reactor to control continuous fermentation. The system was successfully operated for 2 to 3 weeks and MLF was successfully conducted. Contrarily to cell-recycle bioreactors, no NAD^+ depletion and inhibition by lactic acid were recorded.

2.4.2 BIOREACTORS BASED ON IMMOBILIZED CELLS

Several studies demonstrated the possibility of achieving control over MLF by immobilized bacteria (Diviès et al. 1994). Immobilization may increase productivity due to greater packing density or by providing a more protective environment, and also improves subsequent cell separation. Starter cultures may be reused and the fermentation induced and halted at any moment.

Immobilization techniques applied to induce MLF in wine include entrapment and adsorption/attachment (reviewed by Maicas 2001). In the case of entrapment, cells are held either within the interstices of porous materials, such as a sponge of fibrous matrix, or by the physical restraints of membranes or encapsulating gel matrices. Entrapment of *O. oeni* for wine deacidification has been studied using alginates, polyacrylamide and κ-carrageenan. Immobilization via adsorption begins with a sterilized support inoculated with cell suspensions. A biofilm subsequently develops

upon exposure to growth medium. Janssen et al. (1993) assessed the feasibility of *O. oeni* cells immobilized by adsorption onto oak chips for continuous MLF. Recently, Maicas et al. (2001) reported on the adsorption of *O. oeni* on positively charged cellulose sponges.

Although these techniques proved to be successful in decreasing L-malate, most of the materials are rejected by wine producers due to toxicity, pre-fermentation preparation, requirements of additional chemicals, or mechanical instability in the presence of medium components. Other disadvantages include a decrease in cell viability and malolactic activity upon prolonged use, infection by phages, and the risk of modifying the organoleptic properties of wine.

2.4.3 BIOREACTORS BASED ON ENZYMES

A cell-free membrane reactor consisting of free *O. oeni* MLE and cofactors was developed by Formisyn et al. (1997). Complete and rapid consumption of L-malate was, however, not efficiently achieved. The efficiency of the conversion is furthermore dependent on strict pH regulation, leading to wine dilution.

2.4.4 MALATE DEGRADATION BY RECOMBINANT STRAINS OF *S. CEREVISIAE*

The ability of genetically engineered yeast strains to conduct MLF has been studied by various research groups (Ansanay et al. 1996; Bauer et al. 2003a; Bony et al. 1997; Denayrolles et al. 1995; Volschenk et al. 1997a, b). Wild type strains of *S. cerevisiae* metabolize insignificant amounts of malate during alcoholic fermentation due to the absence of an active transport system for malate (Van Vuuren et al. 1995) and the low substrate affinity of its malic enzyme (Fuck et al. 1973). On the other hand, efficient malo-ethanolic fermentation by *Schizosaccharomyces pombe* is accomplished under anaerobic conditions (Osothsilp et al. 1986) through the constitutive synthesis of malate permease, encoded by the *mae1* gene (Grobler et al. 1995), and the malic enzyme, encoded by the *mae2* gene (Viljoen et al. 1994). Volckscenk et al. (1997a) constructed a malolactic yeast strain by co-expressing the *mae1* gene and the *Lactococcus lactis* malolactic gene (*mleS*) in *S. cerevisisae.* This recombinant strain showed rapid growth at very low pH, at conditions even the acid tolerant *O. oeni* are unable to survive (Kunkee et al. 1997). The strain completed MLF within three days in Cabernet Sauvignon and Shiraz grape musts at 20°C (Volschenk et al. 1997a). At 15°C, MLF in Chardonnay grape must was completed within seven days. Apart from a more rapid MLF, compared to the bacterial process, the use of malolactic strains of *S. cerevisiae* as starter cultures should prevent stuck or sluggish MLF, the production of biogenic amines and unwanted flavors. However, compared to fermentation by *O. oeni*, such wines would contain high levels of micronutrients, rendering the wine microbiologically unstable. Aromatic compounds derived from bacterial metabolism would also be missing.

Replacement of malolactic bacteria with genetically engineered yeast in all cases is thus doubtful.

Bauer et al. (2003a) co-expressed the *S. pombe mae1* gene with the malolactic gene of either *P. damnosus* NCFB 1832 (*mleD*), *Lactococcus lactis* (*mleS*) or *O. oeni* (*mleA*) in *S. cerevisiae* and compared the efficiency of malolactic conversion. Rapid conversion of 4.5 g/L of L-malate to L-lactate, reaching L-malate concentrations of below 0.3 g/L within 3 days under fermentative conditions in synthetic grape must media, was achieved with all three malolactic enzymes. However the strain with the *mleD* gene produced significantly lower levels of L-lacate (LA). After four days, 2.8 g/L L-lacate was produced with the recombinant yeast strain harbouring *mleD*, compared to 3.3 g/L produced by the same strain containing *mleS* or *mleA*.

Volschenk et al. (2001) investigated an alternative pathway to reduce the levels of L-malate in wines. The malic enzyme of *S. pombe* decarboxylates L-malate to pyruvate and CO_2 intracellularly. Under fermentative conditions, pyruvate is further metabolized to ethanol and CO_2 resulting in the so-called malo-ethanolic fermentation. However, strains of *S. pombe* produce off-flavors. This and the fact that *S. pombe* requires higher growth temperature, renders this yeast unsuitable for vinification. Volschenk et al. (2001) constructed a *S. cerevisiae* strain containing the *S. pombe mae1* and *mae2* genes integrated in the genome, degrading 5 g/L of L-malate in synthetic and Chenin Blanc grape must. Recombinant malo-alcoholic strains of *S. cerevisiae* produced, however, higher levels of ethanol during fermentation.

2.5 PREVENTION OF MLF

Although MLF is occasionally difficult to induce, prevention of the development of LAB is likewise difficult. Several methods have been implemented with varying degrees of success. Fumaric acid inhibits malolactic fermentation, but is metabolized by yeast and lactic acid bacteria, rendering it unstable (Ough and Kunkee 1974). Dimethyldicarbonate (DMDC) is lethal against yeast and bacteria, and can be used to sterilize wine (Terrell et al. 1993). DMDC is hydrolyzed to CO_2 and a toxic compound, methanol. A further concern is that no activity is left to protect the bottled product. The winemaking process relies on the use of SO_2 to inhibit microbial growth. However, its use is strictly regulated due to associate health risks and organoleptic changes. Mounting consumer demands for safe alternatives to chemical preservatives has led researchers to focus on natural antimicrobial compounds from plants, e.g. phenolics; animals, e.g. enzymes such as lysozyme; and microorganisms, e.g. bacteriocins (Abee et al. 1995). Lysozyme and bacteriocins, such as pediocin PD-1 and nisin, are interesting candidates for wine preservation. Lysozyme is bacteriolytic against most gram-positive bacteria (Gould 1996), but has no effect on yeast (Fugelsang 1997). The activity of lysozyme is not affected by alcohol and it is active in the pH range of wine (Fugelsang 1997). The

Office International de la Vigne et du Vin (OIV) has recently approved the application of lysozyme in winemaking, but the high cost of using lysozyme is still a limiting factor.

2.6 BACTERIOCINS AND THEIR ROLE IN MLF

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins. LAB isolated from wine are capable of producing bacteriocins and they may be responsible for some of the antagonistic effects observed amongst LAB during vinification (Lonvaud-Funel and Joyeux 1993; Strasser de Saad and Manca de Nadra 1993). Bacteriocin production in grape must or wine may have a significant impact on the completion of MLF. Bauer et al. (2003b), however, have shown that grape must does not contain the required growth factors needed for production of pediocin PD-1, a bacteriocin produced by *P. damnosous* NCFB 1832. Whether this is true for other bacteriocins, have to be assessed on individual bases. To our knowledge, no papers have been published on the presence of bacteriocins in finished wines.

Bacteriocins are odorless, colorless, and nontoxic (Hansen 1994). Contrary to lysozyme and antibiotics, bacteriocins are very specific and only affect a small group of microorganisms. Bacteriocins are introduced into foods by either direct addition of the peptide (usually in a purified form), adding of the culture supernatant (a crude extract of the peptide), or by using a bacteriocin-producing starter culture in fermented foods. Although several bacteriocins with novel applications in the food industry have been developed (Ross et al. 1999), nisin is the only purified bacteriocin currently allowed in food (van Kraaij et al.1999). Nisin is a bacteriocin isolated from *Lactococcus lactis* of non-oenological origin. The effectiveness of nisin (Radler 1990a) and pediocin PD-1 (Bauer et al. 2003b) in preventing the growth of LAB in wine has been demonstrated. The peptides have a bactericidal mode of action against a number of LAB, including malolactic strains of *Lactobacillus, Leuconostoc* and *Oenococcus* spp. (Bauer et al. 1993c). Unlike pediocin PD-1, nisin is also inhibitory towards pediococci. These peptides are stable under winemaking conditions and do not effect yeast growth (Bauer et al. 2003b; Radler 1990b). Nel et al, (2002) have shown that pediocin PD-1, when compared with nisin and plantaricin 423, is the most effective in removal of an established biofilm of *O. oeni* from stainless steel surfaces in Chardonnay must. Adherence of malolactic bacteria to surfaces may have a pronounced affect on the ability of malolactic bacteria to survive during alcoholic fermentation and conduct spontaneous MLF. On the other hand, biofilms may be the source of bacterial contamination in wine or even lead to the development of bacteriophages which may cause stuck or sluggish MLF. The addition of nisin and pediocin PD-1 into wine is, however, not yet authorized and cost considerations will play a major role in the acceptance of peptide-based wine preservation strategies. An additional threat to the future application of antimicrobial

agents lies in the development of resistance, which has already been reported for nisin in a variety of Gram-positive bacteria (Verheul et al. 1997).

Since bacteriocins are encoded by genes, a variety of structural analogs of the natural peptide may be constructed through genetic engineering. This opens new possibilities to engineer *S. cerevisiae* wine yeast strains to produce these peptides and to control bacterial populations in wine. The feasibility of this concept has already been demonstrated (Schoeman et al. 1999; van Reenen et al. 2002), but production efficiency will have to be optimized. Schoeman et al. (1999) have cloned pediocin PA-1, a bacteriocin produced by *Pediociccus acidilactici* into *S. cerevisiae*. The bacteriocin is active against most wine spoilage LAB, with the exception of *O. oeni*, and would therefore be ideal as a preservative in wine where MLF, conducted by *O. oeni*, is wanted. Van Reenen et al. (2002) cloned a homologous bacteriocin, plantaricin 423, produced by *L. plantarum* into *S. cerevisiae*. Although pediocin PA-1 and plantaricin 423 belong to the same subclass of bacteriocins, the pediocin-like peptides, and the mature peptides are approx. 40% identical, the spectrum of inhibition differs. Plantaricin 423, contrarily to pediocin AcH, is very active against *O. oeni* (Nel et al. 2002). Pediocin PD-1 and nisin, on the other hand, belong to the lantibiotic family of bacteriocins (Bauer et al. 2003c). Unlike pediocin-like peptides and other Class II bacteriocins, lantibiotics are posttranslationally modified. The engineering of lantibiotics is less straightforward than that of unmodified proteins, since expression systems have to be developed not only for the structural genes, but also for genes encoding the biosynthetic enzymes and regulatory proteins. The cloning of lantibiotic genes in *S. cerevisiae* and its expression is an exiting challenge. Yeast strains expressing pediocin PD-1, nisin or plantaricin 423, would be applicable in wines where MLF is unwanted. The construction of recombinant wine yeast strains expressing bacteriocin genes together with a malolactic gene would be useful in wines where concurrent alcoholic and malolactic fermentation is required, without the effects associated with bacterial metabolism.

2.7 CONCLUSIONS

Sensory and chemical analysis have shown that LAB influence wine quality not only through MLF, but also through other metabolic pathways. Bacterial growth, survival and metabolism in wine depends on a multitude of wine components, environmental conditions, strain specific enzymatic activities and the availability of fermentable substrate. Although our knowledge has increased considerably over the past 10 years, many questions remain unanswered of which the most evident concerns the natural adaptation of wine LAB to such a harsh medium. Three cellular mechanisms play a key role in survival of *O. oeni* in wine: MLF, the plasma membrane-bound ATPase systems, and synthesis of specific stress proteins.

The control of MLF may be governed in several ways. It can be promoted through (a) strain selection; (b) starter culture development and improved reactivation; (c) development of malolactic reactors with free or immobilized bacteria, or enzymes; or (d) the construction of recombinant wine yeast strains conducting concurrent alcoholic fermentation and MLF. MLF can be prevented by (a) employing antimicrobial compounds as wine preservatives and through (b) genetic modification of yeast strains to produce antimicrobial agents such as bacteriocins. Considering the advantages of biological systems over classical chemical control methods there is little doubt that once such systems have been developed, it will impose a healthier and more ecologically friendly alternative. Systematic studies on natural antimicrobials, such as lysozyme and bacteriocin, in synergistic combination with classical preservation agents will also have an increasing role to play in the future. While "naturalness" alone is not a sufficient objective for these developments, the use of natural inhibitors that will improve preservation strategies, with advantages in product quality and safety, merits further research (Gould et al. 1996).

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CHAPTER 3

RESEARCH RESULTS

Cloning and expression of the malolactic gene of *Pediococcus damnosus* NCFB 1832 in *Saccharomyces cerevisiae*

RESEARCH RESULTS

Cloning and Expression of the Malolactic Gene of *Pediococcus damnosus* NCFB1832 in *Saccharomyces cerevisiae*

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ABSTRACT

Wine production is characterized by a primary alcoholic fermentation, conducted by *Saccharomyces cerevisiae*, followed by a secondary malolactic fermentation (MLF). Although most lactic acid bacteria (LAB) have the ability to metabolize L-malate, only a few species are able to survive the high ethanol and SO_2 levels in wine. Wines produced in colder viticultural regions have a lower pH than wines produced in warmer regions. The decarboxylation of L-malate in these wines leads to an increase in pH, more organoleptic complexity and microbiological stability. MLF is, however, difficult to control and problems often occur during filtering of such wines. *Pediococcus* spp. are known to occur in high pH wines and have strong malolactic activity. However, some pediococci strains synthesize exocellular polysaccharides, which may result in abnormal viscosity in wine. Cloning of the malolactic gene into *S. cerevisiae* could result in a malolactic fermentation conducted by the yeast, without running the risk of bacterial spoilage. The malolactic gene from *Pediococcus damnosus* NCFB1832 (*mleD*) was cloned into a laboratory strain of *Saccharomyces cerevisiae* and co-expressed with the malate permease gene (*mae1*) of *Schizosaccharomyces pombe*. Expression of the *mleD* gene was compared to the expression of two other malolactic genes, *mleS* from *Lactococcus lactis* MG1363 and *mleA* from *Oenococcus oeni* Lal1, in the same yeast strain. All three recombinant strains of *S. cerevisiae* converted L-malate to L-lactate in synthetic grape must, reaching L-malate concentrations of below 0.3 g/L within 3 days. However, a lower conversion rate was observed with *mleD* and it was consistently found that the *mleD* gene resulted in a significant lower final L-lactate level.

3.1 INTRODUCTION

Winemaking normally involves two fermentation processes, i.e. an alcoholic fermentation conducted by *Saccharomyces cerevisiae*, followed by a malolactic fermentation (MLF). This secondary fermentation is conducted by species of the genera *Pediococcus*, *Lactobacillus*, *Leuconostoc*, and *Oenococcus oeni*, previously *Leuconostoc oenos* [9], and involves the conversion of L-malate to L-lactate. The direct consequence of this conversion is a decrease in total acidity, but MLF also plays a part in microbial stabilization, while the metabolic activity of the bacteria contributes to the organoleptic complexity of the wine.

The NAD^+/Mn^{2+}-dependent malolactic enzyme (MLE) transforms the C_4 dicarboxylic acid L-malate to the C_3 monocarboxylic acid L-lactate as a direct decarboxylation reaction without any free intermediates [15]. This is contrary to the malic enzyme that converts L-malate to pyruvate, which can be further converted to L-lactate [26]. The MLE has two or four identical subunits of 60-70 kDa [3, 21, 23, 31] and is homologous to the malic enzymes recorded for a number of other organisms. The amino acid sequences deduced from partially sequenced malolactic genes (*mle*) of *Pediococcus, Leuconostoc, Oenococcus, Lactobacillus* and *Lactococcus* spp. aligned with sequences recorded for 22 malic enzymes, but formed a distinct cluster with the malic enzymes of yeast and *E. coli* [13]. Thus far, only the *mle* genes from *Lactococcus lactis* subsp. *lactis* (*mleS*) and *O. oeni* (*mleA*) have been completely sequenced [6, 18].

Wild type *S. cerevisiae* metabolizes insignificant amounts of L-malate during alcoholic fermentation due to the absence of an active transport system for L-malate [32] and the low substrate affinity of its malic enzyme [10]. *Schizocaccharomyces pombe,* on the other hand, conducts an efficient malo-ethanolic fermentation under anaerobic conditions [24] through the constitutive synthesis of malate permease, encoded by the *mae1* gene [12], and the malic enzyme, encoded by the *mae2* gene [33]. Volschenk and co-workers [35] increased the ability of *S. cerevisiae* to metabolize L-malate, by co-expressing the *mae1* gene from *S. pombe* and the *L. lactis* malolactic gene (*mleS*). The recombinant yeast grew at pH values below that recorded for *O. oeni* [17] and metabolized L-malate to L-lactate within three days at 20°C in Cabernet Sauvignon and Shiraz grape musts [34]. In Chardonnay grape must, MLF was completed within seven days at 15°C.

In this paper we describe the cloning, sequencing and heterologous expression of the *mle* gene from *P. damnosus* (*mleD*) in a laboratory strain of *S. cerevisiae*. The ability of the newly constructed strain to conduct MLF was compared with cells of the same yeast strain that have been transformed with the *mle* genes from *L. lactis* subsp. *lactis* (*mleS*) and *O. oeni* (*mleA*), respectively.

3.2 MATERIALS AND METHODS

3.2.1 BACTERIAL STRAINS, PLASMIDS AND CULTURE CONDITIONS

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was used as recipient strain for plasmid maintenance and library construction. The shuttle vector pTRKL$_2$ was used for construction of the genome library. *E. coli* was cultured as described by Sambrook et al. [29] and transformants were selected on either BHI agar (Difco, Difco Laboratories, Detroit, MI), supplemented with 150 µg/mL erythromycin (Ery), or LB agar (Difco) containing 100 µg/mL ampicillin. X-gal and IPTG were added at 70 µg/mL and 40 µg/mL, respectively.

P. damnosus NCFB1832 and *L. lactis* subsp. *lactis* MG1363 were grown at 30°C in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa). *O. oeni* strain Lal1 was isolated from a commercial malolactic starter pack (Lallemand, Saint-Simon, France) and was cultured in acidic grape (AG) broth [9] at 30°C. *S. cerevisiae* was cultured at 30°C in YPD broth, as described by Sambrook et al. [29]. Yeast transformants were isolated on selective YNB agar plates, supplemented with the required amino acids [34].

Table 1 Strains and plasmids used

Strain	Description	Reference/Source
P. damnosus NCFB1832		[11]
L. lactis MG1363		Department of Microbiology, Stellenbosch University, Stellenbosch, SA
O. oeni Lal1		Lallemand, Saint-Simon, France
E. coli DH5α	*recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 *hsdR*17 *supE*44 Φ80 *lacZΔM*15	[14]
S. cerevisiae YPH259	α *ura3-52*, *lys2-801*^{amber}, *ade2-101*^{ochre}, *his3Δ200*, *leu2-Δ1*	[30]
S. pombe IVPT 2010		Institute for Wine Biotechnology, Department of Enology and Viticulture, Stellenbosch University
Plasmids		
pTRKL$_2$	Containing the *lacZ*-gene and Ery resistant marker	[25]
pHVX2	YEplac181 (LEU2) containing the *PGK1* promoter and terminator sequences.	[35]
YEP352-PGK1pt	YYCplac33 (URA3) containing the *PGK1* promoter and terminator sequences.	Institute for Wine Biotechnology, Department of Enology and Viticulture, Stellenbosch University

3.2.2 DNA ISOLATION

Genomic DNA of the LAB and *S. pombe* were isolated according to the methods described by Dellaglio et al. [5] and Hoffman and Winston [16], respectively. Plasmid DNA from *E. coli* was isolated by the method of Lee and Rasheed [19] and purified through CsCl density gradient centrifugation.

3.2.3 CONSTRUCTION OF THE *P. DAMNOSUS* GENOMIC LIBRARY

Genomic DNA was isolated from *P. damnosus* NCFB1832 as described before and partially digested with *Sau*3A (2.8 units to 125 µg DNA in a 500 µl reaction mixture) at 37°C for 2, 3, 5, 7 and 10 min, respectively. The samples were heated at 65°C for 15 minutes, followed by phenol-chloroform extraction and ethanol precipitation. Pooled samples of partially digested DNA were separated in a 10 to 40% linear sucrose gradient [1]. Fragments between 4 and 10 kb were selected and ligated into plasmid pTRKL$_2$ that has been linearized with *Bam*HI. The constructs were transformed into *E. coli* DH5α that were made competent by using the RbCl-procedure, as adapted from a protocol obtained from the John Innes Institute, Norwich, England. The transformed cells were plated onto BHI Agar (Difco), supplemented with 150 µg/mL Ery, X-gal and IPTG as described before, and incubated at 37°C.

Transformants were handpicked with sterile toothpicks (50 white colonies per plate) and replica-plated on BHI agar supplemented with 150 µg/mL Ery, and incubated overnight at 37°C. Colonies were then washed from the agar surface and samples from each plate were stored in 40% (v/v) glycerol at -20°C. Cells collected from all plates were then pooled and incubated at 37°C for 3.5 hr in BHI, supplemented with 150 µg/mL Ery. A glycerol freeze-culture prepared from this culture represented the genomic library. The number of transformants required to represent the entire genome (N) was calculated by using the equation N = Ln(1-p)/Ln(1-F/G), with p = the confidence limit set at 99.99%, G = genome size, and F = the average size of DNA fragments ligated. The average size of the cloned fragments was determined by digesting the plasmid DNA, isolated from 50 transformants, with *Hin*dIII. Recombinant plasmid DNA was isolated and purified by CsCl density gradient centrifugation.

3.2.4 SCREENING OF THE *P. DAMNOSUS* GENOME LIBRARY FOR A *MLED* GENE

All oligonucleotides were synthesized on a Beckman Oligo 1000M DNA synthesizer (Beckman Instrument, California Avenue, Calif.). Primers for PCR were designed from two conserved sequence regions (boxes I and III) in the *mle* genes of *L. lactis* IL1441 [6] and *O. oeni* IOEB 8413 [18]. The corresponding nucleotide sequences were 5'-ATC CAG TTG TTT ATG ATC (mle-oligo1F) (sense) and 5'-CAG TTC CTT GAA TRT CAT CRT T (R = A or G) (mle-oligo1R) (antisense). The DNA fragment

amplified from the genome of *P. damnosus* NCFB1832 was labeled with [α-^{32}P]dATP (Amersham, Braunschweig, Germany) to be used as a probe for colony hybridization. Serial dilutions of the *E. coli* transformants containing the *P. damnosus* library were made and the cells plated on selective BHI agar, as described before. Colony hybridization was performed with the radiolabeled probe under stringent conditions on MSI Nylon membranes (Osmonics, USA, Westborough) as recommended by the suppliers.

3.2.5 AMPLIFICATION OF THE *MAE*1 AND *MLE* GENES

Standard recombinant DNA techniques were used, as described by Ausubel et al. [1]. The *mae1* gene was amplified from the genome of *S. pombe* by PCR, using oligonucleotides 5'-GAT CGA ATT CAT GGG TGA AAC TCA AGG AAA TC and 5'-GAT CAG ATC TTT AAA CGC TTT CAT GTT CAC T, based on the sequence of *mae1* [12]. The primers introduced a *EcoR*I and *Bgl*II site at the 5' and 3' ends of the amplified *mae1*, respectively. PCR amplification of the *L. lactis* subsp. *lactis* MG1363 malolactic gene (*mleS*) was achieved by using the primers 5'-GAT CAG ATC TGA GGT TGT ACG ATG CGT GCA C and 5'-GAT CCT CGA GCC CTT AGT ACT CTG GAT ACC AT, derived from sequences of *mleS* [7]. The primers introduced a *Bgl*II and *Xho*I site at the 5' and 3' ends, respectively. The malolactic gene of *O. oeni* Lal1 (*mleA*) was amplified by PCR using the oligonucleotide pairs 5'-GAT CGA ATT CGA GGA GAA AAT ATG ACA GAT CC and 5'-GAT CCT CGA GGC ATT CAT TAG TAT TTC GGA TC, derived from sequences of *mleA* [18]. These primers introduced a *EcoR*I and *Xho*I site at the 5' and 3' ends, respectively. The *P. damnosus* NCFB1832 malolactic gene (*mleD*) was amplified from an *E. coli* transformant, containing *P. damnosus* genomic DNA, that was identified through colony hybridization. The primers 5'-GAT CCT CGA GTG GAG GCT ATA AAT ATG GCA A and 5'-GAT CCT CGA GAG CTC ATG TAC TAT CTC TTA C were used, which introduced a *Xho*I site at the 5' and 3' of the *mleD* gene.

3.2.6 CLONING OF THE *MAE*1 AND *MLE* GENES INTO *S. CEREVISIAE*

Standard recombinant DNA techniques were performed as described by Ausubel et al. [1]. The amplified *mae1* fragment was digested with *EcoR*I and *Bgl*II, and cloned into the yeast plasmid YEP352-PGK1pt to yield plasmid pRBMAE1. The PCR products representing the three malolactic genes *mleD, mleA* and *mleS*, were digested with corresponding restriction enzymes that were introduced at their 5' and 3' ends and cloned into the shuttle vector pHVX2 to yield plasmids pRBMleD, pRBMleA and pRBMleS, respectively. Orientation of the cloned genes was determined by sequencing. *S. cerevisiae* was co-transformed with pBRMAE1 and either pRBMleD, pRBMleA or pRBMleS using the lithium acetate procedure [1].

3.2.7 SEQUENCING OF THE CLONED FRAGMENTS

DNA isolated with the miniprep method was sequenced by using the dideoxy chain termination method. An ABI Prism GigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI Prism 377 DNA sequencer (PE Applied Biosystem, Foster City, Calif.) were used. Computer analysis of the sequences was performed by using DNA-MAN for Windows® (Lynnon Biosoft, Canada). Database searches were performed by using the BLASTN and BLASTX programs of the National Center for Biotechnology Information, Bethesda, Md. (http://www.ncbi.nlm.nih.gov). Plasmid DNA from *E. coli* transformants containing *P. damnosus* genomic DNA that hybridized with the malolactic probes were sequenced with the PCR oligonucleotide mle-oligo1F. Plasmid pHVX2 containing the *L. lactis* MG1363 and *O. oeni* Lal1 *mle* enzymes were sequenced with oligonucleotides based on sequences of the *PGK1* promoter (5'-GTT TAG TAG AAC CTC GT) and *PGK1* terminator (5'-AGC GTA AAG GAT GGG). Additional primers were synthesized based on new sequences generated. The nucleotide sequences of *mleD* and *mleS* have been submitted to GenBank with accession numbers AY450551 and AY450550.

3.2.8 MALOLACTIC FERMENTATION BY *S. CEREVISIAE*

Conversion of L-malate to L-lactate by recombinant strains of *S. cerevisiae* was determined by inoculating synthetic grape must [7], supplemented with amino acids and adjusted to a pH of 3.3 [35], with 10^6 cfu/ml. Cultures were incubated at 22°C until MLF was complete, with two shakings per day. The L-malate and L-lactate concentrations during fermentation were measured enzymatically using the L-Malic Acid and L-Lactic Acid Test Kits (Roche Diagnostics, Mannheim, Germany), respectively.

3.3 RESULTS

3.3.1 QUALITY OF THE *P. DAMNOSUS* GENOMIC LIBRARY

The size of the genome of *Pediococcus* spp. is approx. 1500 kb [4]. Since the average size of fragments cloned was 6 kb, and all the transformants contain inserts, the entire genome of *P. damnosus* NCFB1832 would be represented by 2 303 transformants. A total of 3 500 transformants were collected after *E. coli* transformation with the genomic library.

Plasmid DNA representing the entire library was digested with *Hind*III. *Hind*III cuts $pTRKL_2$ in the multiple cloning site (MCS) and twice at known positions outside the MCS. Two distinct bands were observed with a background smear between 1 and 10 kb (results not shown), suggesting that the library is representative. The plasmid $pTRKL_2$ contains an *Eco*RI recognition site in the MCS and one site outside.

Digestion of library DNA with *Eco*RI yielded one distinct band with a background smear, as expected (results not shown).

3.3.2 CLONING OF THE *MLED* GENE OF *P. DAMNOSUS*

PCR amplification of the genomic DNA of *P. damnosus* with primers mle-oligo1F and mle-oligo1R, based on conserved sequences in other *mle* genes, generated fragments of approx. 580 bp, which corresponded to the expected size. To clone a full genomic copy of the gene, the PCR product was radiolabeled and used as a probe to screen the *P. damnosus* genomic library. Three *E. coli* transformants out of approx. 1500 screened, hybridized with this probe. Restriction enzyme digests of plasmid DNA isolated from these *E. coli* transformants differed from each other, but shared bands of similar sizes, suggesting that they contained partially overlapping genomic DNA inserts. One of the clones was sequenced by first using the two PCR primers (mle-oligo1F and mle-oligo1R) and then primers generated along the identified sequence. The entire *mleD* gene and flanking regions were sequenced. The other two clones contained the same gene, which confirmed that the genomic library was representative.

3.3.3 NUCLEOTIDE SEQUENCE ANALYSIS OF *MLED*

The complete nucleotide sequence of the *P. damnosus* NCFB1832 *mleD* gene was established by sequencing both strands of the insert in $pTRKL_2$. A 1629-nucleotide open reading frame starting with an ATG initiation codon at position 334 and extending to the TAG stop codon at position 1960 was identified (Figure 1). The open reading frame encoded a putative protein of 542 amino acids with a calculated molecular mass of 59 271 Da and a pI of 4.52. A potential ribosome binding site (RBS) at position 321 was identified.

The *mle* genes of *O. oeni* Lal1 (*mleA*) and *L. lactis* MG 1363 (*mleS*) were also cloned into plasmid $pTRKL_2$ and sequenced. The nucleotide sequence of *mleA* was identical to that of *O. oeni* IOEB 8413. A pairwise comparison of nucleotide sequences of *mleS* and the *mle* gene described for *L. lactis* IL1441 revealed 96.8% homology. Amino acid sequence comparison revealed 99.1% homology. Pairwise comparison of the amino acid sequences revealed identity values of 72.5 and 64.4% for MleDp with MleAp and MleSp, respectively (Figure 2). MleAp and MleSp are 67.1% identical (Figure 2).

```
1       CCAAGTATTCCGCAAAGCCGCCAATGACACCCGAAATTACCTTGTCTGATGATTTTGTTA
61      ATTTTTTATTCATGATATTCCTCTTTTCCGTTATTCATAAATTTTCCTCTAAGTTTACA
121     AACTTTCTTGATACTTTTATTATAACTTTTTGGACTATTAAATCCCATTAAAAAGTGCTA
181     AAAATAGGACACCAGATAATCCCTTGAAAACACAGAGTTTCTGGAACCAGTTATTACCTT
241     TTTTAATACTTTCTTAAACGTTTGACTATAGTTTTTTTGACCGCGTATACTGGCGCTGTT
301     GATTAGAAAAGAAAGTAATTGGAGGCTATAAATATGGCAAAAAGTCCAGTAGAAATTTTG
                              RBS          M  A  K  S  P  V  E  I  L
361     AACGATCCATTTTTAAATAAGGGGACCGCATTTACAAAAGAAGAGCGTCAATCACTTGGT
         N  D  P  F  L  N  K  G  T  A  F  T  K  E  E  R  Q  S  L  G
421     TTGGTTGGGTTATTACCACCTTACGTCCAAACATTAGATGAACAAGCTGCGCAAACGTAT
         L  V  G  L  L  P  P  Y  V  Q  T  L  D  E  Q  A  A  Q  T  Y
481     GCGCAAGTCGAATCAAAAACAAGTAGTTTAGAAAAACGAATTTTCTTAATGGAAATCTTT
         A  Q  V  E  S  K  T  S  S  L  E  K  R  I  F  L  M  E  I  F
541     AATGAAAATCGCGTATTATTCTATAAATTATTTAGCCAGCACGTAGTTGAATTCATGCCA
         N  E  N  R  V  L  F  Y  K  L  F  S  Q  H  V  V  E  F  M  P
601     ATCGTGTATGATCCAACCATTGCCGACACAATTGAAAACTATAGTGAGCTGTTTGTTCAG
         I  V  Y  D  P  T  I  A  D  T  I  E  N  Y  S  E  L  F  V  Q
661     CCCCAAAATGCAACATTCTTATCAATTGATGATCCAGACAGCATGAAGGATTCTTTGAAA
         P  Q  N  A  T  F  L  S  I  D  D  P  D  S  M  K  D  S  L  K
721     AACGCTGCTGATGGTCGCGATATTCGTTTGATCGTGGTCACAGATGCGGAAGGAATTCTA
         N  A  A  D  G  R  D  I  R  L  I  V  V  T  D  A  E  G  I  L
781     GGAATTGGTGACTGGGGCACAAATGGTGTCGACATCTCTGTTGGAAAACTAATGGTTTAT
         G  I  G  D  W  G  T  N  G  V  D  I  S  V  G  K  L  M  V  Y
841     ACAGCGGCTGCCGGAATCGATCCTAGCCAGGTTATGCCAGTTGCTTTAGATACTGGAACA
         T  A  A  A  G  I  D  P  S  Q  V  M  P  V  A  L  D  T  G  T
901     AATAATCAAGATTTGTTAAAAGATCCAATGTACCTTGGCAATCGTCACGAACGCGTAACT
         N  N  Q  D  L  L  K  D  P  M  Y  L  G  N  R  H  E  R  V  T
961     GGTGATCGTTACTACAAATTTGTGGATCAGTTTGTAAACACGGCTGAAGATCTTTTTCCA
         G  D  R  Y  Y  K  F  V  D  Q  F  V  N  T  A  E  D  L  F  P
1021    AAACTTTATCTTCATTTTGAAGATTTTGCTCGGGATAATGATGCTAATATCTTAAATACG
         K  L  Y  L  H  F  E  D  F  A  R  D  N  D  A  N  I  L  N  T
1081    TACAAAGACAAAATCACGACATTTAATGATGACATCCAGGGAACTGGAATTATTGTTTTG
         Y  K  D  K  I  T  T  F  N  D  D  I  Q  G  T  G  I  I  V  L
1141    GCTGGGATTTTGGGTGCTTTGAATATTTCTAAACAAAAAATGACGGATCAAGTTTATCTT
         A  G  I  L  G  A  L  N  I  S  K  Q  K  M  T  D  Q  V  Y  L
1201    TCATTTGGTGCTGGAACTGCTGGCGCTGGAATCACGAAACGGATTTATGATGAATTCTTG
         S  F  G  A  G  T  A  G  A  G  I  T  K  R  I  Y  D  E  F  L
1261    CAACAAGGCTTATCTGCAGATGAAGCAAAGAAACACTTCTATTTGGTTGATAAACAAGGC
         Q  Q  G  L  S  A  D  E  A  K  K  H  F  Y  L  V  D  K  Q  G
1321    TTGTTGTTTGATGATGACAAGACGTTGACACCAGAACAAAAGCCATTTGCACGTTCTCGT
         L  L  F  D  D  D  K  T  L  T  P  E  Q  K  P  F  A  R  S  R
1381    TCTGAATTTGCCAATGCCGATGATTTAACAACTTTAGAAGCGGCTGTAAAGGCAATTCAC
         S  E  F  A  N  A  D  D  L  T  T  L  E  A  A  V  K  A  I  H
1441    CCAACGATTTTGGTGGGAACTTCAACTCAACCAGGAACGTTCACAGAATCAATTGTGAAA
         P  T  I  L  V  G  T  S  T  Q  P  G  T  F  T  E  S  I  V  K
1501    GAAATGGCTGCTCATACTGACCGCCCAATTATTTTCCCGTTATCAAATCCAACTAAATTA
         E  M  A  A  H  T  D  R  P  I  I  F  P  L  S  N  P  T  K  L
1561    GCAGAAGCTAAAGCTGAAGATTTGATTAAATGGTCTGATGGGAAGGCTTTAGTAGCAACA
         A  E  A  K  A  E  D  L  I  K  W  S  D  G  K  A  L  V  A  T
1621    GGAATTCCAGCAGATGACGTTGAATACAATGGCGTCACTTACCAAATTGGTCAGGCTAAC
         G  I  P  A  D  D  V  E  Y  N  G  V  T  Y  Q  I  G  Q  A  N
1681    AATGCGTTAGTTTATCCCGGTTTAGGATTAGGTGTGATTGCTTCCACTGCTAAAGTTTTA
         N  A  L  V  Y  P  G  L  G  L  G  V  I  A  S  T  A  K  V  L
1741    AATGATGAAATGATTTCAAAGGCTGCCCATTCATTAGGTGGAATTGTGGATGGTAACAAG
         N  D  E  M  I  S  K  A  A  H  S  L  G  G  I  V  D  G  N  K
1801    GCTGGTGCTGCTGTATTACCACCAGTTTCAAAGCTCGATCAATTCTCATACACGGTGGCA
         A  G  A  A  V  L  P  P  V  S  K  L  D  Q  F  S  Y  T  V  A
1861    AAGGCTGCAGCGCAGAGTGCGGTTGATCAAAAATTGAATCAGGAACCAATTTCAGATGTT
         K  A  A  A  Q  S  A  V  D  Q  K  L  N  Q  E  P  I  S  D  V
1921    GATAAGGCAATTACCGATATGAAGTGGGAACCAAAGTATTAGGATGTTTTCGAAAGTGAG
         D  K  A  I  T  D  M  K  W  E  P  K  Y
```

Fig. 1. Nucleotide and deduced amino acid sequences of *mleD*, and flanking DNA from *Pediococcus damnosus* NCFB 1832. The putative RBS is underlined. Numbers on the left represent the nucleotide positions.

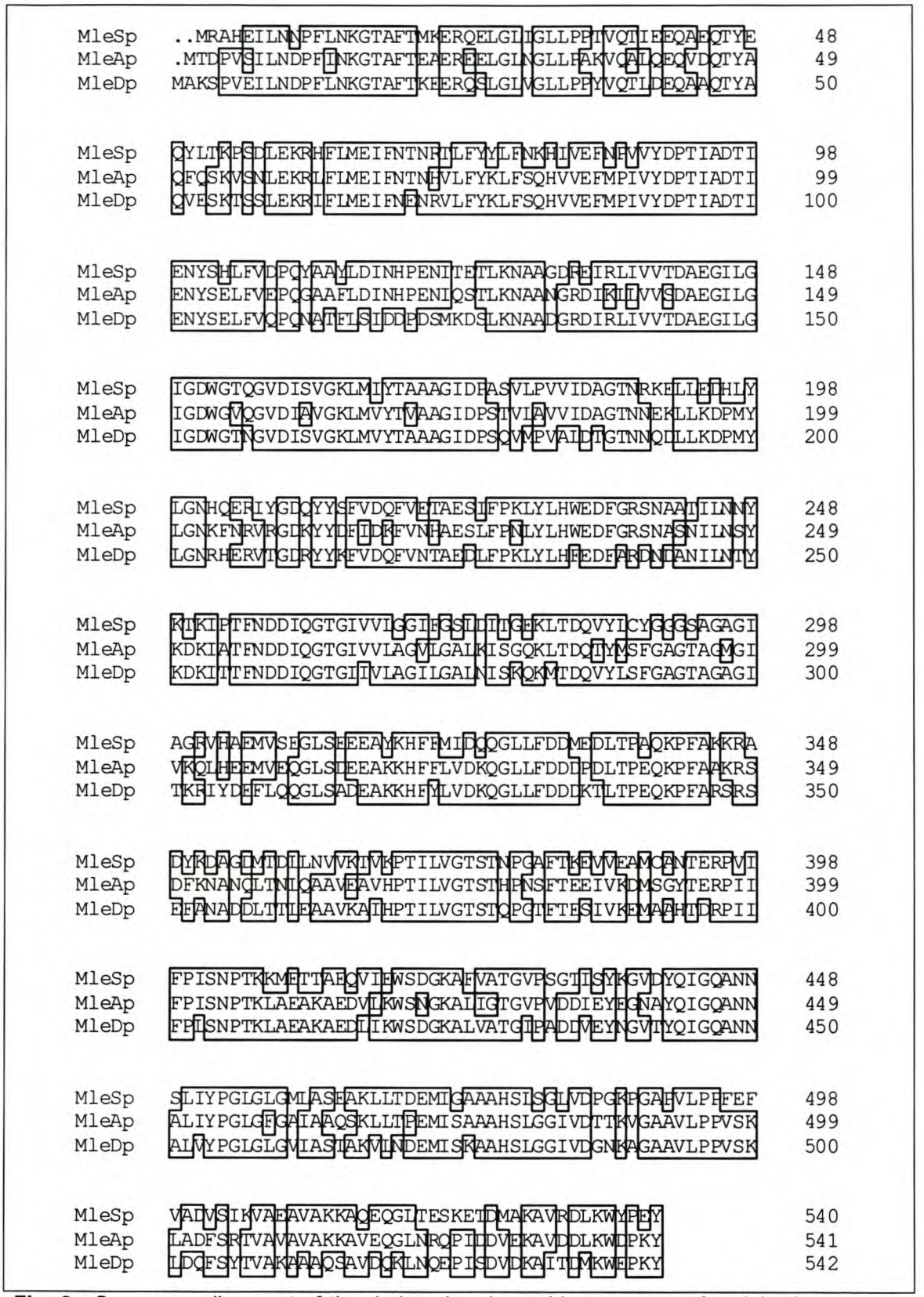

Fig. 2. Sequence alignment of the deduced amino acid sequences of malolactic enzymes produced by *Lactococcus lactis* MG 1363 (MleSp), *Ooenococcus oeni* Lal1 (MleAp) and *Pediococcus damnosus* NCFB 1832 (MleDp). Identical regions are enclosed in boxes.

3.3.4 EXPRESSION OF *MLED* IN YEAST

The *mleD* ORF was cloned into the yeast expression vector pHVX2 described by Volschenk et al. [35], between the promoter and terminator sequences of the *PGK1*-gene to yield plasmid pRBMleD. The *mle* gene of *O. oeni* Lal1 (*mleA*) and *L. lactis* MG 1363 (*mleS*) were also cloned into the same vector leading to the plasmids pRBMleA and pRBMleS, respectively. Similarly, the *S. pombe* malate permease gene (*mae1*) was placed under control of the *PGK1* promoter in the shuttle vector YEP352-PGK1pt. A recombinant strain of *S. cerevisiae* containing the malolactic gene of *P. damnosus* (*mleD*) and *mae1* was constructed by co-transformation of plasmid pRBMleD and plasmid pRBMae1 into *S. cerevisiae* YPH259. Recombinant *S. cerevisiae* strains co-expressing the malolactic enzymes of *L. lactis* MG 1363 and *O. oeni* Lal1 and malate permease were constructed following the same strategy.

3.3.5 MALOLACTIC FERMENTATION BY *S. CEREVISIAE*

Malolactic fermentation is regarded complete when the concentration of L-malate reaches 0.3 g/L [22]. *S. cerevisiae* transformants with the *mleD* gene converted L-malate to L-lactate within 3 days, reaching L-malate concentrations of below 0.3 g/L (Figure 3). However, after 4 days of fermentation in grape must, only 2.8 g/L L-lactic was produced, compared to 3.3g /L formed by transformants with *mleA* and *mleS* genes (Figure 3). Yeast strains containing only the *PGK1*-expression cassette (pHVX2 or YEP352-PGK1pt), the *mle* genes or the *mae1* gene under control of the *PGK1* promotor, were unable to convert a significant level of L-malate to L-lactate (results not shown).

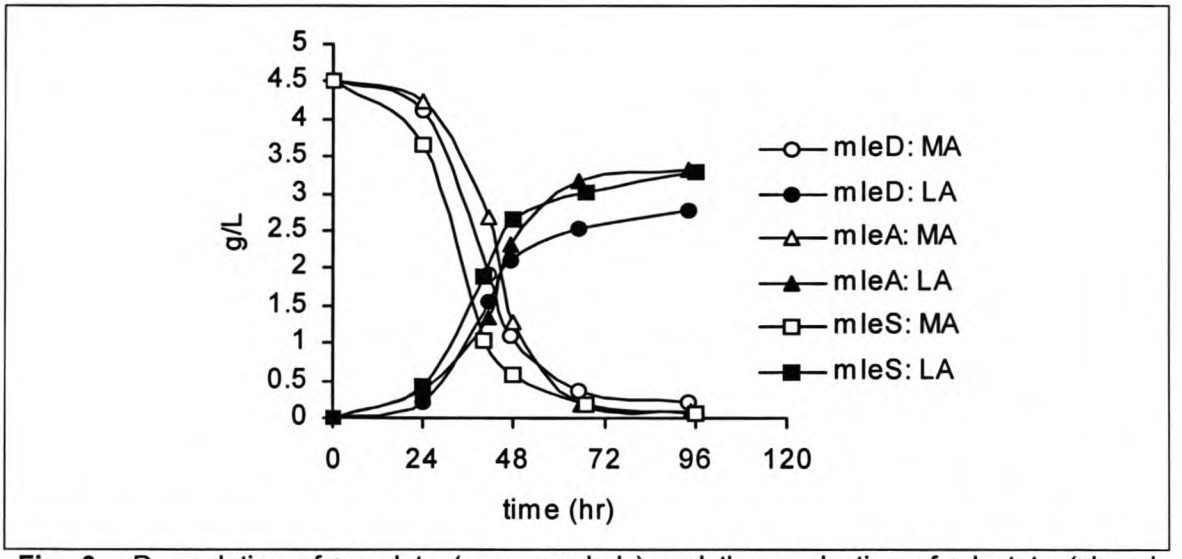

Fig. 3. Degradation of L-malate (open symbols) and the production of L-lactate (closed symbols) in synthetic grape must fermented by recombinant strains of *Saccharomyces cerevisiae* co-expressing *mae1* and the malolactic genes *mleD*, *mleA* and *mleS*, respectively. All data represent an average of three repeats. The values recorded in each experiment did not vary by more than 5%. Single data points are, therefore, presented in the figures without standard deviation bars.

3.4 DISCUSSION

This is the first report of a malolactic gene from *P. damnosus* cloned into and expressed by *S. cerevisiae*. Pairwise comparison of the amino acid sequences of mleDp with MleAp of *O. oeni* Lal1 and MleSp of *L. lactis* MG1363 revealed similarity values of 72% and 64%, respectively. The *mleS* nucleotide sequences of *L. lactis* MG1363 and *L. lactis* IL1441 revealed a high degree of homology (96.8%), while the nucleotide sequence recorded for the MLE of *O. oeni* Lal1 was identical to that recorded for the MLE of *O. oeni* IOEB 8413. A partial sequence covering 36% of the *mle* gene of *O. oeni* ATCC 23279^T was also found to be identical to the corresponding nucleotide sequence of *mleA* [13]. The homology among the malolactic enzymes of *O. oeni* suggests that the species consist of a genetically homogeneous collection of strains.

Analysis of a fragment representing 36% of the *mle* genes of 13 strains of lactic acid bacteria suggested that the gene evolved more rapidly than the 16S gene [13]. The 16S rRNA sequence tree revealed three distinct groups of lactic acid bacteria, *viz. Lactococcus*, *Leuc. mesenteroides* and *O. oeni*, and other species. *Pediococcus* and *Lactobacillus* spp. were intermixed in the 16S rRNA tree, whereas they were separated in the *mle* sequence phylogenetic tree. Although *Leuc. mesenteroides* and *O. oeni* were distinct from other species in the 16S rRNA tree, they were intermixed with *Lactobacillus* species and *L. lactis* in the *mle* tree. The *mle* of *O. oeni* is the closest related to that of *L. lactis* and the genus *Pediococcus*, with *P. acidilactici* and *P. parvulus*, was separated from the other LAB. After now having sequenced the *mle* gene of *P. damnosus* in its entirety, complete sequences are available for malolactic genes representing each of the three groups of LAB. Based on our results, the MLE of *O. oeni* is closer related to *P. damnosus*.

A complete and rapid malolactic conversion by *S. cerevisiae* can be achieved by co-expressing the *P. damnosus mleD* and *S. pombe mae1* genes in this yeast. The removal of L-malate, one of the main organic acids of grape must, is essential for deacidification and stabilization of wine. Even with the use of bacterial starter cultures, stuck or sluggish MLF often cause delays in cellar operations. Furthermore, reduced sulfite levels in wine may lead to proliferation of spoilage organisms, producing off-odors and biogenic amines [20]. Malolactic strains of *S. cerevisiae* that degrade malate efficiently during alcoholic fermentation should prevent problems experienced with bacterial strains. We compared the kinetics of MLF between recombinant yeast strains expressing *mleD*, *mleA* and *mleS*. All three engineered *S. cerevisiae* strains efficiently converted L-malate to L-lactate. However, a lower conversion rate was observed with *mleD* and it was consistently found that the *mleD* gene resulted in a significant lower final L-lactate level. This may be due to a lower substrate affinity of its MLE. A study determining the K_m-values of the three enzymes might explain this phenomenon. Obtaining information of new MLE's is an important prerequisite for future genetic engineering and improvement of MLF. Sequence comparison may also resolve questions e.g., explaining why the MLE, unlike the

malic enzyme, transform malate to lactate without the release of intermediate products, such as pyruvate or NADH.

In the production of aromatic wines, such as Sauvignon Blanc, Riesling and Gewürztraminer, the reduction of L-malate is often required without the effects that the growth of LAB have on the organoleptic profile of the wine. However, *O. oeni* is known to be responsible for spontaneous MLF, especially since increased consumer demand for lower SO_2 levels in wine. Bacteriocins, ribosomaly synthesized antimicrobial peptides or proteins, are interesting candidates for wine preservation and could provide the winemaking community with a safe alternative to SO_2 or at least replace part of the SO_2 currently added to must and wine [2]. The effectiveness of the bacteriocins nisin [27] and pediocin PD-1 [2] in preventing the growth of LAB in wine has been demonstrated. The peptides are produced by *L. lactis* spp. and *P. damnosus* NCFB1832 respectively. They display a bactericidal mode of action against a number of LAB, including malolactic strains of *Lactobacillus, Leuconostoc* and *Oenococcus* spp. Both peptides are stable under wine-making conditions and do not effect yeast growth [2, 28]. Microbial contamination and spontaneous MLF can be overcome by the construction of recombinant wine yeast strains expressing bacteriocin genes, such as pediocin PD-1 or nisin, together with the malolactic gene. These strains would be useful in wines where concurrent alcoholic and malolactic fermentation is required, but the flavorful end products from LAB metabolism are not wanted. Unlike nisin, the nucleotide sequence of pediocin PD-1 remains to be elucidated. The *P. damnosus* library constructed in this study will be an invaluable tool in realizing this.

3.5 CONCLUSIONS

We show that a new bacterial *mle* gene from *P. damnosus* (*mleD*), introduced into yeast *S. cerevisiae* results in a rapid and complete transformation of L-malate into L-lactate. This is also the first report of the functional expression of the malolactic gene from *O. oeni* in *S. cerevisiae* that results in complete MLF by the transgenic yeast strain. Malolactic strains of *S. cerevisiae* that degrade malate efficiently during alcoholic fermentation should prevent problems experienced with bacterial strains, such as stuck or sluggish MLF or the production of biogenic amines or unwanted flavors. Another advantage of this concurrent alcoholic and malolactic fermentation is the completion of MLF early in the cycle to allow application of cellar operations for wine storage and aging. Whether these new yeast strains can replace MLF in all cases is however doubtful. Compared to fermentations by *O. oeni*, the engineered strains would produce wine retaining high concentrations of micronutrients, rendering the wine microbiologically less stable under certain conditions. Flavorful by-products derived from bacterial metabolism would also be missing.

Control of acidity is crucial to warrant wine quality. Lower conversion rate with *mleD* opens the possibility to adjust lactate levels according to specific needs. Small

differences in malolactic conversion may have a significant effect on the organoleptic quality of the wine and the decrease in intracellular malate concentration may affect the formation of other organoleptic by-products. The availability of new malolactic yeast strains enlarges the range of strains that could be used for biological correction of excess acidity in wine.

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CHAPTER 4

RESEARCH RESULTS

Pediocin PD-1 as a method to control the growth of *Oenococcus oeni* in wine

RESEARCH RESULTS

Pediocin PD-1 as a Method to Control the Growth of *Oenococcus oeni* in Wine

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ABSTRACT

Malolactic bacteria are known to stabilize wine and change its organoleptic properties. However, uncontrolled malolactic fermentation, especially in wines with a high pH, which is typical to warmer viticultural regions, may cause spoilage and make the wine unpalatable. Use of SO_2 to inhibit microbial growth is strictly regulated, and mounting consumer demands for safe alternatives to chemical preservatives has led to an increased interest in natural antimicrobial substances. Pediocin PD-1, an antimicrobial peptide produced by *Pediococcus damnosus* NCFB 1832, is active against a number of lactic acid bacteria, including malolactic strains of *Lactobacillus, Leuconostoc,* and *Oenococcus* spp. Antimicrobial activity of pediocin PD-1 remained constant (1600 AU/mL) for 28 days in dry-fermented (yeast fermented) grape must adjusted to pH 3 and 4, respectively. However, antimicrobial activity decreased to 800 AU/mL and 600 AU/mL after 48 hrs in the same must when adjusted to pH 6 and 7, respectively. A complete loss in antimicrobial activity was recorded after 48 hrs of incubation in grape must with a pH of 8 or 9. No change in antimicrobial activity was detected when pediocin PD-1 was incubated in must containing 15% (v/v) ethanol, 100 mg/L SO_2, or a combination thereof. When *Oenococcus oeni* was cultured in Chardonnay must (pH 3.8) and must supplemented with yeast extract (pH 3.8) the viable cell numbers of *O. oeni* decreased from $1x10^6$ cfu/mL to less than 10 CFU/mL after 4 days of incubation in the presence of 30 AU/mL pediocin PD-1. Pediocin PD-1 did not inhibit the growth of a commercial starter culture strain of *Saccharomyces cerevisiae* or a mutant with an impaired cell wall structure caused by deletion of the *CWP2* gene (*cwp2*Δ). Concluded from results obtained in this study, pediocin PD-1 effectively inhibited the growth of *O. oeni* and may provide an alternative to chemical preservatives.

4.1 INTRODUCTION

Malolactic fermentation (MLF) entails the decarboxylation of L-malic acid to L-lactic acid and CO_2, resulting in a decrease in total acidity. Although wines that undergo MLF are considered to be microbiologically stable, many winemakers prefer to control the fermentation process to prevent a further increase in pH, especially in wines from warmer viticultural regions [15]. Several species of LAB have been identified in grape must, with *Oenococcus oeni* being the most dominant due to its acidophilic nature and resistance to the alcohol and SO_2 content of wine [14].

Spontaneous MLF is unpredictable, since it may occur during, or many months after, the completion of alcoholic fermentation [12], rendering the wine unpalatable. Some of the undesirable changes may include a reduction in color (as high as 30% in red wines) or the formation of biogenic amines, as recorded for certain strains of *O. oeni* [16].

Although MLF is occasionally difficult to induce, prevention of the development of lactic acid bacteria (LAB) is likewise difficult. Several methods have been implemented with varying degrees of success. Fumaric acid has been shown to inhibit malolactic fermentation, but it is metabolized by yeast and lactic acid bacteria, rendering it unstable [22]. Dimethyldicarbonate (DMDC) is lethal against yeast and bacteria, and can be used to sterilize wine [28]. Since DMDC is hydrolyzed to CO_2 and methanol, it may be that no activity is left to protect the bottled product. The winemaking process relies on the use of SO_2 to inhibit microbial growth. However, its use is strictly regulated due to associated health risks and organoleptic changes. Mounting consumer demands for safe alternatives to chemical preservatives has led to an increased interest in different solutions. For this reason, natural antimicrobial compounds from plants (such as phenolics), animals (for example enzymes such as lysozyme) and microorganisms (for example bacteriocins such as nisin and pediocin) have been the focus of research in recent years [1].

The bacteriolytic enzyme lysozyme is active against most gram-positive bacteria [9] and act synergistically with the bacteriocin nisin under certain conditions [18]. Lysozyme has no effect on yeast, is not affected by alcohol and is active in the pH range of wine [8]. The Office International de la Vigne of du Vin (OIV) has recently approved the addition of lysozyme to the winemaking process, but the economic implications of using lysozyme are still a limiting factor.

Bacteriocins, ribosomal synthesized antimicrobial peptides or proteins, are odorless, colorless, and nontoxic [11]. Furthermore, since they are encoded by genes, a variety of structural analogs of the natural peptide may be constructed through genetic engineering. This may also open new possibilities to engineer wine strains of *S. cerevisiae* to produce these peptides and to biologically control bacterial populations in wine. The feasibility of this concept has already been demonstrated by Schoeman et al. [26], but levels of production will have to be optimized. Bacteriocins are introduced into foods by either direct addition of the peptide (usually

in a purified form), addition of the culture supernatant (that is a crude extract of the peptide), or by using a bacteriocin-producing starter culture in fermented foods.

Although several bacteriocins with novel applications in the food industry have been developed [25], problems such as low production levels and instability of bacteriocins need to be addressed. To date, nisin is the only purified bacteriocin (lantibiotic) allowed in food [29]. The effectiveness of nisin in preventing the growth of lactic acid bacteria in beer [21] and wine [23, 24] has been demonstrated. However, nisin has not yet been approved for use in the wine industry. Besides these regulatory reasons, cost considerations may play a major role in the acceptance of peptide-based wine preservation strategies. Considering the advantages of a biological system over classical chemical control methods, however, there is little doubt that once these systems have been developed sufficiently, they will be considered as healthier and more ecologically friendly alternatives. However, an additional threat to the future application of antimicrobial agents lies in the development of resistance, which has already been reported for nisin in a variety of gram-positive bacteria [30]. For this reason, it is clear that alternative systems have to be investigated and developed. Systematic studies on such preservation systems in synergistic combination with classical preservation agents will have an increasing role to play in the future.

Pediocin PD-1, first described by Green et al. [10] is a bacteriocin produced by *Pediococcus damnosus* NCFB 1832, a strain isolated from beer. Pediocin PD-1 (< 3.5 kDa) is smaller than pediocin-like bacteriocins and it's inhibitory spectrum differs from that of nisin and the pediocin-like family of bacteriocins [10]. Unlike nisin, pediocin PD-1 is active over a broad pH range, which suggest applications in non-acid foods. The peptide is active against a number of LAB, including malolactic strains of *Lactobacillus, Leuconostoc* and *Oenococcus* spp. [10]. In one of our previous studies [20], when compared with nisin and plantaricin 423, we have shown that pediocin PD-1 is the most effective in removal of an established biofilm of *O. oeni* from stainless steel surfaces in Chardonnay must.

In this study we determined the conditions needed to produce pediocin PD-1 in Chardonnay grape must, studied the stability of the peptide in dry-fermented (that is post yeast fermentation) grape must, and determined the levels of antimicrobial activity needed to effectively control the growth of *O. oeni* in the must.

4.2 MATERIALS AND METHODS

4.2.1 STRAINS AND CULTURE CONDITIONS

P. damnosus NCFB 1832, producer strain of pediocin PD-1, was cultured as described previously [19]. The strain of *O. oeni*, previously *Leuconostoc oenos* [4] was isolated from a commercial malolactic starter culture (Lallemand, Saint-Simon, France) and grown in acidic grape (AG) broth [5]. The wild type *S. cerevisiae* S288C

(MATa *leu*2-3, 112 *his*4-519 *can*1) and an isogenetic strain with impaired cell wall structure due the deletion of the *CWP2* gene (Δ*cwp2*) were used. The wine strains of *S. cerevisiae* included in this study were isolated from commercial samples of active dry yeast (VIN13 and N96). All yeast strains were cultured in YPD medium (Difco, Sparks, USA) at 30°C.

4.2.2 PARTIAL PURIFICATION OF PEDIOCIN PD-1

P. damnosus NCFB 1832 was cultured in de Man-Rogosa-Sharpe (MRS) broth (Biolab, Midrand, South Africa) at 30°C to early stationary phase. Pediocin PD-1 was isolated from the culture by harvesting the cells (8000 x *g*, 5 min, 4°C) and precipitating the proteins from the cell-free supernatant with ammonium sulfate (70% saturation). The precipitate was pelleted by centrifugation (7000 x *g*, 20 min, 4°C), dissolved in deionized water, adjusted to pH 5 with 3N NaOH and dialyzed against deionized water (Spectra/Por Membrane, 1000 Da molecular weight cut-off). The dialyzed sample was stirred for 1 hr (at 4°C) in 30 volumes of a methanol-chloroform mixture (1:2, v/v) to remove fatty acids, including residual Tween 80 [3]. After centrifugation (10000 x *g*, 20 min, 4°C) the pellet was dissolved in sterile distilled water and stored at –20°C. Protein concentrations were determined spectrophotometrically at 280 nm. Nisin, prepared as a 10% (w/v) suspension from NISAPLIN™ (1×10^6 I.U./g, Aplin & Barrett Ltd., Trowbrige, Wilts, England), was included in this study to investigate the effect on a mutant with an impaired cell wall structure. Samples were heat-treated for 10 min at 100°C and then tested for antimicrobial activity according to the spot-on-lawn method as described by Nel et al. [19].

4.2.3 PEDIOCIN PD-1 PRODUCTION IN GRAPE MUST

Chardonnay grape must (GM) with a sugar content of 25°Balling (B), pH 3.1 and 6.98 g/L total titratable acid was centrifuged (9800 x *g*, 10 min, 4°C) and used as such or diluted with distilled water (3:1) to a final sugar content of approximately 18°B. GM was supplemented with 1% (w/v) yeast extract (YE), 1% (w/v) bacteriological peptone (BP) and 2% (w/v) BP, respectively, to yield a final sugar content of approximately 18°B. GM was also supplemented with 1% (w/v) BP, 0.5% (v/v) Tween 80 and 0.014% (w/v) $MnSO_4$ (named complex GM, 18°B). All GM-based media were adjusted to pH 6 with 3N NaOH, 200 mL of each dispersed into 250 mL screw-capped glass bottles and pasteurized (100°C, 5 min). Each medium was inoculated with 1% (v/v) *P. damnosus* NCFB 1832 (OD_{600} = 0.8) and incubated at 25°C. Samples were removed at specific time intervals and examined for bacterial growth (OD_{600}) and the production of pediocin PD-1 by measuring antimicrobial activity against *O. oeni*, as described before.

4.2.4 EFFECT OF INITIAL PH ON THE ANTIMICROBIAL ACTIVITY OF PEDIOCIN PD-1

The effect of pH on the antimicrobial activity of pediocin PD-1 was determined by studying the influence of the peptide on viable cells of *O. oeni* in supplemented GM [1% (w/v) YE, 1% (w/v) BP], with a final sugar content of approximately 18°B, over a period of two months. Five sets of GM-based media were prepared, with each set consisting of six equal volumes of media (200 mL per 250 mL screw-capped glass bottle). Each of the media in a set was adjusted to a specific pH and then pasteurized, as described previously. The pH values in each set ranged from 3.5 to 6.0 (with intervals of pH 0.5). The first set of media served as control, that is with no pediocin PD-1 added. To each of four sets was added 20 AU/mL, 30 AU/mL, 40 AU/mL and 80 AU/mL pediocin PD-1 (heat-treated, as described before). Each of the media was inoculated with *O. oeni* to yield a final cell count of 1×10^6 cfu/mL. In a duplicate experiment with the exact same sets and conditions the media were inoculated to yield a final *O. oeni* cell count of 1×10^3 cfu/mL. Incubation was for two months at 25°C. Every third day the growth of *O. oeni* was monitored by optical density (OD) readings at 600 nm.

4.2.5 STABILITY OF PEDIOCIN PD-1 IN FERMENTED GRAPE MUST AT DIFFERENT PH VALUES

Chardonnay must (GM) fermented dry with *S. cerevisiae* (VIN13) and aged on the lees for one month was adjusted with 3N NaOH or 2N HCl to pH levels ranging from 2 to 9 in intervals of one pH unit. To each pH-adjusted sample (5 mL), pediocin PD-1 was added to a final concentration of 1600 AU/mL. Control samples received no bacteriocin. Samples were maintained at 4°C and 30°C and, at specific time intervals, tested for antimicrobial activity against *O. oeni,* as described before.

4.2.6 EFFECT OF ETHANOL, SO_2 AND TEMPERATURE

The same dry-fermented grape must as in the pH-stability tests was used. Volumes of 50 mL were supplemented with 15% (v/v) ethanol, 100 mg/L SO_2 (potassium metabisulfite) and a combination of ethanol and SO_2, respectively. Pediocin PD-1 was then added to each sample (final concentration 1600 AU/mL), incubated at 30°C and the antimicrobial activity determined at specific time intervals, as described before. The control media contained ethanol, SO_2 and a combination of both, but no pediocin PD-1. Samples which had been adjusted to pH 2, 3, 4 and 5, and to which pediocin PD-1 was added, were also heated for 20 min at 100°C and then tested for antimicrobial activity.

4.2.7 EFFECT OF PEDIOCIN PD-1 ON THE GROWTH OF *O. OENI* IN GM

Different concentrations of heat treated pediocin PD-1 (0, 10, 30, 60 and 80 AU/mL) were added to three sets of pasteurized GM media (200 mL per 250mL screw-

capped glass bottle) and adjusted to pH 3.8. The media used were GM diluted with water to yield a final sugar content of 20°B, GM (20°B), supplemented with 0.5% (w/v) YE (GM + YE), and AG-broth. Each of the media were inoculated with *O. oeni* to yield a final cell count of 1 x 10^6 cfu/mL and incubated at 25°C. Samples from the vessels were analyzed for viable bacterial counts over 25 days.

4.2.8 FERMENTATIONS

Fermentations were conducted in 200 mL GM (25°B) or GM (20°B), supplemented with 0.5% (w/v) yeast extract (GM + YE), in 250 mL screw-capped glass bottles fitted with fermentation locks. All fermentations were conducted at pH 3.8 by adjusting the must with sterile 3N NaOH. After inoculation with different strains of *S. cerevisiae*, or a combination of yeast and *O. oeni*, bacteriocin (pediocin PD-1 or nisin) were added at different concentrations and the cultures incubated at 25°C for approximately two months. Viable cells of *O. oeni* and yeast were detected by plating onto AG agar, supplemented with DELVOCID® (Gist-brocades, Delft, The Netherlands) (85 mg/L), and YPD-agar, respectively. All plates were incubated at 30°C. Loss in CO_2 was determined by weighing the fermentation vessels.

4.3 RESULTS AND DISCUSSION

All data represents an average of three repeats. The optical density readings and antimicrobial activity tests did not vary by more than 5%. In the present study, 1 AU nisin corresponded to 1.95 μg NISAPLIN™ and 1 AU pediocin PD-1 to 0.85 μg total protein.

4.3.1 GROWTH OF *P. DAMNOSUS* IN MODIFIED GRAPE MUST AND PRODUCTION OF PEDIOCIN PD-1

P. damnosus NCFB 1832 reached stationary growth after 100 hrs in GM supplemented with 1% (w/v) YE, 1% (w/v) BP, 2% (w/v) BP, or a combination of BP (1%, w/v), Tween 80 (0.5%, v/v) and $MnSO_4$ (0.014%, w/v) (Figure 1A). The optical density readings of cells in all four of these GM-based media were similar (OD_{max} at 600nm = 1.4 to 1.7), suggesting that the growth rates of the cells were approximately the same. No significant growth was recorded in non-supplemented GM (data not shown).

Grape must supplemented with a combination of BP, $MnSO_4$ and Tween 80 (complex GM) resulted in higher levels of pediocin PD-1 production (1600 AU/mL), compared to GM supplemented with 1% (w/v) and 2% (w/v) BP, which yielded activity levels of 150 and 300 AU/mL, respectively (Figure 1B). Furthermore, in complex GM pediocin PD-1 production increased throughout logarithmic growth (that is for 100 hrs), whereas in GM supplemented with 1 or 2% BP, production increased for 48 hrs only (Figure 1B). The levels of pediocin PD-1 activity remained constant for at least 10 days (Figure 1B), after which it declined to 800 AU/mL in complex GM

and to undetectable low levels in GM supplemented with BP (Figure 1B). No pediocin PD-1 activity was recorded in must supplemented with YE only.

The pH of the must decreased from 6.0 to 3.5 within the first 100 hrs of fermentation, with no significant variation recorded for strains cultured in the different grape must media (Figure 1C).

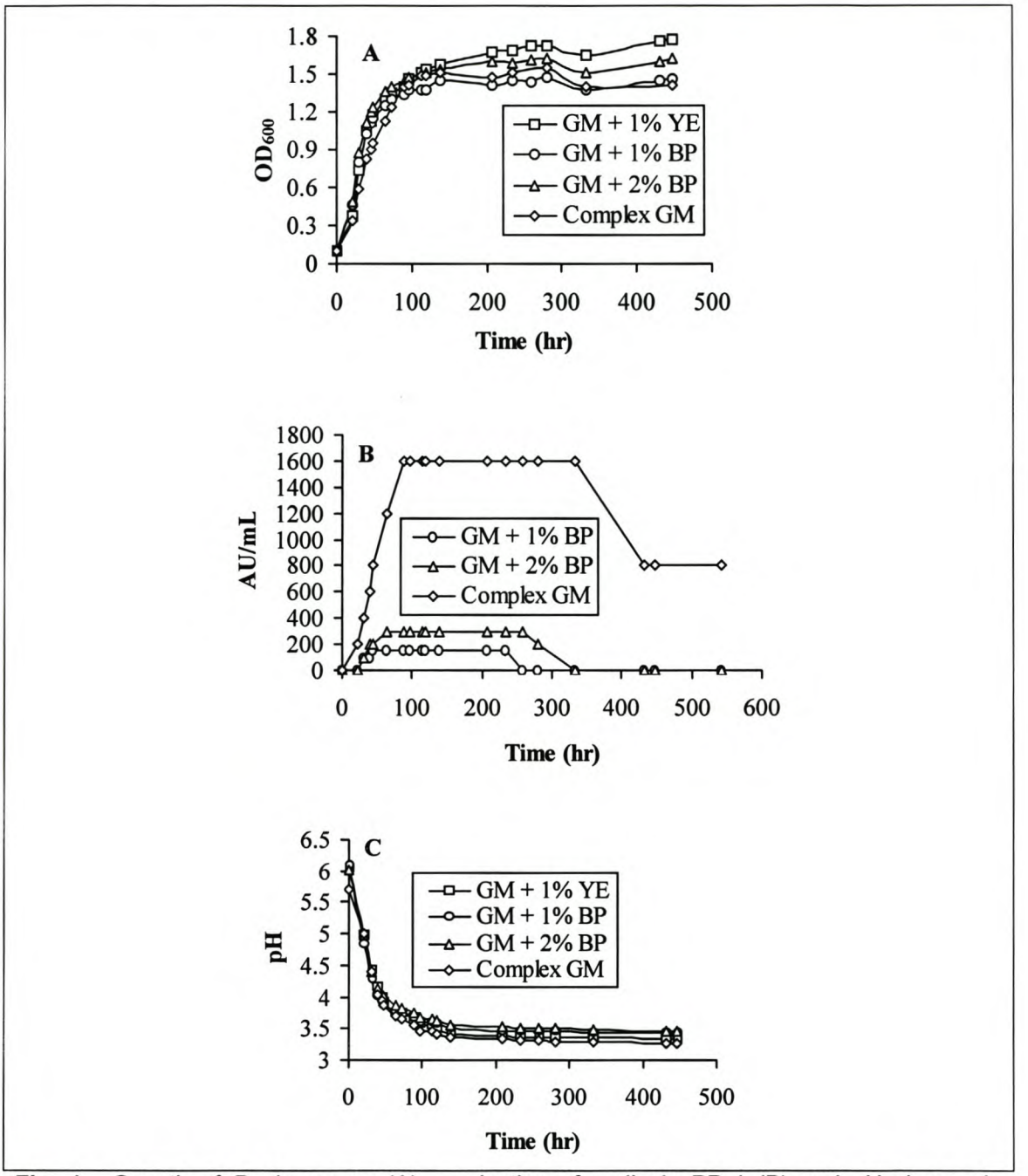

Fig. 1. Growth of *P. damnosus* (A), production of pediocin PD-1 (B) and pH change in growth media (C) as recorded in grape must (GM) supplemented with yeast extract (YE), bacteriological peptone (BP), and a combination of BP, $MnSO_4$ and Tween 80 (complex GM).

In a previous study [19], we have shown that production of pediocin PD-1 is influenced by the initial medium pH and complexity of the growth medium. Despite

the addition of Tween 80 and $MnSO_4$ to GM, as in complex GM, the maximum specific activity recorded was 1000 AU/mL/OD_{600}, which is approximately 80% lower than the activity recorded in modified MRS [19]. Grape must enriched with yeast extract only supported the growth of *P. damnosus* NCFB 1832 (Figure 1A), but without production of active pediocin PD-1. However, addition of pediocin PD-1 to GM that contained yeast extract led to the inhibition of *O. oeni*. This excluded the possibility of inactivation of pediocin PD-1 as a result of peptide binding to negatively charged groups in YE. LAB may be responsible for some of the antagonistic effects observed amongst LAB during vinification, and production of bacteriocins in grape must or wine may have a significant impact on the completion of MLF [17, 27]. Although pediocin PD-1 is stable in GM, the results suggest that GM without supplements does not contain the required factors for pediocin PD-1 production.

4.3.2 EFFECT OF PH ON PEDIOCIN PD-1 ACTIVITY IN SUPPLEMENTED GM

The influence of initial pH on the growth–inhibiting activity of pediocin PD-1 in GM supplemented with YE is shown in Table 1. In the absence of pediocin PD-1, the best growth of *O. oeni* was obtained at pH 5.0 and 5.5 (at day 12). In the presence of 20 AU/mL pediocin PD-1 no viable cells were detected after three days, indicating that the peptide inhibited the growth of *O. oeni* (data not shown). However, after 12 days, growth was recorded in most cultures, although the final biomass was still dependant on initial pH and in a pattern similar to that observed after day three. A concentration of 30 AU/mL completely inhibited the growth of *O. oeni* at an initial pH of 3.5, while 40 AU/mL were required at an initial pH of 6.0. A concentration of 80AU/mL pediocin PD-1 killed cells of *O. oeni* cultured at all the initial growth pH values studied (data not shown). With lower cell numbers of *O. oeni* (10^3 cfu/mL), 10 AU/mL pediocin PD-1 resulted in complete growth inhibition at an initial growth pH of 5.0, 5.5, and 6.0 (data not shown), which suggested an increased activity of pediocin PD-1 at this pH range. Under these conditions, a concentration of 20 AU/mL pediocin PD-1 resulted in the complete inhibition of cellular growth at all of the pH values studied. After two-months no viable cells of *O. oeni* were detected in samples where cellular growth had been completely inhibited during the first twelve day period.

Table 1. Effect of pediocin PD-1 concentration and initial pH on the growth (OD_{600}) of *O. oeni*. Innoculum: 10^6 cfu/mL.

Time	Pediocin PD-1	pH					
	(AU/mL)	3.5	4	4.5	5	5.5	6
Day 3	0	0.15	0.43	0.56	0.52	0.19	0
Day 12	0	0.83	1.05	1.15	1.27	1.24	1.06
	20	0.41	0.72	0.95	0.61	0.53	0.26
	30	0.03	0.64	0.91	0.49	0.47	0.06
	40	0.03	0.44	0.54	0.40	0.16	0.01

4.3.3 THE EFFECT OF PH, SO_2, ETHANOL AND TEMPERATURE ON THE STABILITY OF PEDIOCIN PD-1

At a temperature of 30°C the antimicrobial activity of pediocin PD-1 remained constant (1600 AU/mL) in dry-fermented GM at pH 2, 3, 4 and 5 for 48 hr (Table 2). Lower activity levels (1200 AU/mL) were recorded in the same must when the pH was adjusted to 6, 7, 8 and 9, respectively (Table 2). The activity levels decreased as incubation continued, with a marked lowering at higher pH values. After 48 hrs of incubation, no pediocin PD-1 activity could be recorded at pH 8 and 9. No loss in pediocin PD-1 activity was recorded after 28 days (672 hrs) at pH 3 and 4 (Table 2). Pediocin PD-1 was more stable, especially at higher pH levels, when incubated in the same medium, but at 4°C (Table 2). No change in the activity pattern was observed when SO_2, ethanol or a combination of ethanol and SO_2 were added (data not shown). No zones of growth inhibition were detected with control samples, that is in the absence of pediocin PD-1. Even in the presence of ethanol and SO_2, the peptide was stable at pH values ranging from 2 to 5 and for at least 20 min at 100°C (data not shown). A repeat of these experiments in pH-adjusted $NaPO_4$ buffer (0.1 M) yielded similar results (data not shown).

Table 2. Pediocin PD-1 stability in wine at different pH values at 30°C and 4°C.

Temp	Time (hrs)	pH							
		2	3	4	5	6	7	8	9
30°C	0	1600	1600	1600	1600	1600	1600	1600	1600
	3	1600	1600	1600	1600	1200	1200	1200	1200
	24	1600	1600	1600	1600	800	800	800	800
	48	1600	1600	1600	1600	800	600	0	0
	72	1600	1600	1600	800	800	400	0	0
	120	800	1600	1600	800	400	200	0	0
	672	800	1600	1600	400	200	0	0	0
4°C	0	1600	1600	1600	1600	1600	1600	1600	1600
	24	1600	1600	1600	1600	1200	1200	800	800
	96	1600	1600	1600	800	800	800	200	200
	672	1200	1600	1600	400	400	400	100	100

4.3.4 THE EFFECT OF PEDIOCIN PD-1 AGAINST *O. OENI* IN GM

After inoculation with *O. oeni*, different concentrations of pediocin PD-1 were added to GM, GM + YE and AG-broth adjusted to pH 3.8. Although the cells were rapidly killed by 30 AU/mL pediocin PD-1, a population of 4.8×10^3 cfu/mL was reached in GM after 8 days of incubation (Table 3). At a concentration of 60 AU/mL all cells grown in GM were killed, compared to 80 AU/mL required in AG-broth. Surprisingly only 30 AU/mL was required to kill *O. oeni* grown in GM + YE.

Table 3. Effect of different concentrations of pediocin PD-1 on the growth of *O. oeni* in grape must (GM), grape must supplemented with yeat extract (GM + YE) and acidic grape (AG) medium. Data presented as log cfu/mL. Innoculum: 10^6 cfu/mL *O. oeni*.

Growth Medium	Days	Pediocin PD-1 Activity Units (AU) per mL				
		0	10	30	60	80
GM	0.125	6.00	4.90	3.88	3.56	2.30
	4	7.00	3.70	0	0	0
	8	6.48	7.32	3.68	0	0
	17	6.28	5.95	3.72	0	0
	25	5.24	6.36	6.20	0	0
GM + YE	0.125	6.00	4.85	4.00	2.78	2.20
	4	6.48	2.68	0	0	0
	8	8.79	4.11	0	0	0
	17	8.70	6.78	0	0	0
	25	8.38	6.40	0	0	0
AG	0.125	6.00		5.18	3.11	2.85
	4	8.53		3.53	0	0
	8	8.56		6.49	3.08	0
	17	8.00		8.85	6.40	0
	25	7.56		7.76	6.46	0

Although the results suggest that Chardonnay GM has no significant inhibitory effect on pediocin PD-1 activity, it would be recommended to wait until after clarification before addition of the peptide. However, since pediocin PD-1 is heat stable at acidic pHs, it may be added to the must before pasteurization. Bacterial growth has been suggested as a possible cause of sluggish or stuck alcoholic fermentation [13]. Addition of pediocin PD-1 in the must will prevent bacterial growth during the course of alcoholic fermentation and may prevent fermentation problems associated with bacterial contamination in wines with reduced sulfites.

4.3.5 EFFECT OF PEDIOCIN PD-1 ON THE GROWTH AND FERMENTATION RATE OF *S. CEREVISIAE*

Yeast and filamentous fungi have a rigid cell wall, a complex structure consisting of glucan cross-linked with chitin and cell wall proteins [2]. Since mannoproteins are generally considered to be one of the key cell wall components determining cell wall porosity, they may represent the major barrier preventing free permeation of bacteriocin through the cell wall and thus access to the cytosolic membrane. Dielbandhoesing et al. [6] concluded that the cell wall protein Cwp2p plays a prominent role in protection of cells against antimicrobial peptides.

Although a high concentration of pediocin PD-1 and nisin was added to the must (1000 AU/mL), neither of these peptides effected the growth kinetics or fermentation rate of wine yeasts VIN13 and N96 (data not shown). Furthermore, at a final concentration of 1000 AU/mL, nisin and pediocin PD-1 had no effect on the growth

kinetics or fermentation rate of a yeast mutant with impaired cell wall structure (Δ*cwp2*) when compared to the parent strain (results not shown). The results suggest that, under winemaking conditions, bacteriocins have no inhibitory effect against yeast even if the cell wall is damaged.

4.3.6 THE EFFECT OF PEDIOCIN PD-1 ON THE SIMULTANEOUS DEVELOPMENT OF *O. OENI* AND YEASTS IN GRAPE MUST

Although grape must usually contains viable populations of 10^2 to 10^3 cells/mL of LAB and acetic acid bacteria [7, 8, 15], generally only *O. oeni* is isolated at the end of alcoholic fermentation (usually about 10^2 to 10^3 cfu/mL) [15].

In this study GM (pH 3.8) and GM + YE (pH 3.8) were inoculated simultaneously with a wine yeast strain (VIN13) and *O. oeni*. Depending on the vigor of growth of *O. oeni*, growth arrest of yeast occurred after 6 to 14 days in GM and 3 to 6 days in GM + YE, followed by a significant drop in the number of viable cells (Table 4). At an inoculum of 10^6 cfu/mL the bacteria reached the highest cell density after about two weeks, after which the number of viable cells started to decrease. In GM, in the presence of 25 AU/mL pediocin PD-1, no viable cells of *O. oeni* could be detected after two weeks of fermentation (detection limit: 10 cfu/mL). However, after about four weeks bacteria were detected. Screening of colonies that grew after pediocin PD-1 exposure revealed no increased resistance to the antimicrobial peptide. This suggests that at low pediocin PD-1 concentrations not all cells were killed. Over time the bacterial population increased from a few cells that have survived the treatment to a high density. No bacterial growth was observed in GM with 100 AU/mL of pediocin PD-1. In GM +YE *O. oeni* reached higher cell numbers and survived the presence of 100 AU/mL of pediocin PD-1. No growth occurred in the presence of 150 AU/mL of pediocin PD-1. At 10^3 cfu/mL, 25 AU/mL of pediocin PD-1 killed all cells of *O. oeni*, even in the presence of YE (data not shown).

4.4 CONCLUSIONS

The antimicrobial activity of SO_2 is significantly restricted at pH levels above 4. Although results suggest an increased efficiency in pediocin PD-1 activity at pH 5 and above, the stability of the peptide at pH values between 3 and 4 renders it suitable as a method to control *O. oeni* during MLF. The peptide is not sensitive to proteases or enzymes released during the aging of wine on the lees and are not effected by SO_2 or ethanol at concentrations required for vinification. We have shown that the addition of low concentrations of pediocin PD-1 to GM with mixed cultures of yeast and *O. oeni* leads to the inhibition of *O. oeni* without affecting the growth of yeast. At present, the addition of bacteriocin to wine is not authorized. However, pediocin PD-1 could provide the winemaking community with a safe alternative to SO_2 or at least replace part of the SO_2 currently added to must and wine.

Table 4. Effect of pediocin PD-1 on the development of wine yeast (VIN 13) and *O. oeni* when innoculated jointly into grape must (GM) and grape must supplemented with yeast extract (GM + YE). Data presented as log cfu/mL. Innoculum: 10^6 cfu/mL VIN 13, 10^6 cfu/ml *O. oeni.*

Growth Media	Days	*O. oeni*			VIN 13			
		Pediocin PD-1 Activity Units (AU) per mL						
		0	25	100	0	25	100	150
GM	0.125	6.30	4.00	2.70	5.58	5.76	5.45	
	3	6.78	1.70	0	7.64	7.30	7.00	
	6	7.48	1.48	0	7.52	7.75	7.66	
	14	8.04	0	0	7.95	7.11	7.95	
	20	7.58	0	0	5.85	6.00	5.98	
	27	5.62	2.60	0	4.3	4.70	4.48	
	34			0				
	65	6.00	5.20	0	0	0	0	
GM +YE	0.125	5.90	4.30	2.78	5.41			5.58
	3	5.69	2.91	1.00	7.52			7.70
	6	8.30	4.87	2.63	7.53			7.00
	14	8.70			5.85			6.90
	20	8.00			4.48			6.08
	23		7.08	7.00				
	27	6.51			4.46			6.00
	34							
	55	6.40	6.38	6.90	4.48			4.48

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CHAPTER 5

LITERATURE REVIEW

Mode of action of lipid II-targeting lantibiotics

LITERATURE REVIEW

Mode of action of lipid II-targeting lantibiotics

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ABSTRACT

The antimicrobial action of bacteriocins from Gram-positive bacteria is based on interaction with the cytoplasmic membrane of sensitive bacteria. Models based on studies with artificial membrane systems propose that nisin forms wedge-like poration complexes in the membrane based on electrostatic interaction between the positively charged C terminus of the peptide and anionic membrane phospholipids. Nisin can also permeabilise membranes via a targeted mechanism by using lipid II, the bactoprenol-bound precursor of the bacterial cell wall, as a docking molecule. Another consequence of binding with lipid II, is the inhibition of peptidoglycan biosynthesis. Mersacidine and actagardine also form a complex with lipid II, but binding only blocks the incorporation of lipid II into peptidoglycan, resulting in slow cell lysis rather than pore formation. Both peptides share a conserved sequence motif with plantaricin C and pediocin PD-1, which is most probably involved in the binding of these bacteriocins to lipid II. Although pediocin PD-1 and plantaricin C may inhibit peptidoglycan biosynthesis, pore formation is rather due to electrostatic interaction between the positively charged unbridged N-terminus and anionic phospholipids in the cytoplasmic membrane of sensitive cells. In the light of increased antibiotic resistance, this review focuses on the mode of action of lantibiotics that involve lipid II, possible candidates for the development of new generation novel antibiotic drugs.

5.1 INTRODUCTION

Lactic acid bacteria are the organisms of choice in numerous food fermentations, because they are safe to consume and bring about beneficial sensory changes. In addition to their fermentative abilities, they produce a variety of substances, such as bacteriocins, with antimicrobial activity. Bacteriocins are ribosomally synthesized peptides or proteins and are usually acting against closely related species. Since bacteriocins are odorless, colorless and non-toxic these, they adhere to the requirements set out for food preservatives (Abee et al., 1995).

Bacteriocins have been grouped into three classes based on their structure (Sahl et al., 1995). The lantibiotics (class I) are small heat-resistant peptides that undergo

post-translational modifications, leading to the formation of rings through reaction between dehydrated serine and threonine residues with the sulfhydryl group of cysteine (Jung et al., 1991). The group is further divided into two sub-groups, *viz.* the elongated, amphipathic, pore-forming type A lantibiotics (e.g. nisin), and the rigid, globular peptides of the type B category, e.g. mersacidin and actagardine (Sahl et al., 1995). Class II consists of small (<15 kDa), heat-stable, membrane-active, unmodified peptides (Sablon et al., 2000). Included in this class are the pediocin-like peptides, named after the first and most widely studied pediocin PA-1, produced by *Pediococcus acidilactici*. All pediocin-like bacteriocins have a consensus amino acid sequence motif in the N-terminal part of the mature peptide. Class III is represented by heat-labile proteins larger than 15 kDa.

The mode of action of bacteriocins is not yet fully understood. It is generally assumed that the type A subgroup of lantibiotics and the Class II bacteriocins disturb the integrity of the cell membrane, which leads to a rapid efflux of ions or cytoplasmic solutes (Sablon et al., 2000). Depolarization of the cytoplasmic membrane results in an instant termination of all biosynthetic processes. Models for nisin-membrane interactions suggest that the peptides form wedge-like poration complexes in the membrane (Breukink and de Kruijff, 1999). Results obtained with model membrane systems does not, however, explain results obtained with living cells, in particular since *in vivo* nisin minimum inhibitory concentration (MIC) values are much lower than that obtained with model membranes. These differences were explained by the discovery that nisin uses lipid II, a bactoprenol-bound precursor of the bacterial cell wall, as a docking station for pore formation (Brötz et al., 1998b). Binding to lipid II enhances the stability of pore formation, which increases the activity of the peptide (Wiedemann et al., 2001).

Type-B lantibiotics exerts their activity by binding to specific lipids in the cell membrane (Brötz et al., 1998b; Sahl and Bierbaum, 1998). Within this group, the mersacidine subtype was shown to inhibit peptidoglycan biosynthesis by forming a complex with lipid II (Breukink et al., 1999). The mechanism of action of the class III bacteriocins remains unclear (Klaenhammer, 1993).

5.2 STRUCTURAL ASPECTS OF LANTIBIOTICS

Lantibiotics are characterized by the presence of a high proportion of unusual amino acids, synthesized by post-translational side-chain modifications of ribosomally produced precursor peptides. The most prominent modifications include the thioether amino acids, lanthionine (Lan) and β-methyllanthionine (MeLan), and a number of dehydrated amino acids, such as the α,β-unsaturated amino acids, dehydroalanine (Dha) and dehydrobutyrine (Dhb) (Ingram et al., 1969). Dha and Dhb, resulting from the sequence-specific dehydration of serine and threonine residues, respectively, have electrophilic centers, which react with neighboring nucleofilic groups. Lan is formed when the double bond in Dha is coupled to the thiol

(-SH) group of an upstream cysteine residue. MeLan results when the reaction partner is Dhb. Due to these thioether bridges, a number of intra-molecular rings are formed, conferring a polycyclic structure to lantibiotics (Fig. 1). The chemistry of lantibiotics and the biosynthesis pathways have been discussed in several reviews (Sahl et al., 1995; Sahl and Bierbaum, 1998; Guder et al., 2000).

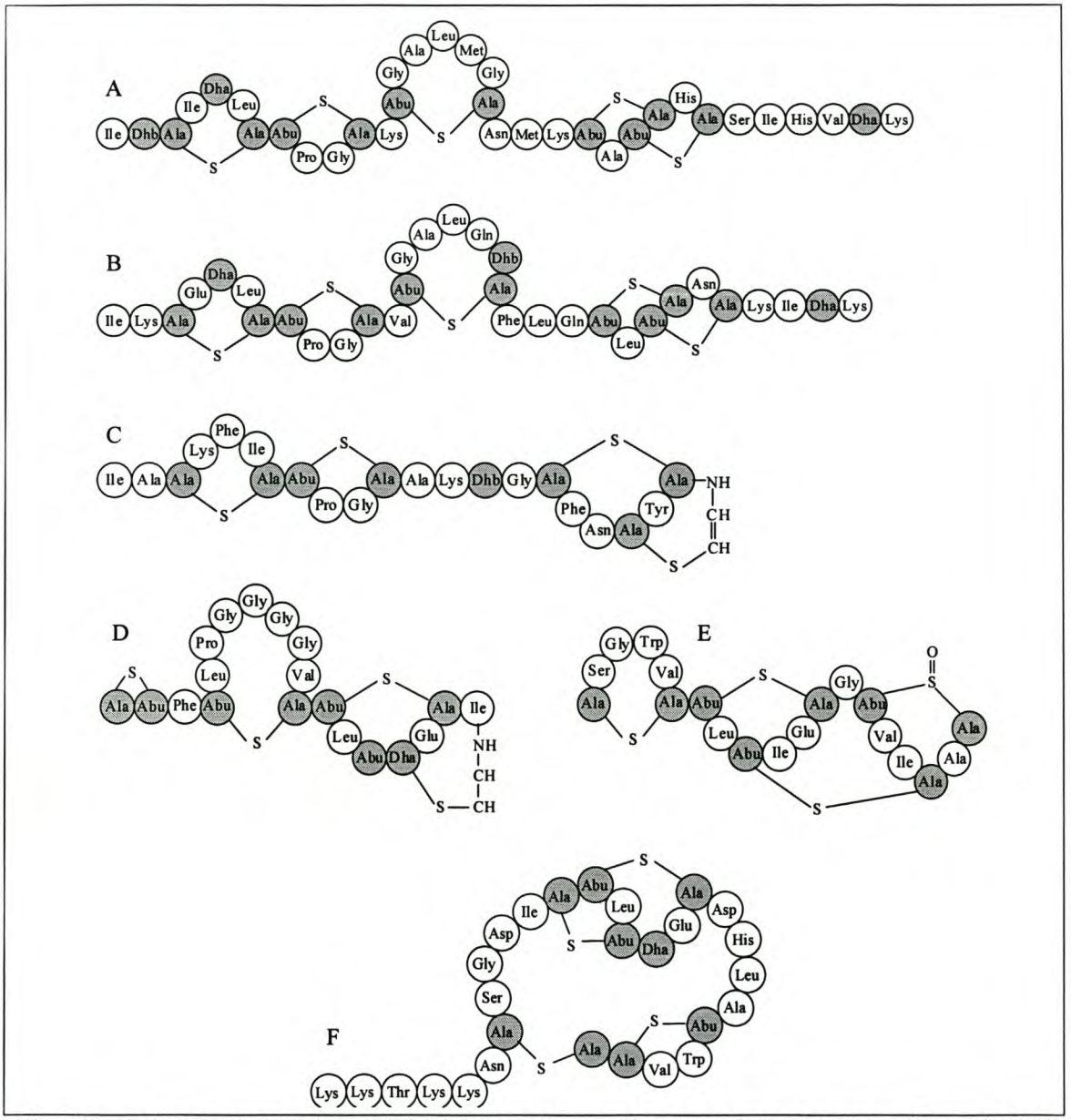

Fig. 1. Primary structures and bridging patterns of selected lantibiotics. The shaded residues indicate amino acids which have undergone post-translational modification. Ala-S-Ala: lanthionine; Abu-S-Ala: β-methyllanthionine; Dha: 2,3-didehydroalanine; Dhb: 2,3-didehydrobutyrine. Nisin A (A), subtilin (B) and epidermin (C) are typical elongated flexible peptides. Unlike the type A lantibiotics, the structure of mersacidin (D) and actagardine (E) is compact and globular. The bridging arrangement of plantaricin C (F) is not conclusive, it represent the most probable pattern of linkages. The peptide has a crossbridge C-terminus and a unbridged N-terminal part.

5.3 TYPE A LANTIBIOTICS

A typical type A lantibiotic is a flexible, elongated peptide with a net positive charge (Jung et al., 1991). The prototype is nisin, produced by *Lactococcus lactis* subsp. *lactis*. Other members of this group is the nisin-like peptide subtilin, produced by *Bacillus subtilis* ATCC 6633, and the lantibiotics epidermin, Pep5 and epilancin K7, all produced by *Staphylococcus epidermidis* (Sahl et al., 1995; Sahl and Bierbaum, 1998).

Nisin is a penta-cyclic peptide consisting of 34 amino acid residues of which 13 have been posttranslationally modified (Fig. 1). Two naturally occurring nisin variants, nisin A and nisin Z, are produced by lactococci (Buchman et al., 1988; Mulders et al., 1991). Nisin A differs from nisin Z in a single amino acid residue at position 27, being histidine in nisin A and asparagine in nisin Z. The structure of the nisin molecule, as determined by NMR, suggests that the peptide contains two well-defined amphipathic domains (Van den Hooven et al., 1996b). The N-terminal domain, amino acids 1 to 19, forms the first three lanthionine rings (A, B, and C). A flexible hinge region connects this domain with the second domain formed by residues 23-28. The second domain consists of intertwined rings D and E and is followed by a flexible region of six C-terminal amino acids.

5.3.1 BIOLOGICAL ACTIVITY OF NISIN

Nisin is bactericidal against a wide range of Gram-positive bacteria (Gross and Morell, 1971). It is also active against Gram-negative bacteria such as *Escherichia coli* and *Salmonella* spp., provided that the outer membrane is damaged (Stevens et al., 1991). Experiments with intact bacterial cells and isolated plasma membrane vesicles have shown that nisin molecules form pores in the membrane, which results in the rapid efflux of small cytoplasmic compounds, e.g. amino acids, potassium, inorganic phosphate, pre-accumulated rubidium, glutamate and ATP (Abee et al., 1994; Ruhr and Sahl, 1985). From the efflux of ATP, which has no transport system in the sensitive cells studied, and glutamate, which is not transported by a proton motif force (PMF)-driven system, it was concluded that small substances diffuse through nisin pores (Ruhr and Sahl, 1985; Sahl, 1991).

The increase in membrane permeability results in the collapse of vital ion gradients and in complete dissipation of both components of the PMF, i.e. transmembrane potential ($\Delta\psi$) and pH gradient (ΔpH), leading to a rapid cessation of all biosynthetic processes (Bruno and Montville, 1993; Ruhr and Sahl, 1985). The PMF drives ATP synthesis and the accumulation of ions and other metabolites through PMF-driven transport systems in the membrane (Harold, 1986). Collapse of the PMF leads to cell death through cessation of energy-requiring reactions. Nisin also inhibits bacterial cell wall biosynthesis *in vitro* (Linnett and Strominger, 1973; Reisinger, et al., 1980). The latter is, however, a comparatively slow process and pore formation is still considered the primary mode of action.

Nisin may also induce autolysis of susceptible staphylococcal cells (Bierbaum and Sahl., 1985). The peptides replace lytic enzymes from their cell wall intrinsic inhibitors. This apparent non-specific activation of cell-wall hydrolysis by nisin results in extensive cell wall degradation, particularly in the septum area between dividing daughter cells. Cell lysis is encouraged by a combination of increased osmotic pressure, which results from pore formation, and a weakened cell wall.

Another antimicrobial effect of nisin is the inhibition of spore outgrowth, attributed to the didehydroalanine residue in position 5 of the peptide (Chan et al., 1996a; Liu and Hansen, 1993). The double bond interacts with a spore-associated factor essential in sporulation.

Recently, nisin and subtilin have been shown to function as signal molecules for measuring the cell density of a population, a phenomenon called quorum sensing (Upton et al., 2001). The fully modified peptides induce transcription of the genes involved in their own biosynthesis in a pheromone-like manner.

5.3.2 NISIN INTERACTION WITH MODEL MEMBRANE SYSTEMS

A better understanding of the molecular mechanisms of membrane disruption by nisin has been gained by using various physiological and artificial membrane systems, e.g. intact bacterial cells and cytoplasmic membrane vesicles, red blood cells, liposomes, micelles, and planar lipid bilayers (reviewed by Benz et al., 1991; Sahl 1991; Breukink and de Kruijff, 1999). Findings from these studies suggest that nisin forms poration complexes in target cell membranes through a multi-step process that includes binding and insertion. However, precisely how pore complexes are formed is not yet known.

5.3.2.1 NISIN BINDING

The interaction between nisin and membrane components of sensitive cells is considered important in its mode of action. Nisin dissipates the PMF and causes carboxyfluorescein (CF) efflux from lipid vesicles which lack membrane proteins, therefore, a protein receptor is not required for nisin binding (Gao et al., 1991; Garcerá et al., 1993; Winkowski et al., 1996; Breukink et al., 1997). Anionic lipids in the membrane may serve as functional nisin binding sites. The cationic nature of nisin, allows it to bind to lipid bilayers through electrostatic interactions with phospholipid head groups (Driessen et al., 1995; Giffard et al., 1996, Martin et al., 1996; Winkowski et al., 1996; Breukink et al., 1997; Mol et al., 1997; Breukink et al., 2000). In lipid monolayers, nisin has a higher affinity for anionic lipids than for zwitterionic lipids (Demel et al., 1996). In lipid vesicles with varying ratios of anionic phosphatidylglycerol (PG) and zwitterionic phosphatidylcholine (PC), release of potassium and CF increased dramatically when the PG concentration exceeded 40%, suggesting that a large amount of anionic lipids are required for efficient nisin binding (Breukink et al., 1997). Isothermal titration calorimetry (ITC) experiments showed that interaction of nisin with charged membranes, consisting of 25% PG, is

mainly electrostatic, accounting for about 60% of the total free energy of binding (Breukink et al., 2000). However, nisin binds to neutral membranes, and binding under these conditions is hydrophobic.

Studies using fragments of nisin (Giffard et al., 1997), or nisin variants derived by mutagenesis or chemical modification (Breukink et al., 1997; Giffard et al., 1997; Moll et al., 1997), shed light on the structural elements in nisin responsible for membrane binding. The C-terminus of the peptide harbors most of the positive charge carried by the nisin molecule. Replacing valine at position 32 of nisin with a negatively charged glutamate residue drastically reduced the anionic lipid-dependent binding of nisin (Breukink et al., 1997), while binding was improved by replacing valine-32 with lysine, which increased the electrical charge by one unit (Breukink et al., 2000). However, adding more positive charges to the C-terminus appeared to be detrimental for the activity of nisin (van Kraaij et al., 1998). Changes in the N-terminus had only minor effects on nisin binding, and N-terminal fragments (N1-12 and N1-20) only loosely interacted with membranes (Giffard et al., 1997, Moll et al., 1997). Even a major change that resulted in opening of the first lanthionine ring and consequent loss of activity hardly affected the binding of this variant (Moll et al., 1997). These results suggest that the C-terminus plays an important role in binding, initiating electrostatic interaction between nisin and the target membrane.

5.3.2.2 NISIN INSERTION

Due to the amphiphilic properties of the peptide, a nisin molecule inserts its hydrophobic side into the outer leaflet of a bilayer, being located within the head group region (van den Hooven et al., 1996a and b). Although hydrophobic residues, positioned at the opposite side of the hydrophilic residues, are located throughout the nisin molecule, the N-terminus of the peptide is the most hydrophobic (Lins, et al., 1999). Monolayer studies revealed that anionic phospholipids are essential for efficient insertion of nisin in the lipid phase of the membrane (Breukink et al., 1997; Demel et al., 1996) and are maximal in monolayers composed of more than 60% PG (Breukink et al., 1997). Nisin variants with N-terminal extensions or with minor changes in the first ring displayed reduced abilities to insert into lipid monolayers and are paralleled by lower antimicrobial activity, while changes in the C-terminus hardly affected nisin insertion (Breukink et al., 1997; van Kraaij et al., 1998). Membrane insertion and activity is also affected when the flexibility of the hinge region, which connects ring C and D, is reduced (Demel et al., 1996). Mol et al. (1997) suggested that this region might act as a twist that allows nisin to bend the lipid surface.

The insertion step has also been studied using nisin variants containing fluorescent tryptophan residues introduced at positions 1, 17 and 32 of the nisin molecule (Breukink et al., 1998). The N-terminal tryptophan residue had the deepest location in the membrane, while the C-terminal tryptophan was located close to the membrane surface. An increase in the concentration of anionic lipids increased the

depth of insertion. The results also showed that the stable orientation of nisin in the membrane is parallel with respect to the membrane surface.

Together, these results suggest that primarily the N-terminal part of nisin inserts into the lipid phase of the membrane and that nisin does not penetrate the lipid bilayer, but rather binds to the head group region in an orientation parallel to the plane of the bilayer.

5.3.2.3 PORE FORMATION

Lantibiotics form large non-specific pores, mediating the efflux of ions, amino acids and ATP from cells, and ions and carboxyfluorescein (CF) from liposomes. Since lantibiotics are small peptides that can span the membrane only once, it is assumed that several molecules associate with the membrane to form a pore. Several studies have suggested that insertion is followed by aggregation (Garcia-Garcerá et al., 1993; Giffard et al., 1996; Breukink et al., 1997; Breukink et al., 1998). However, according to Sahl et al. (1991), this would imply exposure of charged groups in the lipid phase of the bilayer, which is energetically highly unfavorable. The authors have suggested pre-aggregation of the peptides at the surface of the bilayer. It is unknown how many nisin monomers are required to form a pore, but it is probably a dynamic process in which peptides are joining and leaving the transmembrane oligomer pore complex (McAuliffe et al., 2001).

In black lipid membranes the estimated size of the nisin pore was 1 nm, depending on the lantibiotic concentration and the pH (Benz et al., 1991). The pore lifetime was in the order of milliseconds. Nisin formed similar pores in lipid vesicles (Garcerá et al., 1993; Winkowski et al., 1996). Although the stable orientation of nisin in the membrane is parallel with respect to the membrane surface (Breukink et al., 1998), the pore is assumed to be of transient nature, with nisin obtaining a transmembrane orientation during pore formation (van Kraaij et al., 1998; Breukink and de Kruijff, 1999). The pores appear to be anion-selective, since leakage of negatively charged CF was more efficient than leakage of positively charged potassium ions (Breukink et al., 1997). The authors suggested that anion-selectivity may be dependent on the alignment of positively charged residues in the pore. Studies on a nisin variant with a single mutation, in which lysine at position 12 was replaced with a leucine residue, supports such a hypothesis (Dodd et al. 1996). The removal of a positive charge from the N-terminal domain increased nisin-induced membrane conductance and decreased dependency on a threshold potential. Replacement of lysine with a hydrophobic residue probably facilitates the insertion of nisin. These results suggest that Lys-12 plays an important role in controlling the flow of ions through the nisin pore in lipid bilayers (Giffard et al., 1997).

In the absence of anionic phospholipids, nisin can act as an anion selective carrier (Driesen et al., 1995). After transversing across the membrane of CF-filled liposomes composed of cationic phospholipids, the positively charged nisin molecules bind the negatively charged CF molecules and subsequently traverse

across the membrane as a binary complex with the anion. On the outer membrane surface, the anion is released and nisin returns to bind another molecule on the inside. This activity is strongly inhibited in liposomes composed of anionic phospholipids. Strong electrostatic interaction leads to surface localization of nisin, therefore, little or no anion carrier activity is expected *in vivo*.

One of the unanswered questions about the action of nisin is the role of lipid composition. Although nisin binds better to negatively charged membranes than neutral membranes, the same amount of bound peptide resulted in similar levels of membrane leakage (Breukink et al., 2000). Bonev et al. (2000) have shown that nisin can selectively recruit acidic lipids such as the negatively charged lipid PG and is driven, at least initially, by electrostatic interactions. The enrichment of the lipid environment of nisin in PG, as compared to the overall lipid composition, suggests that the negatively charged lipids in the membrane play a more active role than just providing binding sites (Breukink and de Kruijff, 1999).

Trypsin digestion experiments revealed that at least the C-terminal part of nisin translocates across model membranes upon pore formation (van Kraaij et al., 1998). A likely event after transient transmembrane pore formation would be translocation of the whole nisin molecule to the inside of the membrane (van Kraaij et al., 1998), as has been described for magainin, an antimicrobial peptide of animal origin (Matsuzaki et al., 1995).

5.3.3 ENERGY REQUIREMENTS FOR PORE FORMATION

The energized state of a sensitive cell is critical for the pore-forming activity of nisin (McAucliffe et al., 2001). Energy is required for both formation and opening of pores. The electrical transmembrane potential ($\Delta\psi$), as generated by metabolizing cells, is considered the major driving force for activity. The membrane potential lowers the energy barrier for pore formation (Giffard et al., 1997). Black-lipid membrane experiments revealed that nisin can form pores only when a *trans*-negative (inside negative) electrical potential is applied. A calculated threshold potential of approximately –80mV is required for nisin activity (Sahl et al., 1987; Kordel et al., 1989). However, nisin is not exclusively a voltage-dependant bacteriocin. In the absence of a membrane potential, nisin induced leakage of dye from model membrane vesicles containing high levels of anionic lipids (Benz et al., 1991). Nisin also permeabilized lipid vesicles in the absence of $\Delta\psi$, although the presence of $\Delta\psi$ increased poration (Garcerá et al., 1993; Breukink et al., 1997). Mol et al. (1997) furthermore demonstrated the ability of nisin to dissipate the ΔpH of *Lactococcus lactis* cells in the absence of $\Delta\psi$. However, the rate of ΔpH dissipation depended on the magnitude of the ΔpH (*cis*-acid).

5.3.4 MODELS FOR NON-TARGETED PORE FORMATION

Several tentative models have been proposed for the mechanism of pore formation by lantibiotics. Initially a barrel-stave model was suggested for type A lantibiotics

(Sahl et al., 1987), which was later modified to a wedge model (Driessen et al., 1995; van den Hooven et al., 1996a and b). The models differ with regard to the mode of insertion of the molecule into the target membrane (Montville and Chen, 1998). PMF components enhance (barrel-stave model), or mediate (wedge model) insertion and pore formation. According to the barrel-stave model, inserted nisin monomers aggregate laterally to form pores (Fig. 2A). The non-polar side chains of the peptides interact with the hydrophobic lipid core of the membrane, and the hydrophilic side-chains of the peptides point inward, which results in the formation of a water-filled pore. In the wedge model, pore formation is caused by the local perturbation of the lipid bilayer that occurs when nisin molecules bind. Due to its amphiphilic nature, a nisin molecule not only interacts with the phospholipid head groups via ionic forces, but also inserts its hydrophobic side into the outer leaflet of a bilayer. Several peptide molecules could then form a pore by moving through the membrane in response to the electrical potential, while remaining surface-bound and carrying lipids across. The orientation of nisin molecules relative to the lipid headgroups remains unchanged. Thus, contrarily to the barrel-stave model, the peptide does not make contact the hydrophobic core of the membrane. In both models the hydrophilic sides of the nisin molecules face the lumen of the pore.

Pore formation is summarized in figure 2. Nisin first binds with its C-terminus via electrostatic interactions with the anionic lipids (Fig. 2B, step 1). This is followed by the insertion of the N-terminus into the lipid phase of the membrane while the peptide adopts an overall parallel orientation with respect to the membrane surface (Fig. 2B, step 2). The nisin molecule subsequently obtains a transmembrane orientation without losing contact with the membrane surface and thereby distorts the lipid bilayer to form a short-lived pore. Assuming a situation where the phospholipids are active members of the pore, nisin will follow the wedge model of pore formation as shown in Fig. 2B (step 3). Pore formation is, however, transient, such that the peptides might rapidly flip back to the original parallel orientation in the outer leaflet.

Similar to the wedge model, a wormhole model was proposed for the frog skin defensin peptide magainin (Ludtke et al., 1996). Phospholipids are active members of the pores formed by magainin. The pores resemble a torus, in which the outer monolayer of the membrane folds back to the inner monolayer and the lipids in the pore possess an overall positive curvature (Matsuzaki et al., 1998). Although direct evidence is lacking, Breukink and de Kruijff (1999) speculated on the possibility of nisin possessing a similar pore structure. Upon insertion into the membrane, nisin recruits negatively charged lipids and may furthermore disturb the phopholipids in such a way that it induces a positive interface curvature. Since the N-terminus of nisin is located deeper within the lipid phase of the membrane (Fig. 2C, step 2), it is possible that initiation of pore formation results in translocation of the N-terminus to the inner leaflet of the membrane (Fig. 2C, step 3). After transient pore formation, the whole nisin molecule may be translocated to the inside of the membrane as has been described for magainin (Fig. 2C, step 4).

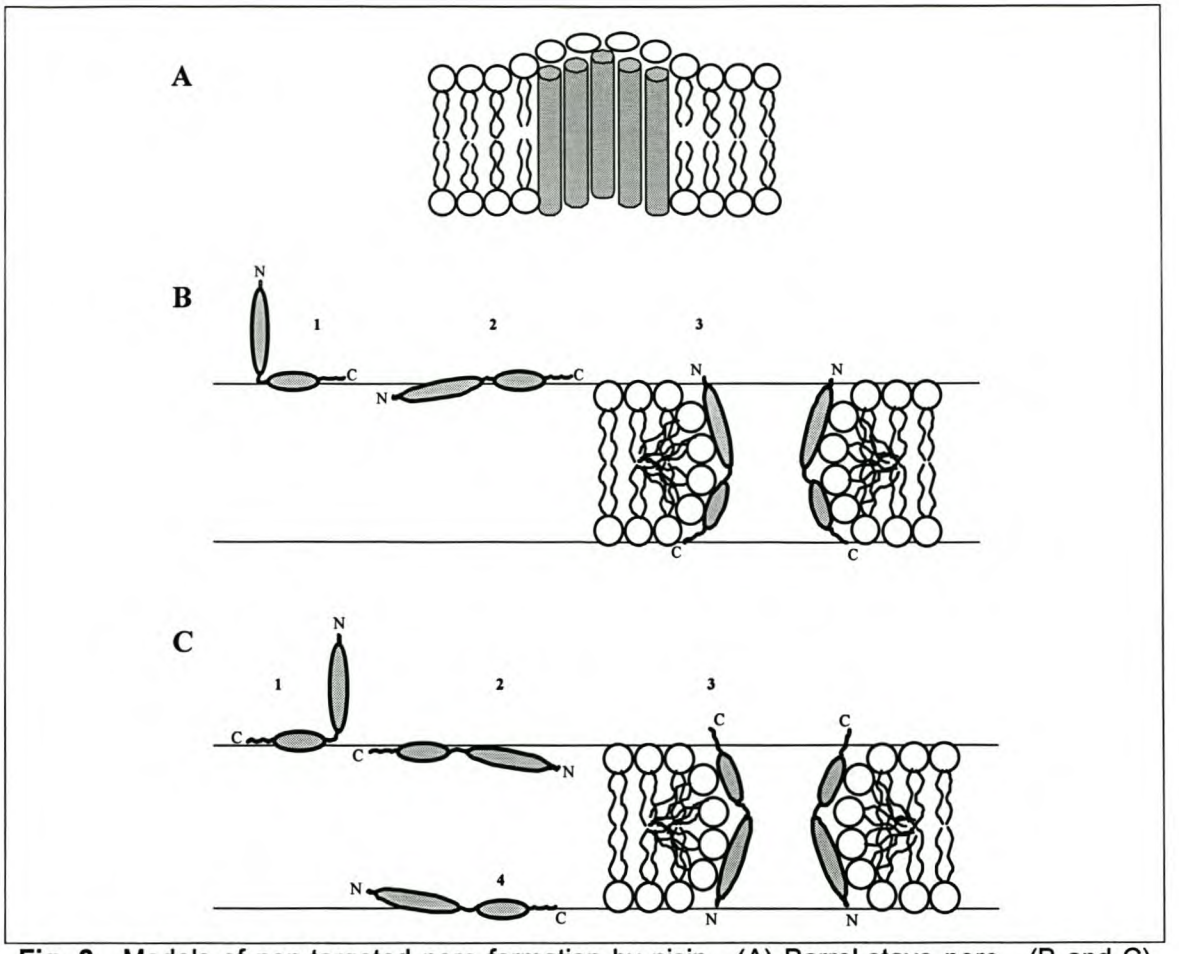

Fig. 2. Models of non-targeted pore formation by nisin. (A) Barrel-stave pore. (B and C) General models for pore formation. Step1: Binding of nisin via its C-terminal; Step2: Insertion of nisin into the membrane. The depth of insertion depends on the percentage of anionic lipids and nisin concentration. Step3: Wedge/Megainin-like pore. Diagram B and C represents pore formation initiated by translocation of the C-terminus and N-terminus, respectively. Step 4: Translocation of the peptide to the inside of the membrane.

How well these models describe pore formation by bacteriocins from other LAB is not known. Contradictory evidence supporting one or the other model reflects the complex nature of pore-formation, and bacteriocin-membrane interactions are far from fully understood. Whether or not phosphoplipids are active members of the pore remains to be determined. Furthermore, none of these models takes specific binding with an integral membrane component into account. The intrinsic binding constant of nisin to model membranes is about a factor of 10 lower than that of magainin (Breukink et al., 2000). This is in correspondence to the higher activity of magainin towards model membrane systems compared to that of nisin (Breukink and de Kruijff, 1999). However, the MIC-values determined against Gram-positive bacteria, are a factor of 100-1000 higher for nisin. Such activity of nisin and related peptides *in vivo* are generally in the nano molar range, while activity in model membrane systems are in the micro molar range (Breukink et al., 1999; Wiedemann et al., 2001). Thus, despite the similar modes of action of nisin and magainin towards

model membranes, there must be a factor present in the membrane of sensitive microbial cells which increases the activity of nisin activity *in vivo*.

5.4 TYPE B LANTIBIOTICS

The rigid, globular Type-B lantibiotics consist of approximately 20 amino acids only and have no charge or are negatively charged. They are further divided into the cinnamycin and mersacidine subtypes (Sahl et al., 1995) and exert their activity by binding to specific membrane lipids (Brötz et al., 1998b). They are also described as inhibitors of a number of important enzymes (Sahl and Bierbaum, 1998). The cinnamycin-like peptides inhibit phospolipases by forming a complex with phosphatidylethanolamide (Hosoda et al., 1996). Treatment of sensitive cells with members of the cinnamycin subtype interfered with several membrane transport systems (Chen and Tai, 1987; Navarro et al., 1985) and treatment of planar membranes resulted in the formation of defined pores (Sheth et al., 1992). Based on their structure and biological activity, mersacidine, produced by a *Bacillus* sp. and actagardine, produced by an *Actinoplanes* sp., were grouped into the mersacidine subtype (Sahl et al, 1995). Both peptides contain four intramolecular thioether bridges, formed predominantly by β-methyllanthionine residues (Fig. 1). In addition, ring B of actagardine (amino acids 5-13) and ring C of mersacidine (amino acid 11-18) are identical, except for one difference (Ile in actagardine is replaced by Dha in mersacidine). These covalent intramolecular junctions impose considerable conformational constraints on the molecules, resulting in a globular shape, restricted flexibility, and stability against protease degradation (Brötz et al., 1997).

Mersacidine and actagardine are of similar sizes (1,825 and 1,890 Da, respectively) and hydrophobicities and have no net charge. Both peptides inhibit cell wall biosynthesis by inhibiting the incorporation of glucose and D-alanine into cell wall material, whereas DNA, RNA and protein synthesis are not affected (Somma et al., 1971; Brötz et al., 1995). Treatment of sensitive cells with mersacidine results in the cessation of growth and a slow induction of lysis rather than pore formation (Brötz et al., 1995). Killing does not occur before the completion of at least one cell cycle. Mersacidine exerts its activity by forming a high affinity complex with lipid II, a membrane-bound precursor in peptidoglycan synthesis (Brötz et al., 1998a and b). Mersacidine has attracted recent attention due to its *in vivo* activity (Limbert et al., 1991; Niu and Neu, 1991). The peptide effectively cured systematic staphylococcal infections in mice, including methicilin-resistant *S. aureus* (MRSA), and subcutaneous staphylococcal abscesses in rats. Since treatment of MRSA is currently only possible with the glycopeptide antibiotics vancomycin and teicoplanin, and resistant strains are emerging (Hiramatsu et al., 1997), this type of lantibiotic is the most promising antimicrobial agent with respect to a potential chemotherapeutical application.

Bauer et al. (2003) tentatively included pediocin PD-1 (2867 Da), produced by *Pediococcus damnosus* NCFB1832, and plantaricin C (2880 Da), produced by *Lactobacillus plantarum* (Turner et al., 1999), into the same group. These peptides share a conserved sequence motif with mersacidine and actagardine that comprises one entire ring structure (see Fig. 1 and Fig. 3). Contrarily to other Type B lantibiotics, plantaricin C and pediocin PD-1 have a net positive charge and in contrast to the mersacidine subtype, these peptides not only induce cell lysis, but also pore formation. Interaction with the cytoplasmic membrane of sensitive cells, leading to pore formation, may depend on the positive charge of the N-terminal end (four out of the first five amino acids are lysines), which would be attracted by the negative charge of the phospholipids. Insertion of the essentially uncharged, hydrophobic C-terminal part of the peptides into the membrane could be accomplished while the N-terminal part remains attached to the phospholipid head groups because of the flexibility that the molecules have around amino acid 6 (Bauer et al., 2004; Turner et al., 1999). In addition to the inhibition of cell wall biosynthesis and pore formation, treatment of actively growing sensitive cells resulted in rapid cell lysis which is characteristic of the induction of autolysis (Bauer et al., 2003).

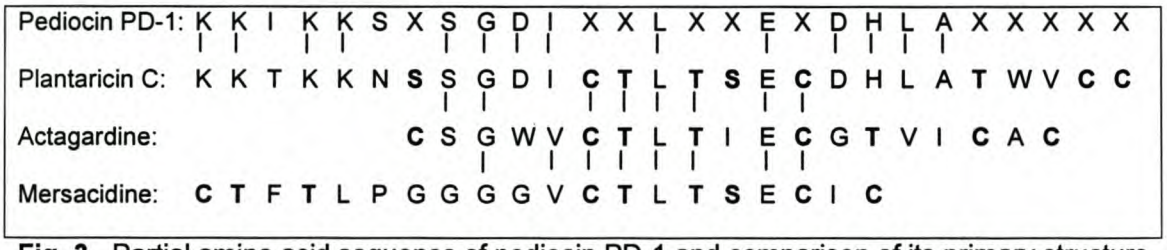

Fig. 3. Partial amino acid sequence of pediocin PD-1 and comparison of its primary structure with homologous bacteriocins. Identical residues are marked, unidentified residues are represented by X, and postulated amino acids before modification are in bold.

5.5 LIPID II INTERACTION

Current models for pore formation may describe the behavior of peptides in pure lipid bilayers, where high peptide concentration are required to induce effects, but it does not explain why *in vivo* nisin MIC values are two to three orders of magnitude lower. The differences in results obtained with living cells were explained by the discovery that nisin uses lipid II (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc), the bactoprenol-bound precursor of the bacterial cell wall, as a docking molecule for subsequent pore formation (Brötz et al., 1998b). Although mersacidine and glycopeptide antibiotics such as vancomycin, use the same target, the molecules do not bind to the same part of lipid II. The complex chemical structure of lipid II provides several possible recognition epitopes. Lipid II is composed of a peptidoglycan head group, MurNAc-(pentapeptide)-GlcNAc, that serves as the basic building block for the cell wall and of a pyrophosphate (PP)-undecaprenyl lipid tail that functions as the carrier for the transport of the peptidoglycan moeity from the cytoplasm to the extracellular domain (Fig. 4).

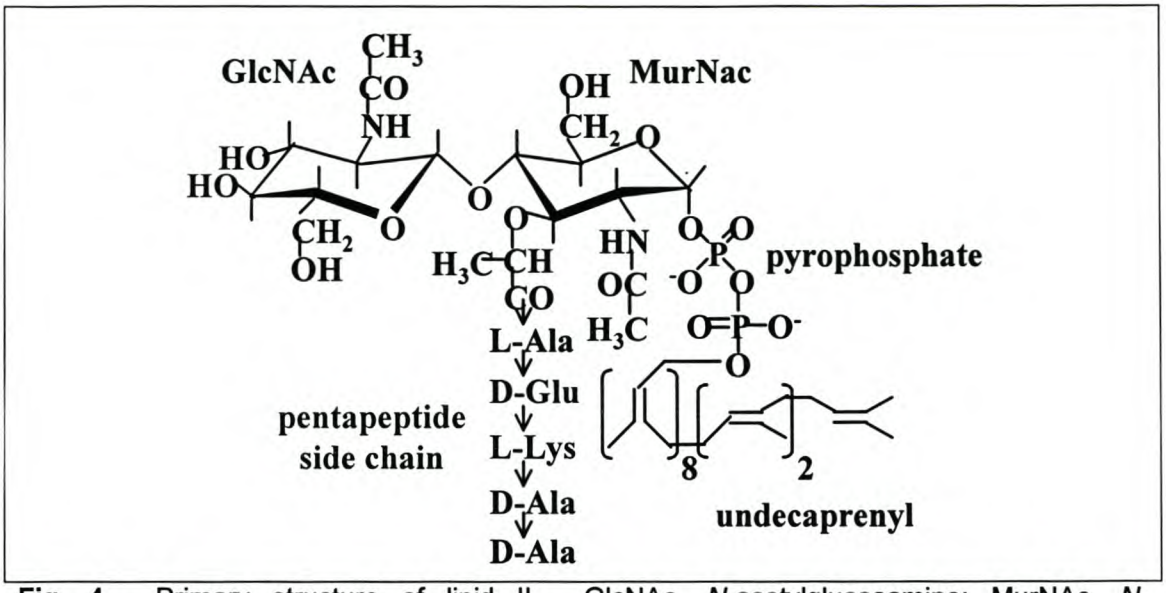

Fig. 4. Primary structure of lipid II. GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid (adapted from Hsu et al., 2002).

5.5.1 PEPTIDOGLYCAN BIOSYNTHESIS CYCLE

The peptidoglycan biosynthesis cycle comprises two lipid-bound intermediates, lipid I and lipid II, synthesized at the cytosolic side of the plasma membrane (Breukink et al., 1999). Lipid I is formed via the transfer of the phospho-*N*-acetyl muramic acid (MurNAc)-pentapeptide group from the uridine 5'-diphosphate (UDP)-activated amino sugar MurNAc-pentapeptide to the membrane-bound lipid carrier undecaprenyl-pyrophosphate. Lipid II results from coupling of a second UDP-activated amino sugar *N*-acetyl-D-glucosamine (GlcNAc) to the MurNAc group of lipid I. Subsequently, lipid II is transported to the exterior side of the membrane. The two amino sugars and the pentapeptide are then coupled to the cell wall and converted into peptidoglycan. The cycle is completed once the remaining undecaprenylpyrophosphate is transported back to the cytosolic side of the membrane where it is dephosphorilated to undecaprenylphosphate.

5.5.2 BINDING OF NISIN TO LIPID II

Nisin and subtilin inhibited bacterial cell wall biosynthesis *in vitro* (Linnet and Strominger, 1973). This was later shown to be due to the formation of a complex between nisin and lipid II (Reisinger et al., 1980). Brötz et al. (1998b) studied the nisin subtype of lantibiotics with respect to their affinity for lipid II. The authors demonstrated that, *in vitro* and *in vivo*, lipid II has a high affinity for nisin and epidermin, but not for Pep5 and epilancin K7. The study suggests that nisin and epidermin use lipid II as a docking station for specific binding to bacterial membranes and that lipid II binding decreases the energy required for nisin insertion into the membrane. Breukink et al. (1999) demonstrated that an increase in lipid II concentration in the range of 0.001 to 0.1 mol% increased the sensitivity of model

membranes to nisin. The activity of nisin in these model systems is in the range of activity against intact cells. Although nisin binds to lipid I and lipid II, nisin activity is dependant only on interaction with lipid II. The diverse sensitivities to nisin displayed by different bacteria may be caused by different concentrations of lipid II in the membrane or differences in the accessibility of lipid II for nisin.

Several groups suggested that the N-terminal ring structures of nisin are important for the interaction of nisin with lipid II (Chan et al., 1996b; Brötz et al., 1998b; Breukink et al., 1999). From comparison of structural features of nisin and epidermin with Pep5 and epilancin K7, Brötz et al. (1998b) suggested that the first two rings are important for lipid II binding. It was subsequently shown that nisin mutants with small variations in the three N-terminal rings displayed not only reduced bactericidal activity, but also strongly influenced interaction with lipid II (Breukink et al., 1999). Chan et al. (1996b) identified an N-terminal nisin fragment (residues 1-12) as a strong antagonizer of nisin towards sensitive cells. The fragment was not, however, able to antagonize the activity of subtilin. This suggests that the variations between the N-terminal domains of nisin and subtilin (Fig. 1) could have increased the affinity of subtilin for lipid II with respect to the affinity of nisin for lipid II (Breukink and de Kruijff, 1999).

With respect to lipid II, the structural requirements for the interaction with nisin, subtilin or epidermin remains to be elucidated. The lipid moiety is not sufficient to promote pore formation, since incorporation of undecaprenol and dodecaprenylphosphate did not render liposomes susceptible to these lantibiotics (Brötz et al., 1998b). The affinity of nisin and epidermin appeared to be the same for lipid I and lipid II, which are distinguished by a mono-(MurNAc) and a disaccharide (MurNAc-GlcNAc) moiety, respectively, suggesting that MurNAc is important for the interaction with these lantibiotics. Breukink et al. (1999) speculated on the possibility of the sugar backbone of MurNAc being the recognition site of nisin. This was based on the similarities between the sugar backbone of lipid II of nisin sensitive *Methanobacterium* spp. and that of eubacteria. The pyrophosphate (PP) and/or the undecaprenyl moiety could, however, be involved in the interaction, since recognition of the sugar alone would mean that the entire cell wall would act as a massive sink for nisin (Breukink and de Kruijff, 1999).

The presence of lipid II resulted in a dramatic enhancement of nisin pore lifetime from milliseconds to several seconds, causing the peptide to be highly active (Wiedeman et al., 2001). Mutations affecting the conformation of rings A through C led to reduced affinity of the peptide for lipid II rather than affecting its pore formation activity. However, nisin peptides mutated in the flexible hinge region with an unaltered ability to bind to lipid II were unable to form pores, but had only a slightly reduced activity *in vivo*. These results suggest that nisin combines two very efficient killing mechanisms, mediated by lipid II, in one molecule. Nisin blocks lipid II from incorporation into peptidoglycan (Fig. 5) and simultaneously uses lipid II for targeted

pore formation. Lipid II mediation increases nisin activity from the micro molar to the nano molar range.

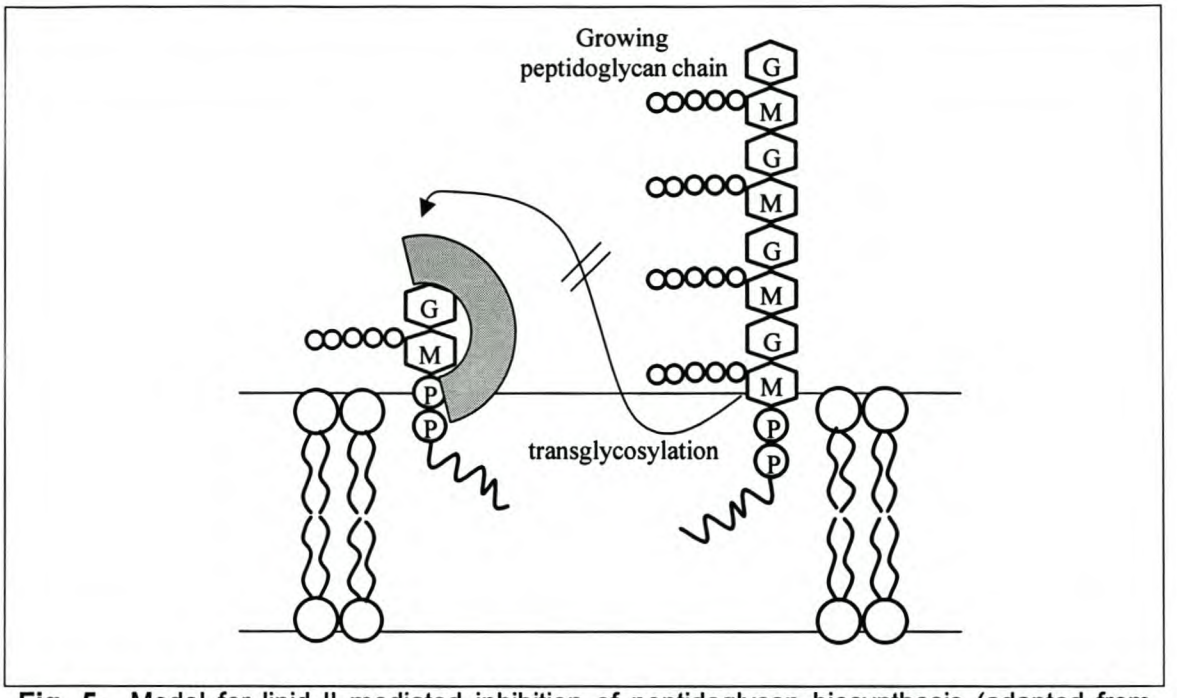

Fig. 5. Model for lipid II mediated inhibition of peptidoglycan biosynthesis (adapted from Guder et al., 2000). Lantibiotics (marked by shading) such as nisin and the mersacidine subtype bind to lipid II, thereby blocking the polymerization of the peptidoglycan. Nisin and mersacidine-like peptides do not bind to the same part of lipid II.

5.5.2.1 MODEL FOR LIPID II-MEDIATED NISIN PORE FORMATION

In the presence of lipid II, nisin can disturb the cytoplasmic bilayer at nanomolar concentrations. Wiedemann et al. (2001) proposed that the N-terminal part of nisin first binds to the outwardly orientated carbohydrate moiety of lipid II in a 1:1 stoichiometry (Fig. 6, step 1). A negative surface charge is not required for this interaction. Binding is followed by aggregation of the nisin-lipid II complexes, which result in conformational rearrangements within the first two N-terminal rings (Hsu et al., 2002). The flexible hinge region, connecting rings C and D, may allow bending of the C-terminal part of the nisin molecule and thus insertion into the membrane. The C-terminus is then assumed to translocate across the membrane and thus the inwardly orientated lipid moiety of lipid II becomes available for interaction with nisin (Fig. 6, step 2). Interaction probably occurs between the ε-amino groups of the lysine side chains of nisin and the pyrophosphate groups of lipid II (Wiedemann et al., 2001). The presence of lipid II enhances pore lifetime dramatically, the pore formation process becomes voltage-independent, and the anion selectivity of the pore is reduced. Interactions with the lipid II moiety therefore stabilize the transmembrane orientation of the nisin peptides. Several lipid II-nisin complexes may assemble to form a functional pore. The position of the lipid moiety of lipid II in the bilayer remains unknown and no information is available regarding the pore opening process. After transmembrane pore formation, the entire nisin molecule

might be translocated to the inside of the membrane, in accordance with possible translocation in the absence of lipid II (van Kraaij et al., 1998).

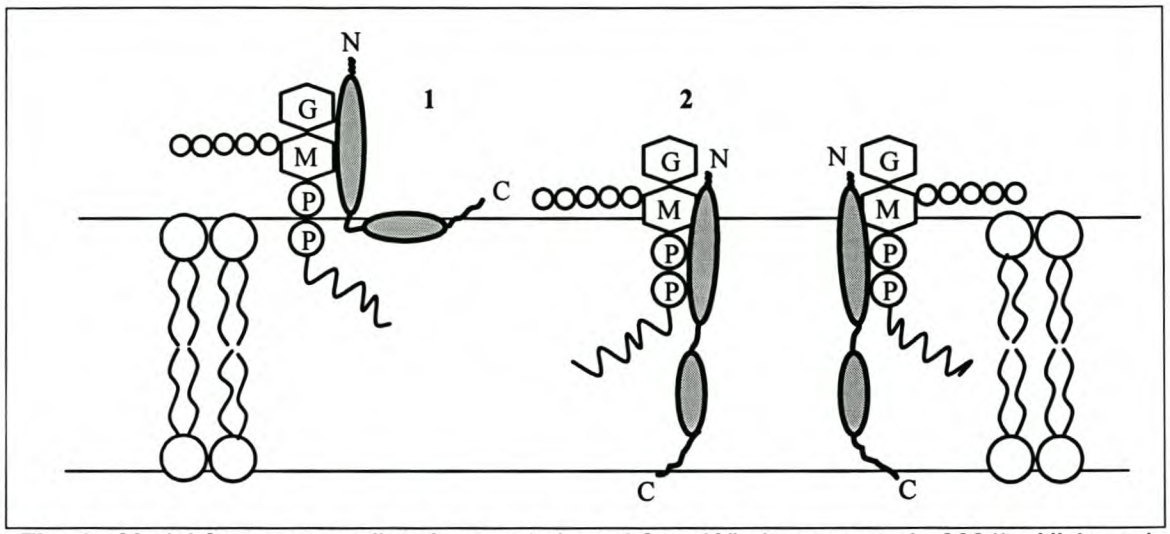

Fig. 6. Model for target-mediated pores (adapted from Wiedemann et al., 2001). Nisin and related peptides use lipid II as a docking molecule for subsequent pore-formation. Step 1: The N-terminal of nisin binds with the first sugar moiety of lipid in a 1:1 stoichiometry. Step 2: Pore formation is initiated by the translocation of the C-terminus across the membrane. Interaction with the pyrophosphate (PP) moiety of lipid II may be involved in stabilizing the transmembrane orientation of the peptides. It is not clear whether pores formed in the presence of lipid II will follow the wedge-like or barrel-stave model.

At high nisin concentrations (micro molar range) pores can be formed without lipid II (Wiedeman *et. al.*, 2001). However, for optimal activity in the absence of lipid II, membranes should at least contain 50-60% negatively charged phospholipids, which will allow the positively charged C-terminus of nisin to bind via electrostatic interactions to anionic lipids. Transmembrane pores with a millisecond lifetime are subsequently formed and the wedge or megainin-like models may best describe the pore forming process. The membrane potential supports pore formation under such conditions and pores are ion-selective.

5.5.3 BINDING OF THE MERSACIDINE SUBTYPE OF LANTIBIOTICS WITH LIPID II

Both mersacidine and actagardine inhibit biosynthesis of peptidoglycan (Brötz et al., 1995; Somma et al., 1971). Mersacidine interferes with the membrane-associated transglycosylation step, the first polymerization reaction in the biosynthetic pathway, resulting in the accumulation of lipid II in the membrane (Brötz et al., 1997). Brötz et al. (1998a) have shown that this is due to the tight interaction of mersacidine with lipid II, and not with the transglycosolase enzyme (Fig. 5). Actagardine competed with mersacidine for the same binding site, while co-incubation with nisin or vancomycin did not. Interaction of vancomycin with lipid II involves the C-terminal D-alanyl-D-alanine moiety of the pentapeptide side chain of MurNAc. Thus, the interaction of mersacidin with lipid II occurs via a binding site that is not targeted by

any antibiotic currently in use. Mersacidine can discriminate lipid I from lipid II, indicating that GlcNAc, the only difference between these two, contains part of the recognition motif (Brötz et al., 1998a). The exchangeable hydroxyl protons and/or the pyrophosphate group (Fig. 4) may also play an important role in binding (Hsu et al., 2003).

Recently, solution nuclear magnetic resonance (NMR) studies of mersacidine-lipid II interactions revealed an unexpected flexible nature of mersacidine, which was based on the ability of mersacidine to adapt to its environment in terms of its three-dimensional structure (Hsu et al., 2003). A small hinge region (Ala-12 and Abu-13), which can open and close the ring structures, governs the conformational changes. When binding to lipid II, mersacidine uses this hinge to adjust the exposure of charge groups, suggesting that electrostatic interactions govern binding mechanisms despite the rather hydrophobic nature of mersacidine. There is not sufficient evidence to assess which charged group is mainly responsible for binding. However, methylation of the N-terminus increased the MIC by two-fold, and biotinylation inactivated mersacidine's activity. Furthermore, replacement of Glu-17, the single negatively charged residue of mersacidine, by Ala or amidation of the carboxyl group of Glu-17 resulted in inactivation of the peptide. The results suggest that the charge of the side chain of Glu-17 is required for binding with lipid II. Moreover, the bioactivity of mersacidine is ion-dependant since the presence of calcium ions enhances its activity *in vivo*, whereas magnesium ions have no effect. Since lipid II is mostly negatively charged, calcium ions may provide a bridge between mersacidine and lipid II. The conserved sequence motif shared by mersacidine, actagardine, plantaricin C and pediocin PD-1 appears, therefore, to play a central role in the binding of these bacteriocins to lipid II. The mentioned bacteriocins have a similar spectrum of inhibition, including *S. aureus* and *Enterococcus* sp. and, acting on a novel target, may prove useful in the treatment of emerging multi-drug-resistant pathogens, such as methicilin-resistant *S. aureus* and vancomycin-resistant enterococci.

Insight into the mode of action of pediocin PD-1 and of plantaricin C, as well as the involvement of lipid II, was obtained through comparing the mode of action of pediocin PD-1 and nisin (Bauer et al., 2003). The trivalent cation gadolinium (Gd^{3+}) has an inhibitory effect on the action of both nisin (Abee et al., 1994; Bauer et al., 2003) and pediocin PD-1 (Bauer et al., 2003). Addition of Gd^{3+} prevents the formation of pores by these peptides and even closes pores if added after the initiation of pore formation. Gd^{3+} at high concentrations (10 mM), completely inhibited the activity of these peptides against *O. oeni* (Bauer et al., 2003). The ions can bind to the negatively charged headgroups of phospholipids in the cytoplasmic membrane and may shield electrostatic interactions between the peptides and the anionic lipids. Lipid II-targeted pore formation is, however, not dependent on a negative surface charge. It has been shown that neutralization of the negative charges induces a condensation of phospholipids, resulting in a more rigid membrane (Harwood and Russell, 1984). This effect may reduce the efficiency of

peptide insertion and therefore pore formation, or reduce the accessibility of lipid II binding sites for the antimicrobial agent. Pore formation in the membranes of actively growing *O. oeni* cells was accompanied with cell lysis, and the inhibition of bacteriocin activity by Gd^{3+} decreased with decreased concentrations of Gd^{3+} (Bauer et al., 2003). At a concentration of 2 mM Gd^{3+} cell death in the presence of nisin was no longer accompanied by cell lysis, while pediocin-induced lysis was not inhibited. While Gd^{3+} interferes with the specific interaction of nisin with lipid II, preventing inhibition of bacterial cell wall biosynthesis, the same appears not to apply to pediocin PD-1. The wedge model may therefor best describe nisin pore formation in the presence of Gd^{3+}. The results of this study furthermore suggest that specific interaction of pediocin PD-1 with lipid II binding sites inhibit peptidoglycan biosynthesis, as observed with mersacidin, but it does not result in the formation of pores. Pediocin PD-1-induced pore formation is rather due to electrostatic interaction between the positively charged N-terminal and anionic lipids. At high concentrations of Gd^{3+}, wedge-like pore formation is inhibited due to the complete shielding of electrostatic interactions. Moreover, the inwardly orientated lipid moiety of lipid II, involved in binding of both nisin and the mersacidine-subtype, may no longer be accessible due to the increased rigidity of the membrane.

5.6 CONCLUSIONS

Nisin can permeabilize membranes via a targeted and a non-targeted mechanism. Through interaction with lipid II nisin not only forms highly specific pores, but also inhibits peptidoglycan biosynthesis. Since the N-terminal double ring system of nisin is instrumental for lipid II interaction and subtilin and epidermin share the general setup of this ring system, it is likely that these lantibiotics act similarly. Mersacidin and actagardine also bind to lipid II, but only block its incorporation into peptidoglycan resulting in slow cell lysis. The bacteriocidal effect of pediocin PD-1 and plantaricin C is primarily due to the generation of small pores, but through interaction with lipid II the peptides may combine this with inhibition of cell wall biosynthesis. Additional antimicrobial effects, such as the apparent induction of autolysis by nisin, plantaricin C and pediocin PD-1 further complicate the mode of action picture. The diversity of antibiotic activities combined in one molecule explains why lantibiotics can be so potent. Although the lipid II-targeted antimicrobial peptides share a common binding molecule, they do not bind to the same part of lipid II. Since antibiotic resistance is on the increase, the diversity of such a targeting action is of great importance. These peptides are excellent candidates for the development of new generation novel antibiotic drugs. Although not yet identified, other lantibiotics may also use membrane components, such as lipid II, as docking molecules for antimicrobial action.

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CHAPTER 6

RESEARCH RESULTS

Purification, partial amino acid sequence and mode of action of pediocin PD-1, a bacteriocin produced by *Pediococcus damnosus* NCFB 1832

RESEARCH RESULTS

Purification, Partial Amino Acid Sequence and Mode of Action of Pediocin PD-1, a Bacteriocin Produced by *Pediococcus damnosus* NCFB 1832

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ABSTRACT

Pediocin PD-1 is a ribosomally synthesized antimicrobial peptide produced by *Pediococcus damnosus* NCFB1832. It inhibits the growth of several food spoilage bacteria, including malolactic bacteria isolated from wine. Pediocin PD-1 is 2866.87 ± 0.4 Da in size, has an iso-electric point (pI) of ca. 9.0 and, on amino acid level, revealed partial homology to the lantibiotic plantaricin C. The highest activity of pediocin PD-1 against cells of *Oenococcus oeni* was observed at an external pH of 5.0 and at 25°C. The primary mode of action of pediocin PD-1 is most probably due to pore formation, as indicated by the efflux of K^+ from metabolically active cells of *O. oeni*. Gadolinium (10 mM Gd^{3+}) prevented pediocin PD-1 from being active against *O. oeni*, suggesting that the mode of action of pediocin PD-1 relies on a net negative charge of the cell surface. We also present data on the activity of the lantibiotic nisin against *O. oeni*. Stationary phase cells and cells in phosphate buffer reacted differently to pediocin PD-1 and nisin than their growing counterparts. In comparison to nisin, pediocin PD-1 is less active against non-growing cells of *O. oeni*.

6.1 INTRODUCTION

Lactic acid bacteria are known to preserve fermented foods and to produce a variety of substances with antimicrobial activity, including antimicrobial peptides collectively known as bacteriocins (11). Many of the bacteriocins inhibit food-borne pathogens and spoilage bacteria (11), including malolactic bacteria in wine (3, 29). Since these

peptides are odorless, colorless and non-toxic, they are considered to be ideal food preservatives (1).

Bacteriocins are ribosomally synthesized and are usually active against genetically related species (19). They have been grouped into three classes based on their structure (24). The lantibiotics (class I) are small heat-resistant peptides that undergo post-translational modifications, which leads to the formation of rings through reaction between dehydrated serine and threonine residues with the sulfhydryl group of cysteine (22). The lantibiotics are further divided into two sub-groups, namely the elongated and amphipathic pore-forming type A peptides (e.g. nisin), and the globular peptides of the type B category, e.g. mersacidin and actagardine (5, 27). Class II consists of small (<15 kDa), heat-stable, membrane-active, unmodified peptides (31). Included in this class are the pediocin-like peptides, named after pediocin PA-1 produced by *Pediococcus acidilactici*. All pediocin-like bacteriocins have a consensus amino acid sequence motif in the N-terminal part of the mature peptide. Class III is represented by heat-labile proteins with sizes in excess of 15 kDa.

The mode of action of bacteriocins is not yet fully understood. Model membrane studies with nisin have shown that lipid II acts as a docking station (37). After binding, nisin wedges itself into the cell membrane to form short-lived pores which disturb the integrity of the cytoplasmic membrane and causes the efflux of ions and other cell components (14, 31). At high concentrations of nisin pore formation may occur in the absence of lipid II, provided the cell membrane contains at least 50% negatively charged phospholipids (37). Under these conditions, the positively charged C-terminus of nisin is important for initial binding and antimicrobial activity. Mersacidin and the antibiotic vancomycin also bind to lipid II, but to a different part of the molecule (4).

Pediocin PD-1 is produced by *Pediococcus damnosus* NCFB 1832, a atrain originally isolated from beer. The peptide differs from pediocin-like bacteriocins in that it is not active against pediococci and has a unique sensitivity pattern to proteolytic enzymes (18). Furthermore, compared to other pediocin-like bacteriocins, pediocin PD-1 is not very active against *Listeria monocytogenes*. The peptide is hydrophobic, resistant to heat (10 min at 121°C), remains active after 30 min of incubation between pH 2.0 and 10.0 (18) and is not affected by 15% (v/v) ethanol and 100 ppm SO_2, or a combination thereof (3). The peptide is active against a range of Gram-positive bacteria, including members of the genera *Clostridium, Bacillus, Staphylococcus, Enterococcus, Propionibacterium*, *Lactobacillus, Leuconostoc* and the species *Oenococcus oeni* (previously *Leuconostoc oenos*; 12). In a previous study (29) it was shown that pediocin PD-1, when compared with nisin and plantaricin 423, is the most effective in removal of an established biofilm of *O. oeni* from stainless steel surfaces in Chardonnay must.

In this paper we report on the amino acid sequence of pediocin PD-1 and the effect of pH and temperature on the activity of the peptide, measured in terms of the rate of potassium efflux from cells of *O. oeni*.

6.2 MATERIALS AND METHODS

6.2.1 STRAINS AND CULTURE CONDITIONS

P. damnosus NCFB 1832, producer of pediocin PD-1, was cultured in MRS broth (Biolab, Midrand, South Africa) at 30°C (29). *O. oeni* strain Lal1 was isolated from a commercial malolactic starter culture (Lallemand, Saint-Simon, France) and cultured in acidic grape (AG) broth (13).

6.2.2 ISOLATION OF PEDIOCIN PD-1 AND DETERMINATION OF ITS ISO-ELECTRIC POINT

P. damnosus NCFB 1832 was cultured in MRS Broth (Biolab) at 30°C to early stationary phase. The cells were harvested (8,000 x *g*, 5 min, 4°C) and the proteins precipitated from the cell-free supernatant by using ammonium sulfate (70% saturation; 32). The precipitate was concentrated by centrifugation (7,000 x *g*, 20 min, 4°C) and resuspended in 50 ml de-ionized water, containing ampholytes with a pH-range of 3 to 10 (Bio-Rad, Hercules, California, USA), and subjected to iso-electrical focusing in a Rotofor electrofocusing cell (Bio-Rad). A constant current of 12 W was applied for 5 h at 8°C. After separation, a small volume from each of the collected fractions was adjusted to pH 7.0 with 3 N NaOH or 3 N HCl and tested for antimicrobial activity against *O. oeni*, as described by Nel et al. (29). The fractions that tested positive were pooled, resuspended in de-ionized water to a total volume of 50 ml and again subjected to electrofocusing.

6.2.3 PURIFICATION OF PEDIOCIN PD-1

Partially purified pediocin PD-1 was prepared as described by Bauer et al. (3), but with the following modifications. The pelleted ammonium sulfate precipitate was dissolved in de-ionized water, adjusted to pH 6.0 with 3 N NaOH and dialyzed against deionized water by using a 1,000 Da molecular weight cut-off dialysis membrane (Spectra/Por, Rancho Domiquez, USA). The dialyzed sample was stirred for 1 h at 4°C in thirty volumes of methanol:chloroform (1:2, vol/vol) to remove fatty acids, including residual Tween 80 (10). The sample was centrifuged (10,000 x *g*, 20 min, 4°C) and the pellet resuspended in 50 mM sodium phosphate buffer, pH 7.6 (buffer A). The protein suspension was applied to a SP-Sepharose Fast Flow (Pharmacia Biotech, Uppsala, Sweden) cation-exchange column equilibrated with the same buffer. Peptides were eluted from the column with a NaCl step-gradient (0.1 to

0.5 M) in buffer A. Active fractions were pooled, dialyzed against deionized water and lyophilized. Protein concentrations were calculated from optical density readings at 280 nm, according to Sambrook et al. (32). The peptides were separated by tricine-SDS-PAGE, according to the method of Schägger and Von Jagow (33). A Rainbow protein molecular mass marker (Amersham International, Little Chalfont, UK) with a size range of 2.35 to 46 kDa was used. One half of the gel was stained with Coomassie Brilliant Blue R-250 (ICN Biomedicals Inc., Ohio, USA). The other half of the gel was overlaid with an active growing culture of *O. oeni* (ca. 10^8 CFU/ml) imbedded in AG agar, as described previously (18). The overlaid gel was incubated at 30°C for 48 h and examined for growth inhibition of *O. oeni.*

6.2.4 MASS SPECTROMETRY AND AMINO ACID SEQUENCING

Lyophilized samples of the active fractions of pediocin PD-1 collected from the SP-Sepharose column were resuspended in distilled water and subjected to MALDI-TOF Mass Spectrometry on a Voyager-DE Pro Biospectrometry Workstation (Perspective Biosystems, Framingham, Massachusetts, USA). Sequencing was done on an Applied Biosystems Procise 491 Protein Sequencing apparatus (PE Biosystems, Warrington, UK).

6.2.5 MUTAGENESIS

P. damnosus NCFB 1832 was cultured in MRS Broth supplemented with acriflavine (5 to 40 μg/ml), novobiocin (110 to 900 μg/ml) and nitrosoguanidine (10 to 500 μg/ml), as described by Ruiz-Barbara et al. (30) and Kuila et al. (25). Dilutions of the treated cells were plated onto MRS Agar (Biolab) and incubated at 30°C. Colonies were selected at random and inoculated onto a second set of MRS agar plates (10 CFU/plate, in duplicate). After overnight incubation at 30°C one set of plates was overlaid with AG soft agar (0.75%, wt/vol, agar), seeded with viable cells of *O. oeni* Lal1 (*ca.* 1×10^8 CFU/ml). The plates were incubated for 48 h at 30°C and examined for colonies of *P. damnosus* that failed to produce zones of growth inhibition. Corresponding colonies from the second set of plates were selected for plasmid isolations. Plasmid DNA was isolated by using the lysozyme-mutanolysine lysis method (8), followed by CsCl–density gradient centrifugation and agarose gel electrophoresis, as described by Sambrook et al. (32).

6.2.6 MINIMUM INHIBITORY CONCENTRATION (MIC) OF PEDIOCIN PD-1

Cells of *O. oeni* Lal1 were harvested (5,000 x *g*, 5 min) during mid-exponential growth (OD_{600} = 0.5 to 0.6; ca. 5 x 10^8 CFU/ml), washed once in sterile AG broth (pH 5.0) and resuspended in the same medium. Serial two-fold dilutions of partially

purified pediocin PD-1 (1.2 x 10^6 AU/g total protein) were prepared in AG-broth (pH 5.0) and 0.2 ml inoculated with active growing cells of *O. oeni* to a final of ca. 1 x 10^9 CFU/ml (OD_{600} = 1.00). After incubation for 24 h at 30°C, 10 µl of each cell suspension was spotted on AG agar plates and incubated at 30°C until growth was observed. Nisin (10%, wt/vol, of 1 x 10^6 I.U./g NISAPLIN™, Aplin & Barrett Ltd., Trowbrige, Wilts, UK) was used as control. The most diluted concentration of pediocin PD-1 that inhibited the growth of *O. oeni* Lal1 was recorded as 1 MIC.

6.2.7 INFLUENCE OF CULTURE PH ON THE ACTIVITY OF PEDIOCIN PD-1

O. oeni Lal1 was grown in 800 ml AG broth (pH 4.8) at 25°C to OD_{600} = 0.5 to 0.6. The culture was divided into four equal volumes (200 ml), harvested by centrifugation (5000 x *g*, 5 min, 4°C), washed with sterile AG broth, pre-adjusted to pH 4.0, 5.0, 6.0 and 7.0, respectively, with 3 N NaOH or 3 N HCl, and then resuspended in the same media of corresponding pH to OD_{600} = 1.00 (ca. 1 x 10^9 CFU/ml). Four test tubes with 9 ml sterile AG-broth (pH 4.0, 5.0, 6.0, and 7.0), were each inoculated with 1 ml of a cell suspension of corresponding pH. Pediocin PD-1 (1 MIC/ml) was added to each of the test tubes. Viable cell numbers were determined after 1 h, 6 h and 24 h of incubation at 25°C, by preparing serial dilutions and plating onto AG agar (pH 4.8). Colonies were counted after 11 days of incubation at 30°C. The plates were sealed with parafilm to prevent them from drying out.

In a separate experiment, the efflux of K^+ from cells of *O. oeni* suspended at different pH values was recorded. *O. oeni* Lal1 was grown in 900 ml AG broth (pH 4.8) at 25°C to OD_{600} = 0.5 to 0.6. The culture was divided into three equal volumes, harvested (5000 x *g*, 5 min, 4°C) and washed with sterile 100 mM sodium phosphate buffer of pH 5.0, 6.0, and 7.0, respectively. The cells were resuspended in sterile phosphate buffer of corresponding pH, supplemented with 0.2% (wt/vol) glucose and 0.5 mM KCl, and adjusted to OD_{600} = 1.00 (ca. 10^9 CFU/ml). The level of K^+ in the medium (K^+_{out}) was recorded with a valinomycin-based potassium-selective electrode (Radiometer, Copenhagen, Denmark). As soon as a steady state of K^+_{out} was maintained for at least 2.5 min, pediocin PD-1 (1 MIC/ml) was added to each of the cell suspensions. Changes in K^+_{out} were recorded every 5 s for a period of 65 min. The intracellular level of K^+ (K^+_{in}) was calculated from the increase recorded in the K^+_{out}-level and results were expressed in nmol K^+_{in} per number of cells. The total content of intracellular K^+ was determined by exposing the cells to lysozyme (10 mg/ml, 30 min, 37°C).

The experiment was repeated with *O. oeni* cells resuspended in glucose-containing phosphate buffer (pH 6.0) and treated with nisin (1 MIC/ml) and lysozyme (10 mg/ml), respectively. In all experiments, the average of three readings was plotted and the standard deviations for each data point determined.

6.2.8 EFFECT OF TEMPERATURE ON PEDIOCIN PD-1 ACTIVITY

O. oeni Lal1 was grown at 8, 15 and 25°C in 800 ml AG-broth (pH 4.8) to mid-exponentially growth (OD_{600} = 0.5 to 0.6). Cells were harvested and washed with sterile AG broth, as described before. Three 10-ml cell suspensions (ca. 10^9 CFU/ml), resuspended in sterile AG broth (pH 5.0) to OD_{600} = 1.00, were treated with pediocin PD-1 (1 MIC/ml). Incubation was at 8°C, 15°C and 25°C, respectively, and viable cell numbers recorded after 1 h, 3 h and 6 h, as described before.

In a separate experiment, the efflux of K^+ from cells of *O. oeni* was recorded. Strain Lal1 was grown at 8°C, 15°C and 25°C to OD_{600} = 0.5 to 0.6, washed with 100 mM sterile sodium phosphate buffer (pH 6.0) and resuspended in the same buffer, supplemented with 0.2% (wt/vol) glucose and 0.5 mM KCl, as described before. K^+_{out} readings were recorded every 5 s for 90 min and the K^+_{in}-levels determined as described before. For each experiment, an average of three readings was plotted and the standard deviations for each data point determined.

6.2.9 EFFECT OF GADOLINIUM (GD^{3+}) ON THE ACTIVITY OF PEDIOCIN PD-1

O. oeni Lal1 was grown in AG broth (pH 4.8) at 25°C to mid-exponential growth (OD_{600} = 0.5 to 0.6). Six 10-ml cell suspensions (ca. 10^9 CFU/ml) were prepared in AG broth (pH 5.0), as described before. To the first cell suspension pediocin PD-1 (1 MIC/ml) and 2 mM gadolinium (Gd^{3+}) were added. To the second cell suspension only pediocin PD-1 (1 MIC/ml) was added. The third and fourth cell suspensions were treated in the same way as the first two cell suspensions, except that pediocin was replaced by nisin (1 MIC/ml). The fifth cell suspension received only 2 mM Gd^{3+}. The sixth cell suspension received neither of the two bacteriocins, nor Gd^{3+}. Cell suspensions with Gd^{3+} were allowed a 10 min contact time at 25°C before the addition of pediocin PD-1 or nisin. Viable cell numbers were determined after 1 h, 3 h, 5 h and 6 h of incubation at 25°C by plating onto AG agar, as before. Cell density was measured at 600 nm over 24 h.

6.2.10 EFFECT OF PEDIOCIN PD-1 ON NON-GROWING *O. OENI* CELLS

O oeni Lal1 was grown in AG-broth (pH 4.8) at 25°C to stationary phase (OD_{600} = 1.3). The pH of the cultures were adjusted to 5 with 3 M NaOH and the cells were treated with pediocin PD-1 (1 MIC/10^9 CFU/ml), followed by incubation at 25°C. Viable cell numbers were determined over a period of 5 h, as described before.

Two sets (3 x 10 ml test tubes each) of mid-exponentially phase *O. oeni* Lal1 cell suspensions were prepared in 100 mM sodium phosphate buffer (pH 6, 4°C) as described before. The first two cell suspensions of the first set were treated with pediocin PD-1 (1 MIC/ml) and nisin (1 MIC/ml) respectively. The third cell suspension received no bacteriocin. The second set was treated as the first set, except that the phosphate buffer was supplemented with 0.2% (wt/vol) glucose. All

cell suspensions were incubated at 4°C. Viable cell numbers and turbidity were measured over a period of 5 h and 24 h respectively, as described before.

6.3 RESULTS

All data represent an average of three repeats. The values recorded in each experiment did not vary by more than 5%. Single data points are, therefore, presented in the figures without standard deviation bars. In the present study, 1 MIC pediocin PD-1 corresponded to 850 μg total protein and 1 MIC nisin to 39 μg NISAPLIN™.

6.3.1 ISO-ELECTRIC POINT OF PEDIOCIN PD-1

Fractions collected between pH 7.5 and 10.0 from the first separation revealed antimicrobial activity against *O. oeni* Lal1. However, the second separation yielded only three active fractions, between pH 8.90 and 9.06. The pI of pediocin PD-1 was therefore estimated to be approx. 9.0.

6.3.2 PROTEIN PURIFICATION AND MOLECULAR SIZE OF PEDIOCIN PD-1

The yield of pediocin PD-1 activity at each stage of purification is shown in Table 1. Fifty-five percent of pediocin PD-1 activity was recovered after lyophilization of the dialyzed ammonium sulfate precipitate. Extraction of fatty acids and other impurities from the precipitate with methanol and chloroform resulted in a further 8% loss of total activity (47% yield). Although only 34% of total pediocin PD-1 activity was recovered after cation exchange chromatography, the specific activity of the purified pediocin PD-1 was 1,700-fold higher than recorded in the culture supernatant (Table 1). Separation by tricine-SDS PAGE yielded an active peptide band of ca. 2.5 kDa (Fig. 1). Mass spectrometry analyses revealed a distinctive peak corresponding to a molecular mass of 2,866.87 ± 0.4 Da (Fig. 2).

TABLE 1. Purification of pediocin PD-1 from cells grown in MRS broth

Purification stage	Vol (ml)	Total protein conc.[a]	Total activity (AU)	Sp act[b]	Increase in sp act	Act yield (%)
Culture supernatant	600.0	24,030	288×10^4	120	1	100
Ammonium sulfate precipitate after:						
- dialyses	97.0	2,765	248×10^4	897	8	86
- lyophilization	15.50	2,400	159×10^4	663	6	55
- methanol-chloroform extraction	6.57	1,143	135×10^4	1,181	11	47
Cation exchange	74.0	4.8	97×10^4	200,000	1,700	34

[a] Total protein concentration refers to protein concentration (mg/ml) multiplied by the volume (ml).
[b] Specific activity is activity units (AU) divided by the total protein concentration.

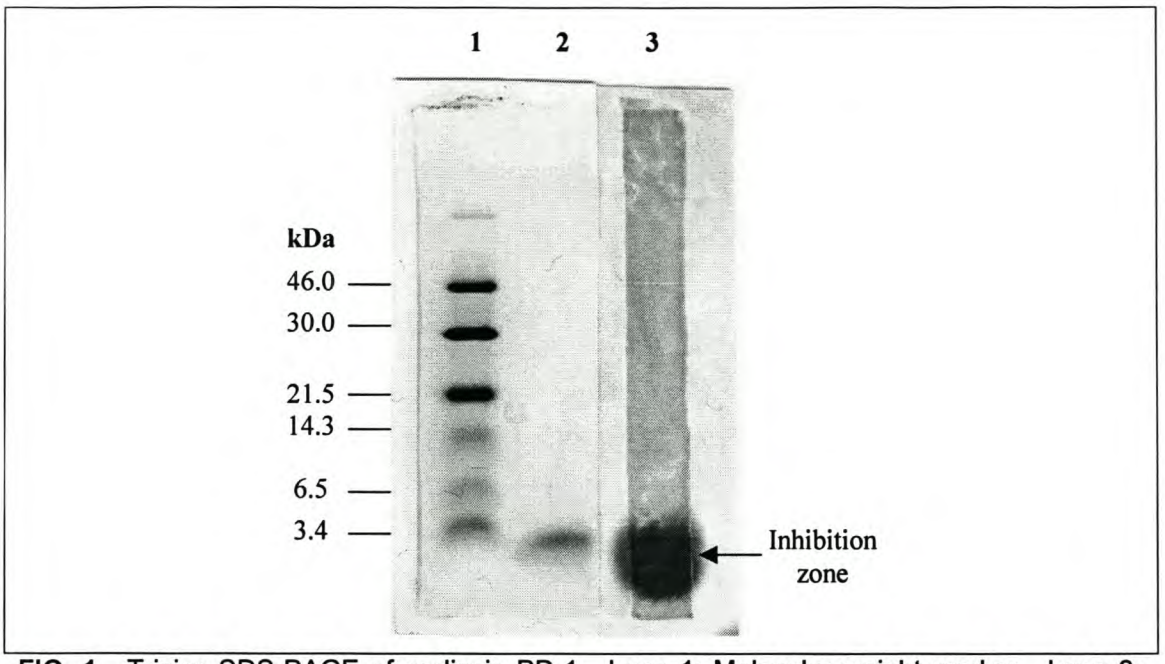

FIG. 1. Tricine-SDS-PAGE of pediocin PD-1. Lane 1: Molecular weight marker. Lane 2: Peptide band stained with Coomassie Blue. Lane 3: Zone of growth inhibition, corresponding to the position of the peptide band in lane 2. The gel was covered with viable cells of *O. oeni* (10^8 CFU/ml), imbedded in AG agar. Incubation was at 30°C for 48 h.

6.3.3 AMINO ACID SEQUENCE

Twenty-two amino acids from the N-terminal of pediocin PD-1 were sequenced by Edman degredation (NH_2-Lys-Lys-Ile-Lys-Lys-Ser-Xaa-Ser-Gly-Asp-Ile-Xaa-Xaa-Leu-Xaa-Xaa-Glu-Xaa-Asp-His-Leu-Ala-). Not only was the sequence blocked after the 22th amino acid, but six of the amino acids (Xaa) in the accessible portion of the sequence could not be identified. Alignment of the amino acid sequence revealed areas of homology to plantaricin C, actagardine and mersacidin (Fig. 3).

6.3.4 MUTAGENESIS

To establish whether the pediocin PD-1 gene was plasmid born, *P. damnosus* NCFB 1832 was treated with plasmid curing agents. The highest concentration of acriflavine and novobiocin at which *P. damnosus* NCFB 1832 could grow was 30 μg/ml and 800 μg/ml, respectively. Screening of 2,000 colonies from the acriflavin-treated culture yielded no mutants with a loss of pediocin PD-1 production. From the 3,500 colonies screened that were treated with novobiocin, only three mutants lost the ability to produce pediocin PD-1. All three mutants retained immunity to pediocin PD-1. In the case of nitrosoguanidine, a concentration of 300μg/ml yielded a 50% survival rate. From the 1,500 colonies that were treated with nitrosoguanidine, only one mutant defective in pediocin PD-1 production was isolated. The mutant was,

however, sensitive to pediocin PD-1. All four mutants generated with novobiocin or nitrosoguanidine retained their plasmids (results not shown). When the sensitive mutant obtained with nitrosoguanidine treatment was grown in the presence of low concentrations of pediocin PD-1 through ten subsequent transfers to fresh media, this variant became resistant. Control cells grown in the absence of bacteriocin remained sensitive to pediocin PD-1. On the other hand, when the pediocin PD-1 resistant mutants were grown in the absence of bacteriocins through successive transfers, they became sensitive.

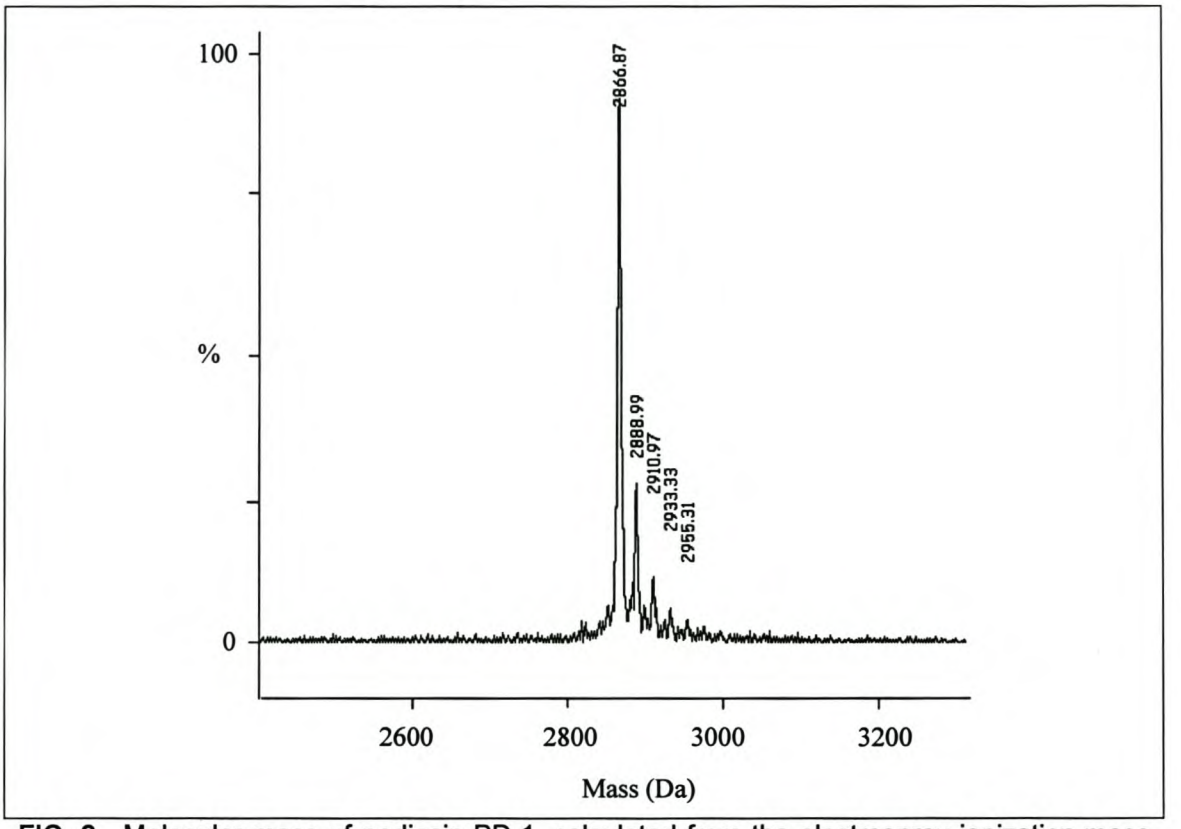

FIG. 2. Molecular mass of pediocin PD-1, calculated from the electrospray ionization-mass spectroscopy multiple charged spectra.

Pediocin PD-1:	K	K	I	K	K	S	X	S	G	D	I	X	X	L	X	X	E	X	D	H	L	A	X	X	X	X	X
Plantaricin C:	K	K	T	K	K	N	**S**	S	G	D	I	**C**	**T**	L	**T**	**S**	E	**C**	D	H	L	A	**T**	W	V	**C**	**C**
Actagardine:							**C**	S	G	W	V	**C**	**T**	L	**T**	I	E	**C**	G	**T**	V	I	**C**	A	**C**		
Mersacidin:	**C**	**T**	F	**T**	L	P	G	G	G	G	V	**C**	**T**	L	**T**	**S**	E	**C**	I	**C**							

FIG. 3. Partial amino acid sequence of pediocin PD-1 and comparison of its primary structure with homologous bacteriocins. Identical residues are marked, unidentified residues are represented by X, and postulated amino acids before modification are in bold.

6.3.5 INFLUENCE OF CULTURE PH ON PEDIOCIN PD-1 ACTIVITY

The effect of pH on the sensitivity of *O. oeni* towards pediocin PD-1 is shown in Fig. 4. After 1 h of incubation at pH 4.0, 5.0, 6.0 and 7.0 at 25°C, the viable cell numbers of *O. oeni* decreased from 10^8 CFU/ml to approx. 10^6 CFU/ml. Variable results were obtained at prolonged incubation. At pH 5.0 no viable cells were detected after 24 h in the presence of pediocin PD-1 (detection limit: 10 cfu/ml). Cells incubated at pH 6.0 and 7.0 were less inhibited and decreased from 1 x 10^8 CFU/ml to between 4 x 10^2 and 2 x 10^3 CFU/ml over 24 h. At pH 4.0, *O. oeni* was the least affected by pediocin PD-1, with cell numbers decreasing to approx. 4 x 10^4 CFU/ml over the same time period.

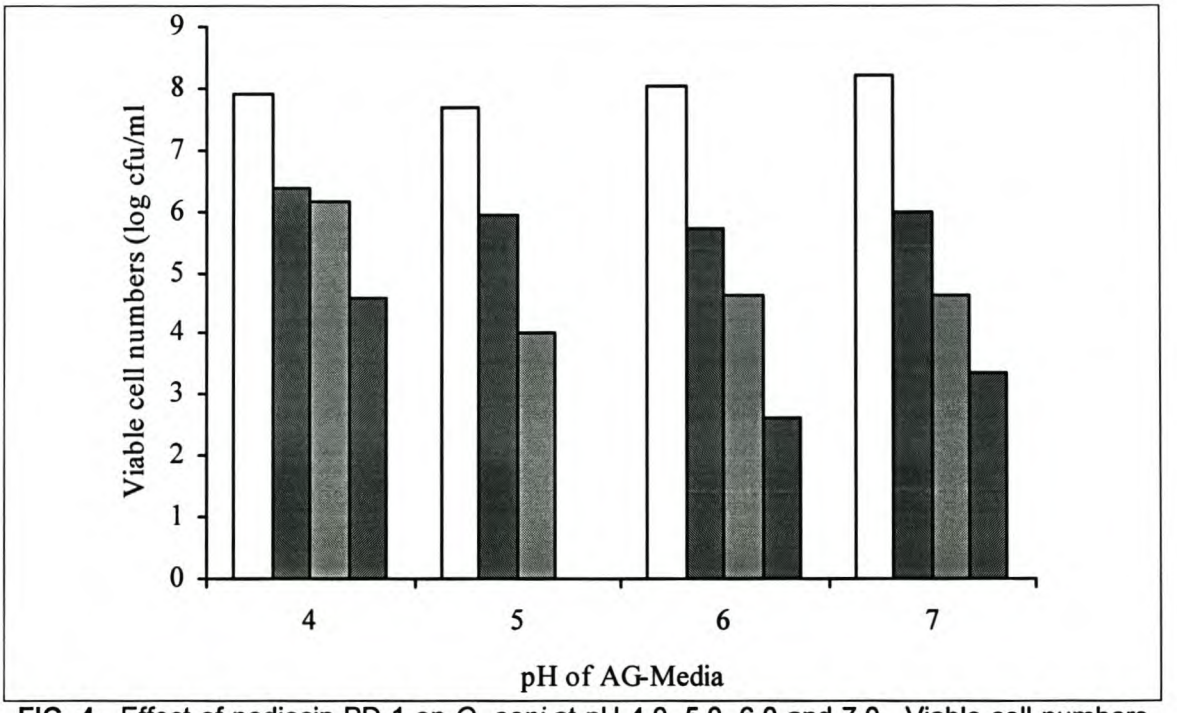

FIG. 4. Effect of pediocin PD-1 on *O. oeni* at pH 4.0, 5.0, 6.0 and 7.0. Viable cell numbers were recorded before the addition of pediocin PD-1 (☐), and after 1 h (▨), 6 h (▦), and 24 h (▨) of pediocin PD-1 addition.

The efflux of K^+ from pediocin PD-1-treated cells, energized with glucose in phosphate buffer, was influenced by external pH (Fig. 5). Freshly prepared *O. oeni* cells lost K^+ when incubated in buffer without KCl (results not shown). However, at 25°C in the presence of glucose and 0.5 mM KCl, K^+ accumulated to between 135 and 155 nmol/10^9 cells (Fig. 5). Efflux of potassium (K^+) occurred rapidly and immediately after the addition of pediocin PD-1 or nisin, followed by a slow release (Fig. 5). Cells incubated at pH 6.0 lost approx. 70 nmol K^+/10^9 cells (from approx. 135 to 65 nmol K^+/10^9 cells) during the first 30 s in the presence of pediocin PD-1, followed by a short recovery period (ca. 3 min) and slow K^+ efflux, reaching an intracellular K^+ concentration of approx. 10 nmol K^+/10^9 cells over the next 55 min

(Fig. 5). Cells incubated at pH 5.0 lost only 35 nmol $K^+/10^9$ cells during the first 30 s, but reached an intracellular K^+ concentration of below 5 nmol $K^+/10^9$ cells over the next 40 min (Fig. 5). The rate of efflux decreased dramatically when the extracellular pH was raised to pH 7.0 (Fig. 5). To determine whether pediocin PD-1 acted bactericidal or bacteriostatic under these conditions, cells were incubated in the presence of pediocin PD-1 for 2 h, serially diluted, plated on AG-agar and incubated at 25°C. Less than 10% of the cells were killed at all pH values tested (results not shown). A higher rate of K^+ efflux was recorded when cells were treated with lysozyme or nisin (Fig. 5).

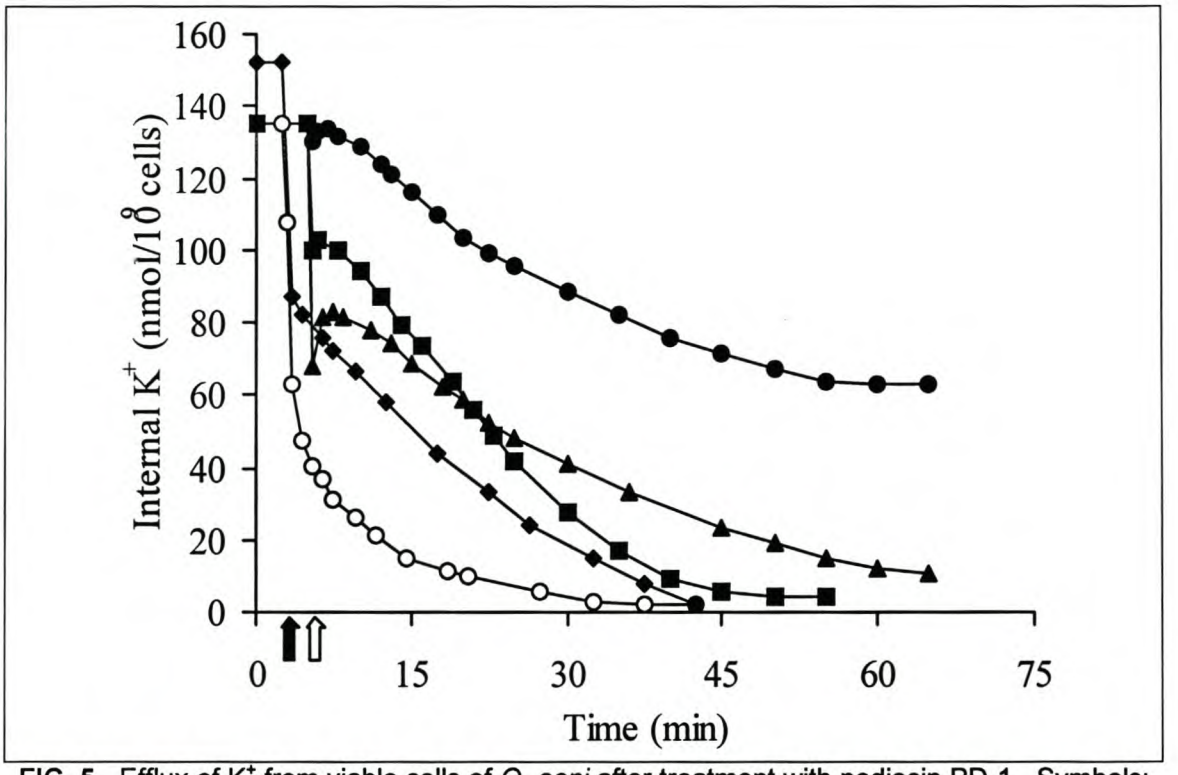

FIG. 5. Efflux of K^+ from viable cells of *O. oeni* after treatment with pediocin PD-1. Symbols: pH 5 (■), pH 6 (▲), and pH 7 (●); nisin-induced K^+ efflux at pH 6 (◆); lysozyme-induced K^+ efflux at pH 6 (○). Nisin and lysozyme were added after 2.5 min (↑), and pediocin PD-1 was added after 5 min (⇧).

6.3.6 EFFECT OF TEMPERATURE ON PEDIOCIN PD-1 ACTIVITY

O. oeni Lal 1 grown at 8°C is more resistant to pediocin PD-1 than cells grown at 15 and 25°C (Fig. 6). After 1h at 25°C the cell numbers decreased from *ca.* 1 x 10^9 CFU/ml to 2 x 10^5 CFU/ml, followed by a further decrease to *ca.* 1 x 10^4 CFU/ml over the next 5 h. At 8°C the cell numbers decreased from ca. 1 x 10^9 CFU/ml to 1 x 10^7 CFU/ml after 6 h and at 15°C from ca. 1 x 10^9 CFU/ml to 4 x 10^5 CFU/ml over the same period. The rate of K^+ efflux from cells incubated at 25°C was also much higher, as recorded by a decrease in intracellular K^+ from 135 nmol/10^9 cells to

75 nmol/10^9 cells within the first 5 min, and a further decrease to approx. 10 nmol/10^9 cells over the next 60 min (Fig. 7). The initial rate of K^+ efflux was less at 8°C and 15°C (Fig. 7). However, similar low levels of intracellular K^+ (less than 10nmol/10^9 cells) were recorded after 70 and 85 min at 15°C and 8°C, respectively (Fig. 7).

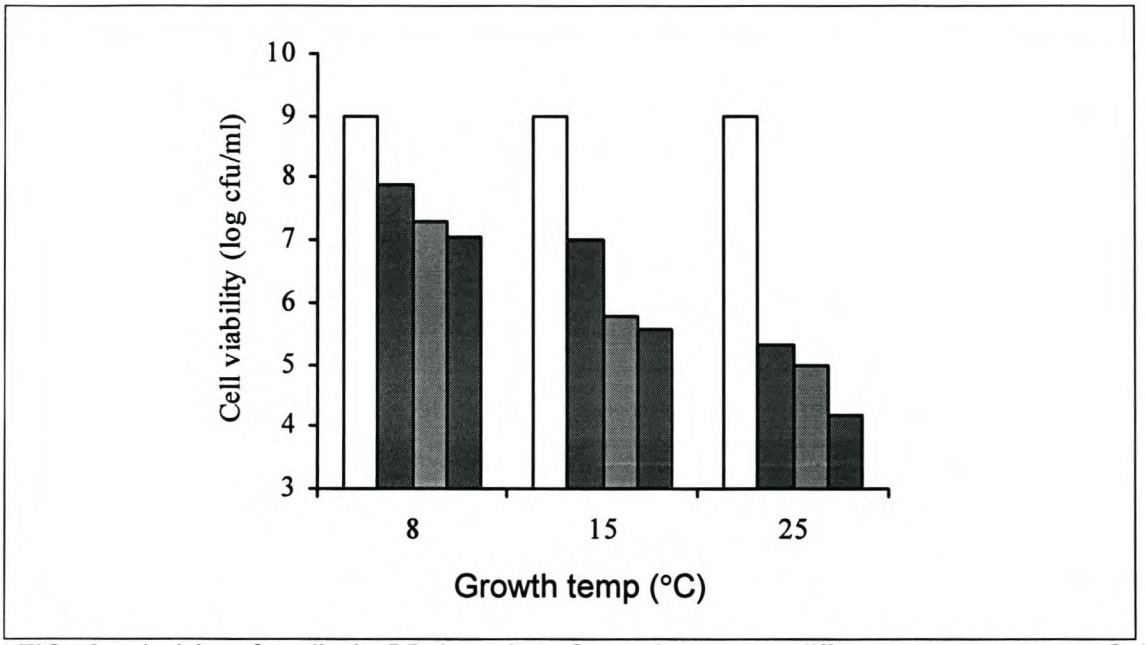

FIG. 6. Activity of pediocin PD-1 against *O. oeni* grown at different temperatures. Cell viability was determined before pediocin PD-1 addition (□), and after 1 h (▨), 3 h (▦), and 6 h (▨) of pediocin PD-1 addition.

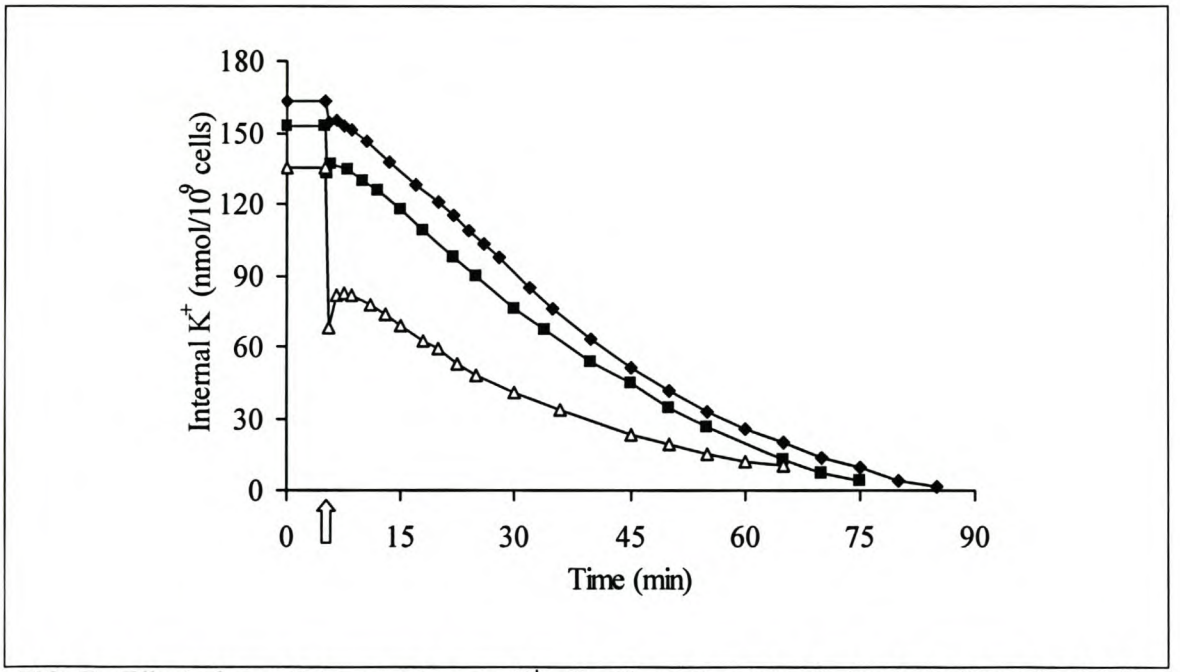

FIG. 7. Effect of growth temperature on K^+ efflux from *O. oeni* cells treated with pediocin PD-1. Symbols: 8°C (◆), 15°C (■), 25°C (△). The arrow indicates pediocin PD-1 addition.

6.3.7 EFFECT OF GD^{3+} ON BACTERIOCIN ACTIVITY

Gadolinium (Gd^{3+}) at 10 mM completely inhibited the activity of pediocin PD-1 and nisin over a period of 24 h at 25°C, as detected by viable cell counts recorded for *O. oeni* (results not presented). Lower concentrations of Gd^{3+} (2 mM) were not as inhibitory to pediocin PD-1 and nisin, as indicated by a decrease in cell numbers (Fig. 8). The cell numbers decreased from ca. 1 x 10^9 CFU/ml to 1 x 10^5 CFU/ml over 5 h in the absence of Gd^{3+}, compared to a decrease from ca. 1 x 10^9 CFU/ml to 1 x 10^6 CFU/ml in the presence of 2 mM Gd^{3+}. Cell death in the presence of nisin and pediocin PD-1 is accompanied with cell lysis as revealed by a decrease in turbidity (OD_{600}) from approx. 1.0 to 0.4 over a period of 6 h (Table 2). At 2 mM Gd^{3+}, pediocin-induced lysis was not inhibited. In the case of nisin, however, cell death was no longer accompanied with cell lysis. In the presence of Gd^{3+}, the cell density of nisin-treated cells did not differ significantly from cells that were not treated with bacteriocin at any point measured over a period of 24 h. The slight decrease in optical density observed for these Gd^{3+}-treated cells could not be linked to a decrease in viable cell numbers, but may have been due to a slight precipitation of cells in response to a high Gd^{3+} concentration.

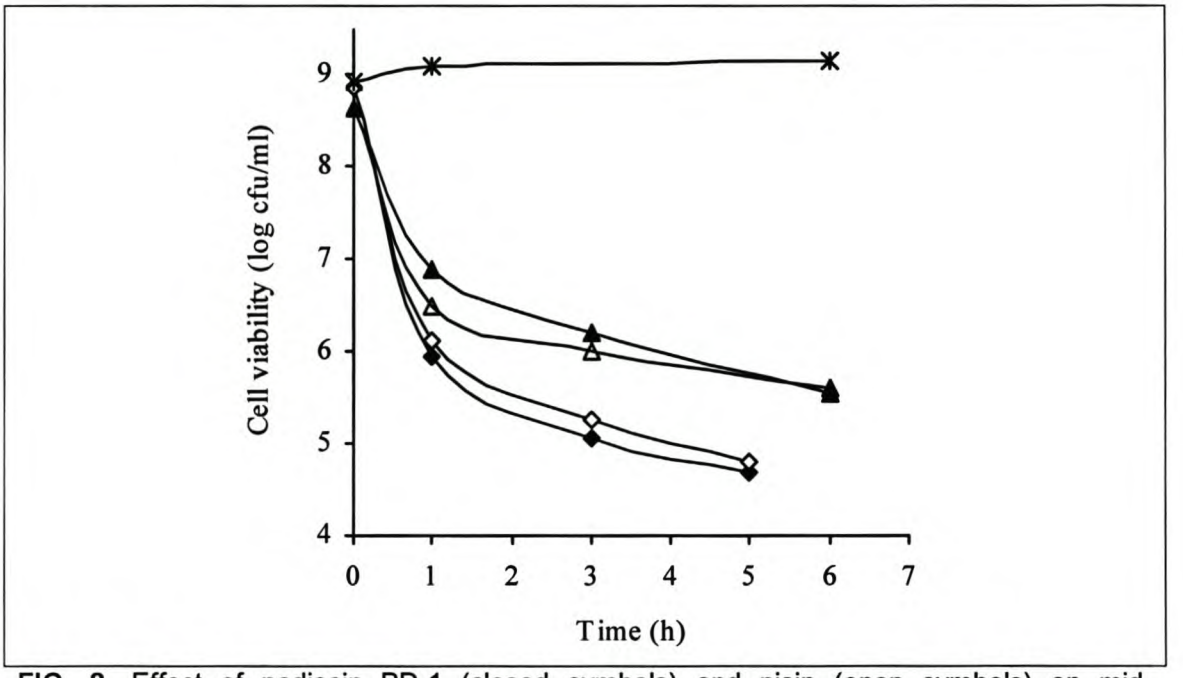

FIG. 8. Effect of pediocin PD-1 (closed symbols) and nisin (open symbols) on mid-exponentially phase cells of *O. oeni* grown in AG broth (pH 5.0) at 25 °C. Results were recorded in the absence and presence of Gd^{3+} (2 mM). Symbols: ∗, Cells grown in the absence of bacteriocin; ◆ and ◊, cells treated with bacteriocin; ▲ and △ cells treated with bacteriocin, in the presence of 2 mM Gd^{3+}.

TABLE 2. Bacteriocin-induced cell lysis and the effect of Gd^{3+} as indicated by changes in optical density readings (OD_{600})

	No bacteriocin		Pediocin PD-1		Nisin	
Time	without Gd^{3+}	2 mM Gd^{3+}	without Gd^{3+}	2 mM Gd^{3+}	without Gd^{3+}	2 mM Gd^{3+}
0	0.98	1.01	1.05	1.04	1.06	1.00
1 h	0.98	0.99	0.81	0.85	0.89	0.99
3 h	1.03	0.95	0.54	0.55	0.58	0.94
6 h	1.07	0.91	0.37	0.42	0.44	0.91
24 h	1.13	0.86	0.28	0.38	0.36	0.85

6.3.8 EFFECT OF BACTERIOCIN ON NON-GROWING *O. OENI* CELLS

The addition of pediocin PD-1 or nisin to mid-exponentially phase *O. oeni* cells in AG-media resulted in a similar drastic decrease in viable cell numbers from ca. 1×10^9 to 1×10^5 CFU/ml within 5 h of treatment (Fig. 8). In contrast, cells from cultures grown to stationary phase decreased from ca. 1×10^9 to 2×10^8 and 9×10^7 over 5 h when treated with pediocin PD-1 or nisin respectively (results not shown). Stationary-phase cells remained at ca. 1×10^9 CFU/ml in the absence of bacteriocins.

As indicated by a reduction in turbidity, the addition of pediocin PD-1 or nisin to growing mid-exponentially phase cells in AG-broth resulted in immediate and drastic cell lysis from an OD_{600} of 1.00 to 0.28 and 0.36 respectively within 24 h (Table 2). However, cell lysis of resting cells in 100 mM $NaPO_4$ buffer (4°C), treated with pediocin PD-1 or nisin, was dramatically reduced, reaching OD_{600} values of 0.90 and 0.75, respectively, over the same period (results not shown). No significant reduction of turbidity was observed after 6 h on treatment of these cells with pediocin PD-1. In the case of nisin, the turbidity decreased from OD_{600} of 1.00 to 0.83 after only 1 h of treatment. For both untreated and bacteriocin treated cells, the turbidity in buffer with or without glucose did not differ significantly from each other at any point. Bacterial counts after exposure to pediocin PD-1 in phosphate buffer (4°C) remained constant at approx. 1.0×10^9 CFU/ml whether the cells were energized with glucose or not (results not shown). In contrast, nisin treatment resulted in an immediate reduction of cell viability, reaching 3.0×10^7 CFU/ml in the absence of glucose, compared to 1.5×10^7 in the presence of glucose, after 1 h of treatment (results not shown). The population declined slowly after the initial nisin-induced shock, reaching ca. 6.0×10^6 CFU/ml in both buffers after 6 h of treatment.

6.4 DISCUSSION

Purification of pediocin PD-1 resulted in a 1700-fold increase of specific activity. The complete amino acid sequence could not be obtained through Edman degradation. Modified amino acids typically result in blank cycles during sequencing

by Edman degradation or result in blockage (35;23). Although further studies are needed to confirm that pediocin PD-1 belongs to the family of lantibiotics, the amino acid sequence is very homologous to plantaricin C, a lantibiotic produced by a *Lactobacillus plantarum* LL441 (35). The positions of the blanc cycles in pediocin PD-1 correspond with the positions of the modified amino acids in plantaricin C (Fig. 3). The homology recorded between plantaricin C and pediocin PD-1 suggests that production of similar bacteriocins are not necessarily limited to specific genera or restricted to organisms sharing a specific environment. Two globular, uncharged, type B lantibiotics, mersacidin and actagardine, contains a conserved sequence motif (amino acids 11 to 18 and 5 to 13, respectively) that comprises one entire ring structure (7). This conserved motive is shared with plantaricin C and possibly pediocin PD-1 (amino acids 12 to 18 for both bacteriocins) (Fig. 3). Plantaricin C comprises two distinct domains: a globular domain similar to that of mersacidin and actagardine, and a linear, positively charged N-terminal domain (Fig. 9). The amino acid sequence alignments strongly suggest that pediocin PD-1 has a primary structure similar to that of plantaricin C.

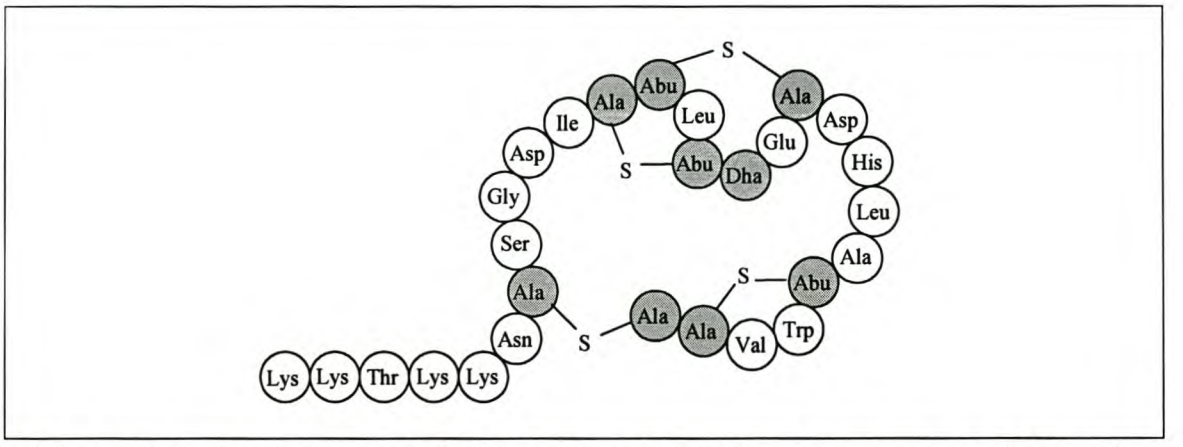

FIG. 9. Primary structure of plantaricin C (adapted from Turner et al., 1999). The shaded residues indicate amino acids which have undergone post-translational modification. Ala-S-Ala: lanthionine; Abu-S-Ala: β-methyllanthionine; Dha: 2,3-didehydroalanine. The bridging arrangement is not conclusive, it represent the most probable pattern of linkages. The peptide has a crossbridge C-terminal domain and an unbridged N-terminus.

Besides actagardine, the mode of action of mersacidin differs from the type-A and other type-B lantibiotics. Treatment with mersacidin results in the cessation of growth and a slow induction of lysis rather than pore formation (6). Mersacidin exerts its bactericidal action by inhibition of peptidoglycan biosynthesis through interaction with lipid II (5). Although the exact binding site for mersacidin remains to be identified, interaction with lipid II occurs via a binding site not recognized by any of the antibiotics currently in use. The conserved sequence motif shared by mersacidin,

actagardine, plantaricin C and pediocin PD-1 may be involved in the binding of these bacteriocins to lipid II. Recently, this was indeed confirmed for mersacidin (20). These bacteriocins have a similar spectrum of inhibition, including *S. aureus* and *Enterococcus* sp. and, acting on a novel target, may prove useful in the treatment of emerging multi-drug-resistant pathogens, such as methicilin-resistant *S. aureus* and vancomycin-resistant enterococci.

Genes encoding bacteriocin production are often located on plasmids (9). Treatment of bacteriocin-producing bacteria with mutagenic agents may result in plasmid curing, accompanied by loss in bacteriocin production. In the case of strain NCFB 1832, all mutants that lost the ability to produce pediocin PD-1 retained their plasmids, suggesting that the genes may be located on the chromosome, or that the genes are located on a plasmid coding for essential biochemical properties and therefore the Bac^- mutation occurred without the plasmid loss. Cells treated with nitrosoguanidine became sensitive to pediocin PD-1, which suggests a possible cluster location of immunity-conferring and pediocin PD-1-producing genes. Attempts to clone the structural gene of pediocin PD-1 by PCR using degenerate primers is complicated by high codon degeneracy. All attempts were unsuccessful. The Bac^- mutants can revert between bacteriocin sensitivity and resistance. *P. damnosus* NCFB 1832 can thus be resistant to pediocin PD-1 due to specific (gene encoded immunity protein in the case of producers) or non-specific reasons (in the case of non-producers). A threat to future application of bacteriocins lies in the development of resistance, which has already been reported for the lantibiotic nisin in a variety of Gram-positive bacteria (36). The mechanisms by which bacteria become resistant to bacteriocin are not understood.

Results obtained from incubation of *O. oeni* Lal 1 in the presence of pediocin PD-1 suggest that the action of the peptide is pH-dependent with an optimum at pH 5.0. The ability of pediocin PD-1 to form pores in sensitive cells of *O. oeni*, as observed by K^+ loss, is also pH dependant and increased when the extracellular pH was lowered from 7.0 to 5.0. Although the rate of pediocin PD-1-induced K^+ loss was the highest at pH 5.0, initial K^+ loss was the highest at pH 6. This suggests that, under physiological conditions, the ΔpH may contribute to pediocin PD-1 action as observed for nisin (28). Antimicrobial activity may be, at least in part, ascribed to a synergistic effect between pediocin PD-1 and reduced pH, and may be species specific. The decrease in pediocin PD-1 activity against *O. oeni* at pH 4.0, compared to pH 5.0, may be due to the decrease in solubility of purified pediocin PD-1 below pH 5.0 (results not shown). The peptide is, however, more stable between pH 2.0 and 5.0, with the best stability observed between pH 3.0 and 4.0 (3).

Efflux of K^+ occurred rapidly and immediately after the addition of pediocin PD-1, followed by a phase of slow release. However, leakage rate *per se* did not directly correlate to cellular death, since it was possible to revive more than 90% of the cells after 2h of pediocin PD-1 treatment in phosphate buffer (pH 5.0 to 7.0). The cells

may recover from pore formation when other environmental parameters are optimal. In contrast to nisin-treated cells, a short period of reaccumulation of K^+ could be observed after the initial rapid release following pediocin PD-1 addition. These results suggest that pediocin PD-1 may not cause leakage of essential metabolites such as ATP, and that the peptide may act in a way similar to that suggested for plantaricin C (16). A delay in the loss of ATP was observed after treatment of sensitive cells with plantaricin C and the gradual depletion of intracellular ATP was attributed to ATP hydrolysis by the proton-pump rather than leakage. It is therefore likely that, while pediocin PD-1 treatment results in an efflux of ions, amino acids and ATP remain intracellular and are utilized to reaccumulate K^+. We therefore propose that cell death is a result of the disruption of the PMF, rather than immediate loss of metabolites. In contrast, permeabilization of the cytoplasmic membrane of *O. oeni* by nisin was followed by a drastic reduction in viable cell numbers. Nisin forms large pores as a result of aggregation of several molecules making the membrane permeable to larger compounds, such as ATP and amino acids (26).

The activity of pediocin PD-1 is dependent on the growth temperature of *O. oeni*. Initial pediocin PD-1-induced K^+ efflux, as observed for cells grown at 25°C, was severely reduced at lower growth temperatures. It is well known that membrane fluidity decreases at lower temperatures (17), which may renders the membrane more resistant to pore formation, as observed for *L. monocytogenes* treated with nisin Z (2). *O. oeni* is capable of regulating its membrane fluidity by altering the lipid and protein content (34). The decrease in K^+ efflux observed at lower temperatures may thus be due to changes in the lipid and/or protein content of the cell membrane of *O. oeni*.

Results of potassium efflux studies were obtained with non-growing cells resuspended in phosphate buffer. Although the data suggest that pediocin PD-1 forms pores in the membranes of non-growing cells, the cells did not lyse and pore formation was not accompanied with immediate reduction in viable cell numbers. However, when pediocin PD-1 was added to actively growing *O. oeni* cells in AG-media, a drastic reduction in cell viability was accompanied with cell lysis. Pediocin PD-1-induced lysis is not observed with all sensitive strains, e.g. *Lactobacillus sake* LMG 13558 (results not shown), suggesting that pore formation may act as the sole mechanism for peptide action. As has been suggested for plantaricin C, insertion of the essentially uncharged, globular, C-terminal part of the peptides into the cytoplasmic membrane may be achieved with the positive N-terminal part (four out of the first five amino acids are lysines) remaining attached to the phospholipid head groups via electrostatic interactions (Fig. 9).

Cations such as Mg^{2+}, Ca^{2+} and Gd^{3+} bind to negatively charged head groups of phosphatidylglycerol and cardiolipin in the cytoplasmic membrane of *L. monocytogenes,* rendering nizin Z less antimicrobial (2). It is known that neutralization of negative charges in bacterial cell membranes induces a

condensation of lipids, resulting in a more rigid membrane (19). In our study, treating of *O. oeni* with Gd^{3+} (10 mM) prevented pediocin PD-1 and nisin activity against *O. oeni*. The inhibition of bacteriocin activity decreased with decreased concentrations of Gd^{3+}. In the presence of 2 mM Gd^{3+} cells treated with nisin did not undergo lysis. In the case of pediocin PD-1, Gd^{3+} (2 mM) did not uncouple cell lysis from cell death. While 2 mM Gd^{3+} interferes with the specific interaction of nisin with lipid II binding sites, preventing inhibition of bacterial cell wall biosynthesis, the same appears not to apply to pediocin PD-1. Although nisin and the mersacidin subtype of lantibiotics share the same target it is known that they do not bind to the same part of lipid II. Lipid II has a complex chemical structure providing several possible recognition epitopes. The structural requirements for interaction remains to be elucidated.

We furthermore observed no induction of potasium efflux from *O. oeni* cells in the presence of 10 mM Gd^{3+} after the addition of pediocin PD-1 or nisin (results not presented). We suggest that pore formation is prevented at high concentrations of Gd^{3+} due to complete shielding of electrostatic interaction between the peptides and anionic lipids. Moreover, lipid II binding sites may no longer be accessible due to increased rigidity of the membrane.

In conclusion, our results suggest that pediocin PD-1 acts on the cytoplasmic membrane of *O. oeni*, and that the antimicrobial activity may occur along two mechanisms. The bactericidal effect is primarily due to the generation of pores in the cell membrane. The pores appear to be small and cell death as a result of pore formation is probably due to the disruption of the PMF, rather than immediate loss of metabolites. However, pediocin PD-1 may combine this with inhibition of cell wall biosynthesis, which leads to cell lysis. As has previously been reported for nisin (15), this study confirms that it is important to examine the effect of antimicrobial peptides under a range of growth phases and conditions before drawing generalized conclusions about their efficacy. When the presence of *O. oeni* in foods and alcoholic beverages is a concern, it is important to differentiate between growing and non-growing cells in order to assure effective control.

6.5 ACKNOWLEDGMENTS

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CHAPTER 7

General discussion and conclusions

GENERAL DISCUSSION AND CONCLUSIONS

Malolactic fermentation (MLF) is conducted by lactic acid bacteria (LAB) which convert L-malate, present in wine after alcoholic fermentation, into L-lactate and CO_2 (Henick-Kling, 1995). This secondary fermentation, catalysed by the malolactic enzyme (MLE), is mainly driven by *Oenococcus oeni,* since this organism is able to grow in such a harsh environment as wine. The removal of L-malate, one of the main organic acids of grape must, is essential for deacidification and stabilisation of a large proportion of wine. The occurrence and completion of MLF is difficult to control, and winemakers are looking for improved malolactic starter cultures or alternative means to either inhibit MLF or to stimulate the process. Here we investigated alternative solutions. It has previously been shown that functional expression of the MLE gene in *Saccharomyces cerevisiae* results in MLF conducted by the yeast, without running the risk of bacterial spoilage. Obtaining information about new MLEs is an important prerequisite for future genetic engineering and improvement of MLF. Additional sequences may also resolve questions related to why the MLE, unlike the malic enzyme, transforms malate to lactate without the release of intermediate products, such as pyruvate or NADH. The malolactic gene of *Pediococcus damnosus* NCFB 1832 (*mle*D) that we have described is 72% and 64% identical to the amino acid sequences of *Oenococcus oeni* Lal1 (MleAp) and *L. lactis* subsp. *lactis* MG1363 (MleSp), respectively (Bauer et al., 2003a). Complete and rapid malolactic conversion by *S. cerevisiae* was achieved by co-expressing these MLE genes with the *Schizosaccharomyces pombe* malate permease (*mae1*) gene. However, a lower conversion rate and a significant lower final L-lactate level were observed with the yeast expressing *mle*D. A study determining the K_m-values of the three enzymes might explain this phenomenon. Lower conversion rate with *mleD* opens the possibility to adjust lactate levels according to specific needs. Small differences in malolactic conversion may have a significant effect on the organoleptic quality of the wine and the decrease in intracellular malate concentration may affect the formation of other organoleptic by-products. This is also the first report of the functional expression of the malolactic gene from *O. oeni* in *S. cerevisiae* that result in complete MLF by the transgenic yeast strain. The availability of new malolactic yeast strains enlarges the range of strains that could be used for biological correction of excess acidity in wine. Malolactic strains of *S. cerevisiae* that degrade malate efficiently during alcoholic fermentation should prevent problems experienced with bacterial strains, such as stuck or sluggish MLF or the production of biogenic amines or unwanted flavours. Another advantage of this concurrent alcoholic and malolactic fermentation is the completion of MLF early in the cycle to allow application of cellar operations for wine storage and ageing. Whether these new recombinant yeast strains can replace MLF in all cases is, however, doubtful. Compared to fermentations by *O. oeni*, the engineered yeast would produce wine retaining high

concentrations of micronutrients, rendering the wine microbiologically less stable under certain conditions. Flavourful by-products from bacterial metabolism would also be missing.

MLF in wine is not always desired and although MLF is occasionally difficult to induce, prevention of the development of LAB is likewise difficult. Uncontrolled MLF, especially in wines with a high pH, which is typical to warmer viticulture regions, may render the wine unpalatable or even cause spoilage. Control of MLF in these wines is thus of paramount importance. The use of SO_2 to inhibit microbial growth is strictly regulated, and mounting consumer demands for safe alternatives to chemical preservatives has led to an increased interest in natural antimicrobial substances. Pediocin PD-1, an antimicrobial peptide produced by *Pediococcus damnosus* NCFB 1832, is active against a number of lactic acid bacteria, including malolactic strains of *Lactobacillus, Leuconostoc* and *Oenococcus* spp. We demonstrated the effectiveness of this peptide in preventing the growth of *O. oeni* in grape must and wine (Bauer et al., 2003b). Pediocin PD-1 is stable under winemaking conditions and does not effect yeast growth. We have furthermore shown that pediocin PD-1, when compared with nisin and plantaricin 423, is the most effective in removal of an established biofilm of *O. oeni* from stainless steel surfaces in Chardonnay must (Nel et al, 2002). Pediocin PD-1 could provide the winemaking community with a safe alternative to SO_2 or at least replace part of the SO_2 currently added to must and wine. The addition of bacteriocins to wine is, however, not yet authorised and cost considerations will play a major role in the acceptance of peptide-based wine preservation strategies. An additional threat to the future application of antimicrobial agents lies in the development of resistance, which has already been reported for nisin in a variety of Gram-positive bacteria (Verheul et al., 1997).

LAB isolated from wine are capable of producing bacteriocins and may be responsible for some of the antagonistic effects observed amongst LAB during vinification (Strasser de Saad and Manca de Nadra, 1993; Lonvaud-Funel and Joyeux, 1993). Bacteriocin production in grape must or wine may have a significant impact on the completion of MLF. We have shown, however, that grape must does not constitute a suitable environment for pediocin PD-1 production by *P. damnosus* (Bauer et al, 2003b). Whether this is true for other bacteriocins will have to be assessed on an individual basis. To our knowledge, no papers have been published on the presence of bacteriocins in finished wines.

Since bacteriocins are encoded by genes, a variety of structural analogues of the natural peptide may be constructed through genetic engineering. It opens new possibilities to engineer *S. cerevisiae* wine yeast strains to produce these peptides and to biologically control bacterial populations in wine. The feasibility of this concept has already been demonstrated (Schoeman et al., 1999; van Reenen et al., 2002), but levels of production will have to be optimised. Schoeman et al. (1999) have cloned pediocin PA-1, a bacteriocin produced by *P. acidilactici* PAC1·0 into *S. cerevisiae*. This bacteriocin is active against most wine spoilage LAB with the

exception of *O. oeni* and would therefore be ideal as a preservative in wine where MLF, conducted by *O. oeni*, is wanted. Van Reenen et al. (2002) cloned a homologous bacteriocin, plantaricin 423, produced by *Lactobacillus plantarum* 423 into *S. cerevisiae*. Although pediocin PA-1 and plantaricin 423 belong to the same subclass of bacteriocins, the pediocin-like peptides, and the mature peptides are approx. 40% identical, the spectrum of inhibition differs. Plantaricin 423, contrarily to pediocin PA-1, is very active against *O. oeni* (Nel et al., 2002). Pediocin PD-1 and nisin, on the other hand, belong to the lantibiotic family of bacteriocins (Bauer et al., 2003c). Unlike the pediocin-like bacteriocins, lantibiotics are posttranslationally modified. The engineering of lantibiotics is less straightforward than that of unmodified proteins, since expression systems have to be developed not only for the structural genes but also for the genes encoding the biosynthetic enzymes and regulatory proteins. The cloning of lantibiotic genes in *S. cerevisiae* and its expression is an exiting challenge. Unlike nisin, the nucleotide sequence of pediocin PD-1 is not known. The *P. damnosus* library that we have constructed (Bauer et al., 2003c) will be an invaluable tool in realising this aim. Yeast strains expressing bacteriocin genes, such as pediocin PD-1, nisin or plantaricin 423, would be useful in wines where MLF is not wanted. The construction of recombinant wine yeast strains expressing bacteriocin genes together with the malolactic gene would be useful in wines where concurrent alcoholic and malolactic fermentation is required without the effects associated with bacterial metabolism.

Effective application of bacteriocins requires an understanding of the molecular mechanisms of these peptides. This is especially true for the lantibiotics, where many questions regarding the function of dehydrated amino acids, the enzymatic reactions responsible for their formation, and mechanism of action remain unanswered. With understanding comes the possibility to enhance the stability and activity of these peptides. Already, the potential of genetic engineering for rational drug design has been demonstrated for substilin, where site-directed mutagenesis resulted in the enhancement of the chemical and antimicrobial properties of the peptide (Liu and Hansen, 1992). Bacteriocins are grouped into different classes and subtypes based on their antimicrobial activity and structure. Although the complete amino acid sequence of pediocin PD-1 could not be obtained through Edman degradation, we tentatively included pediocin PD-1 (2867 Da) and plantaricin C (2880 Da), produced by *Lactobacillus plantarum* LL441 (Turner et al., 1999), into the mersacidine subtype of the Type B lantibiotics (Bauer et al., 2003c). This group is represented by mersacidine, produced by a *Bacillus* sp., and actagardine, produced by an *Actinoplanes* sp. (Sahl et al, 1995). Mersacidine and actagardine are of similar sizes (1825 and 1890 Da, respectively) and hydrophobicities and carry no net charge. These two lantibiotics contain a conserved sequence motif (amino acids 11 to 18 and 5 to 13, respectively) that comprises one entire ring structure (Brötz et al., 1997). This conserved motive is shared with plantaricin C and possibly pediocin PD-1 (amino acids 12 to 18 for both bacteriocins). Mersacidine exerts its bactericidal

action by inhibition of peptidoglycan biosynthesis through interaction with lipid II (Brötz et al., 1998).

Although the lipid II-targeting antimicrobial peptides and vancomycin (a glycopeptide antibiotic) share a common binding molecule, the recognition epitopes among nisin, vancomycin and mersacidin are different (Breukink et al., 1999). As antibiotic resistance is becoming more and more common, the diversity of such a targeting action is of great importance. The conserved sequence motif shared by mersacidine, actagardine, plantaricin C and pediocin PD-1 may be involved in the binding of these bacteriocins to lipid II. Contrarily to mersacidine and actagardine, plantaricin C and pediocin PD-1 carry a net positive charge and these peptides not only induce cell lysis, but also pore formation (Bauer et al., 2003c, González et al., 1996). We propose that specific interaction of pediocin PD-1 with lipid II inhibits peptidoglycan biosynthesis, as observed with mersacidin, while pore formation is rather due to electrostatic interaction between the positively charged unbridged N-terminus and the anionic phospholipids in the cytoplasmic membrane of sensitive cells (Bauer et al., 2003c).

To investigate the potential of pediocin PD-1 to be applied as a natural preservative, we examined the effect of the peptide on cells of *O. oeni* under a range of conditions and growth phases. *O. oeni* was more resistant against pediocin PD-1, when the cells were grown at reduced temperatures (Bauer et al. 2003c). *O. oeni* is capable of regulating its membrane fluidity by altering the membrane's lipid and protein content (Tourdot-Maréchal et al. 2000). These changes may render the membrane more resistant to pore formation. Pediocin PD-1 is active at pH values ranging from 2.0 to 10.0, while highest activity against cells of *O. oeni* was observed at an external pH of 5 (Bauer et al., 2003c). Interesting, optimal growth of *O. oeni* in the absence of pediocin PD-1 was also observed at this pH value. The peptide furthermore displayed little activity against non-growing *O. oeni* cells and cells in stationary phase. It is therefore important to differentiate between growing and non-growing cells in order to assure effective control. Antimicrobial activity may be, at least in part, ascribed to a synergistic effect between pediocin PD-1 action and pH, and the conditions for optimal activity may be species specific. Among considerations of application of bacteriocins are those of stability and solubility. At high concentrations of pediocin PD-1, we observed a decrease in solubility below pH 5. The peptide is, however, more stable between pH 2.0 and 5.0, with best stability observed between pH 3.0 and 4.0 (Bauer et al., 2003b). Since pediocin PD-1 is very potent and will only be applied in low concentrations, solubility related problems should never present itself. In solution, purified pediocin PD-1 is very unstable, but it is remarkably stable in the presence of a high salt buffer or in a dried state. The presence of food or beverage components also protects the peptide. Pediocin PD-1 retained its activity for at least 20 min at 100°C at pH values ranging from 2 to 5 (Bauer et al., 2003b) and even sustained autoclaving (121° for 10 min) at pH 7 (Green et al., 1997).

The application of pediocin PD-1 should not be restricted for use in the wine industry. Staphylococci, clostridia and bacilli are common spoilage organisms found in processed food and in natural cheeses (Delves-Broughton et al., 1996). Species from all these genera are included in the spectrum of inhibition of pediocin PD-1 (Green et al., 1997; R. Bauer, unpublished data). Having a similar spectrum of inhibition as mersacidine and actagardine, including *Staphylococcus aureus* and *Enterococcus* spp. (R. Bauer, unpublished data) and, acting on a novel target, pediocin PD-1 may prove useful in the treatment of emerging multi-drug-resistant pathogens, such as methicilin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci. Treatment of MRSA is currently only possible with glycopeptide antibiotics, vancomycin and teicoplanin, and resistant strains are emerging (Hiramatsu et al., 1997). Low concentrations of mersacidine effectively cured systematic staphylococcal infections in mice (including MRSA strains) and subcutaneous staphylococcal abscesses in rats (Limbert et al., 1991; Niu and Neu, 1991), making this type of lantibiotic the most promising antimicrobial agent with respect to a potential chemotherapeutical application. Bacteriocins such as pediocin PD-1, being strongly bactericidal towards mastitis pathogens (*Staphylococcus* spp. and *Streptococcus* spp.), should also have potential as a therapeutic agent in the treatment of bovine mastitis (R. Bauer, unpublished data). This concept has already been proven in field trails with nisin (Delves-Broughton et al., 1996) and enterocin 1071, a bacteriocin produced by *Enterococcus faecalis 1071* (Davidse et al., 2004, manuscript in preparation). Pediocin PD-1 also displays a strong antimicrobial action against *Propionibacterium* spp. including *Propionibacterium acnes* (R. Bauer, unpublished data). *P. acnes* is an opportunistic pathogen found within the human body and plays a significant role in the skin disease, acne vulgaris. Although *P. thoenii* strains produce bacteriocins active against *P. acnes*, production levels are very low and the bacteriocin is sensitive to proteolytic enzymes (van der Merwe et al., 2003, article in press). Pediocin PD-1, on the other hand, is more stable against protease degradation due to its rigid structure. This, together with the high activity displayed by low concentrations make this peptide a more promising agent with respect to a potential cosmetic application in topical skin treatment of acne.

The main obstacle to the use of bacteriocins in food and beverages lies with regulation. Although the commercial exploitation of bacteriocins to date is mainly restricted to food-related applications of nisin, potential novel applications for lantibiotics and unmodified bacteriocins continue to be developed. Further developments of pediocin PD-1 are likely to include synergistic actions with other bacteriocins, such as nisin, and with enzymes such as lysozyme.

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APPENDIX I

Growth optimization of *Pediococcus damnosus* NCFB 1832 and the influence of pH and nutrients on the production of pediocin PD-1

APPENDIX I

Growth optimization of *Pediococcus damnosus* NCFB 1832 and the influence of pH and nutrients on the production of pediocin PD-1

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AIMS

Optimization of the growth of *Pediococcus damnosus* NCFB 1832 and the production of pediocin PD-1 by traditional fermentation methods.

METHODS AND RESULTS

Fermentation studies were conducted in De Man Rogosa and Sharpe (MRS) broth (Oxoid), pre-adjusted to specific pH values, and in MRS broth supplemented with various nitrogen sources, $MnSO_4$, $MgSO_4$ and Tween 80. The production of pediocin PD-1 closely followed the growth curve of *Ped. damnosus* NCFB 1832. Maximum levels of bacteriocin activity (3249 AU $ml^{-1}/O.D._{max}$) were recorded in MRS broth with an initial pH of 6·7. In media with an initial pH of 4·5 bacteriocin activity as low as 222 AU $ml^{-1}/O.D._{max}$ was recorded. The highest bacteriocin activity was recorded in growth conditions allowing the greatest pH variation (highest ΔpH). The addition of bacteriological peptone at 1·7% (w/v), $MnSO_4$ (0·014%, w/v) and Tween 80 (3%, v/v) to MRS and adjusting of the medium pH to 6·7, resulted in a further increase in activity (from 3249 to 5078 AU $ml^{-1}/O.D._{max}$). The same medium, but an initial pH of 6·2 resulted in a 82.5% decrease in bacteriocin activity.

CONCLUSIONS

Pediocin PD-1 production is not only stimulated by the presence of specific growth factors (e.g. bacteriological peptone, $MnSO_4$ or Tween 80), but may also be stimulated by the lowering in pH during growth (highest ΔpH), and thus also the amount of organic acids produced.

SIGNIFICANCE AND IMPACT OF STUDY

The production of pediocin PD-1 of the wild-type producer strain was significantly improved by using a defined medium and traditional fermentation methods.

A1.1 INTRODUCTION

Bacteriocins are defined as ribosomaly synthesized antimicrobial peptides or proteins produced by bacteria, with antagonistic activity against bacteria genetically closely related to the producer strain (De Vuyst and Vandamme 1994b). The pediocin-like bacteriocins fall into class IIa and are defined as small heat-stable *Listeria*-active peptides (Klaenhammer 1993; Horn *et al.* 1998).

A number of bacteriocins produced by *Pediococcus* spp. have been described, viz. pediocin A produced by *Ped. pentosaceus* FBB-61 (Etchells *et al.* 1964; Flemming *et al.* 1975), PA-1 produced by *Ped. acidilactici* PAC 1·0 (Gonzales and Kunka 1987), AcH produced by *Ped. acidilactici* H (Bhunia *et al.* 1987, 1988), JD produced by *Ped. acidilactici* JD-23 (Richter *et al.* 1989), SJ-1 produced by *Ped. acidilactici* SJ-1 (Schved *et al.* 1993), N5p produced by *Ped. pentosaceus* (Strasser de Saad *et al.* 1995), 5 produced by *Ped. acidilactici* UL5 (Haung *et al.* 1996) and pediocin L50 that has been renamed as enterocin L50 (Cintas *et al.* 1998).

Little research has been done on the bacteriocins of *Pediococcus* spp., despite the fact that they cause spoilage in beer, wine and other high-pH food products. The spectra of antimicrobial activity of the pediocins produced by *Ped. pentosaceus* and *Ped. acidilactici* are very similar and the genes coding for their production share many conserved regions (De Vuyst and Vandamme 1994a). This is not surprising, since the latter two species are phylogenetically closely related (Collins *et al.* 1991). *Ped. damnosus* is, however, phylogenetically distant from *Ped. acidilactici* and *Ped. pentosaceus* (Collins *et al.* 1991). Furthermore, the spectrum of antimicrobial activity of pediocin PD-1, produced by *Ped. damnosus* NCFB 1832, is different from that recorded for other pediocins and unique in the sense that it does not inhibit *Pediococcus* spp. (Green *et al.* 1997).

Most of the research aimed at the increase of bacteriocin production is focussed on genetic manipulation of the producer strain. Little research has been done on fermentation optimization of bacteriocin-producing strains. For optimal bacteriocin production the producer strain usually requires complex nutrients, ions, Tween 80 and well-controlled growth conditions, such as temperature and pH (Biswas *et al.* 1991; De Vuyst and Vandamme 1992; Daba *et al.* 1993; Kaizer and Montville 1993; Parente and Ricciardi 1994; Parente *et al.* 1994; Yang and Ray 1994; Mørtvedt *et al.* 1995; Leroy and De Vuyst 1999; Callewaert and De Vuyst 2000).

Bacteriocin production is usually growth associated, i.e. the level at which the peptide is produced is linked to the biomass formed (De Vuyst and Vandamme 1992; Mørtvedt *et al.* 1995; De Vuyst *et al.* 1996; Kim *et al.* 1997; Aasen *et al.* 2000; Callewaert and De Vuyst 2000). However, production rates do not always correlate

with growth rate or biomass production (Kim *et al.* 1997; Bogovic-Matijasic and Rogelj 1998; Aasen *et al.* 2000). Enhanced production at non-optimum growth pH and/or temperature suggests that low growth rate, unfavorable growth conditions, or other less optimal environmental conditions may also stimulate bacteriocin production (De Vuyst *et al.* 1996; Aasen *et al.* 2000; Callerwaert and De Vuyst 2000).

In this paper we report on the influence of fermentation conditions such as pH, various nitrogen sources, manganese, magnesium and Tween 80 on the growth of *Ped. damnosus* NCFB 1832 and the production of its bacteriocin, pediocin PD-1.

A1.2 MATERIALS AND METHODS

A1.2.1 BACTERIAL STRAINS AND GROWTH CONDITIONS

The pediocin PD-1 producer strain, *Ped. damnosus* NCFB 1832, was grown in De Man Rogosa and Sharpe (MRS) broth (Oxoid) at 30 °C. The indicator strain, *Oenococcus oeni* (previously *Leuconostoc oenos*, Dicks *et al.* 1995) was grown in acidic grape broth (Dicks *et al.* 1990) at 30 °C.

A1.2.2 PRODUCTION OF PEDIOCIN PD-1

Production of pediocin PD-1was followed during fermentation in 2 l MRS broth. The medium was inoculated with 1% (v/v) of a three-day-old culture of *Ped. damnosus* NCFB 1832 ($O.D._{600}$ approx. 1·4). Fermentations were conducted at 30 °C for 115 h. Samples were withdrawn aseptically from the fermentation medium at regular time intervals and analyzed for cell growth ($O.D._{600}$) and bacteriocin production (AU ml^{-1}), as described by Green *et al.* (1997).

A1.2.3 FERMENTATION OPTIMIZATION OF PEDIOCIN PD-1 PRODUCTION

The inoculum size used in all fermentation experiments was 1% (v/v) of a three-day-old ($O.D._{600}$ = 1·4) culture grown in MRS broth. All fermentations were conducted in duplicate, with three repeats of each fermentation run. The average of six pH measurements, optical density readings, and bacteriocin activity tests was thus determined.

A1.2.3.1 Growth pH

The effect of pH on the growth of strain NCFB 1832 and the production of pediocin PD-1 were studied in 250 ml Erlenmeyer flasks. The pH of MRS broth was adjusted to 7·0, 6·7, 6·4 and 6·2 by adding sterile 5 N NaOH. To obtain initial pH values of 6·0, 5·8, 5·4, 5·0 and 4·5, the medium was adjusted with sterile 2 N HCl. All pH adjustments were done after autoclaving. Samples were taken at the end of the fermentation to determine the pH, maximum optical density (600 nm), bacteriocin

activity (AU ml^{-1}) and specific bacteriocin activity as described by Verellen *et al.* (1998).

A1.2.3.2 Effect of nitrogen sources

The influence of different nitrogen sources were tested in MRS broth, supplemented with the following: 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) casamino acids (Oxoid), respectively, as used by Verellen *et al.* (1998). Fermentations were conducted in sterile 2 l Biostat® M fermentors with a maintained agitation speed of 50 rpm, without aeration and at a constant pH of 6·7 by automatic addition of sterile 5 N NaOH. The pH, optical density ($O.D._{600}$) of the culture and antimicrobial activity (AU ml^{-1}) of pediocin PD-1 were determined directly after inoculation, after 5 and 10 h, and then every 7 h of fermentation.

A1.2.3.3 Effect of manganese, magnesium and Tween 80

Fermentations were conducted in 200 ml MRS broth, supplemented with 1·7% (w/v) bacteriological peptone. The medium pH was adjusted to 6·7 by the addition of sterile 2 N NaOH. The concentrations of $MnSO_4 \cdot H_2O$ used were between 0·004 and 0·064% (w/v). Magnesium was added in the form of $MgSO_4 \cdot 7H_2O$ (0·005%, w/v). Medium without $MgSO_4 \cdot 7H_2O$ served as control. Tween 80 was added at levels of 0·05%, 0·1%, 0·2%, 0·35%, 1·0%, 2·0% and 3·0% (v/v). Medium without Tween 80 served as control. Samples were taken after 27, 32, 48, 55, 72 and 96 h of fermentation. The pH, optical density of the culture and bacteriocin activity (AU ml^{-1}) were recorded and the specific bacteriocin activity (AU ml^{-1}/ $O.D._{600}$) calculated as decribed before.

A1.2.3.4 Optimization of growth medium and pediocin PD-1 production

An optimal medium was compiled for the production of pediocin PD-1 based on results obtained from individual experiments. MRS broth was supplemented with 1·7% (w/v) bacteriological peptone, 0·014% (w/v) $MnSO_4$ and 3·0% (v/v) Tween 80 and adjusted to an initial pH of 6·7 with 5N NaOH before autoclaving. MRS broth not supplemented and inoculated at an initial pH of 6·2 served as control. The culture pH, optical cell density and activity (AU ml^{-1}) of pediocin PD-1 were recorded after 27, 32, 38, 55, 72 and 96 h of fermentation.

A1.3 RESULTS

The pH measurements, optical density readings, and bacteriocin activity tests of the six fermentation runs did not vary by more than 5%. The data represents the average of six fermentation runs.

A1.3.1 PEDIOCIN PD-1 PRODUCTION DURING GROWTH IN MRS BROTH

Growth of *Ped. damnosus* NCFB 1832 and the production of pediocin PD-1 were monitored in MRS broth without any additives (Fig. 1). The cells reached stationary phase after 84 h of fermentation. During this time the optical density of the culture increased to 2·13 (measured at 600 nm) and remained at that level for the remainder of the fermentation. Production of pediocin PD-1 more-or-less followed the growth curve and increased to 1600 AU ml^{-1} during the logarithmic phase of growth and remained at this level throughout the stationary phase. The pH decreased from 6·2 to 3·9 during the first 84 h of fermentation, but decreased further to pH 3·6 during stationary growth.

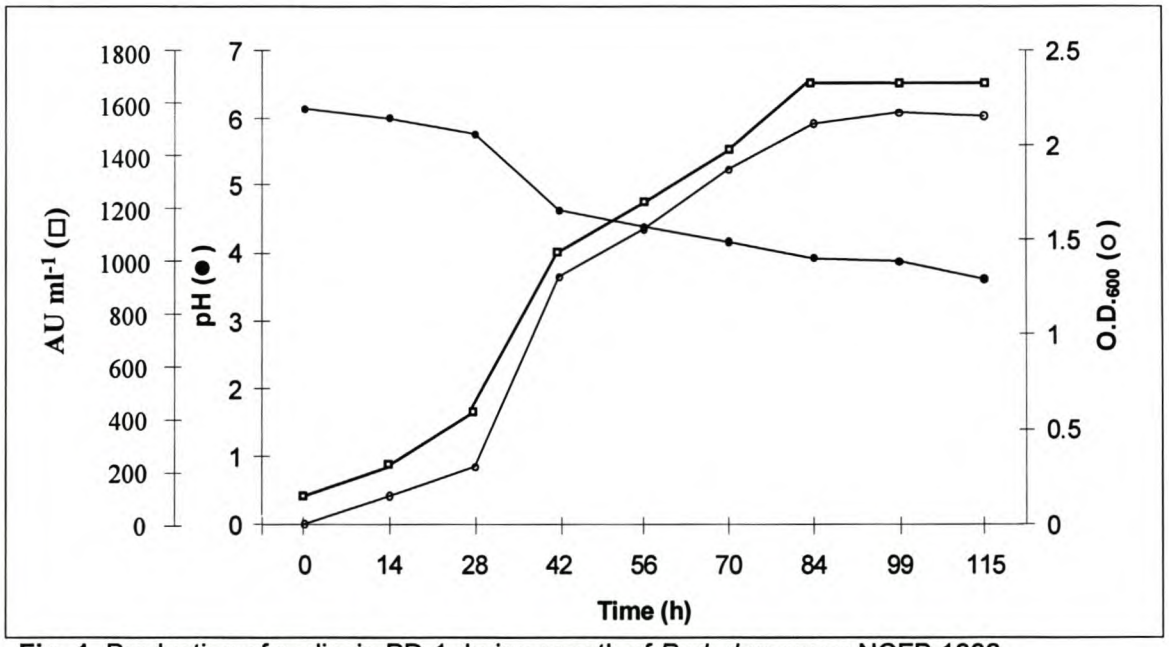

Fig. 1 Production of pediocin PD-1 during growth of *Ped. damnosus* NCFB 1832. Symbols: ●, changes in pH; ○, optical density at 600 nm; □, pediocin PD-1 activity in AU ml^{-1}.

A1.3.2 THE EFFECT OF INITIAL PH ON GROWTH AND PRODUCTION OF PEDIOCIN PD-1

The effect of initial growth pH is shown in Table 1. The highest specific activity was obtained with an initial pH of 6·7, followed by growth at pH 7·0, 6·4, 6·2, 6·0, 5·8, 5·4, 5·0 and 4·5. Changes in pH that were recorded during growth (ΔpH) coincided with the $O.D._{max}$-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. The highest ΔpH (ΔpH 2·56) was obtained for cells that started at an initial growth pH of 6·7.

Table 1 Effect of initial pH on the growth of *Ped. damnosus* NCFB 1832 and the production of pediocin PD-1*

Initial pH	7.00	**6.70**	6.40	6.20	6.00	5.80	5.40	5.00	4.50
Final pH	5.04	**4.14**	4.40	4.37	4.39	4.34	4.29	4.25	4.43
Δ pH	1.96	**2.56**	2.00	1.83	1.61	1.46	1.11	0.75	0.07
$O.D._{max}$	1.039	**1.970**	1.890	1.864	1.721	1.663	1.484	1.008	0.451
Maximum bacteriocin production (AU ml^{-1})	2600	**6400**	3200	1600	1400	1200	800	400	100
Specific bacteriocin production (AU $ml^{-1}/O.D._{max}$)	2502	**3249**	1693	858	814	722	539	397	222

*The values represent the average of two experiments.

A1.3.3 THE EFFECT OF DIFFERENT NITROGEN SOURCES ON THE GROWTH AND PRODUCTION OF PEDIOCIN PD-1

Ped. damnosus NCFB 1832 reached stationary growth phase within 38 h when the medium was supplemented with bacteriological peptone (Fig. 2A). Meat extract provided the second best growth, followed by tryptone, yeast extract and casamino acids. Medium supplemented with bacteriological peptone and meat extract produced the highest level of pediocin PD-1 activity, followed by tryptone and yeast extract (Fig. 2B). Bacteriocin production occurred throughout logarithmic growth, but ceased at the onset of the stationary phase. For the remainder of the fermentation no reduction of bacteriocin activity was observed. All substrates resulted in similar maximum cell density ($O.D._{max}$ between 2·0 and 2·3), except for casamino acids, which did not yield significant growth.

A1.3.4 THE EFFECT OF MANGANESE, MAGNESIUM AND TWEEN 80 ON THE SPECIFIC ACTIVITY OF PEDIOCIN PD-1

To study the effect of different concentrations of $MnSO_4$, $MgSO_4$ and Tween 80 on the growth of *Ped. damnosus* NCFB 1832 and pediocin PD-1 production, readings were taken at six specific time intervals and the results reported as AU $ml^{-1}/O.D._{max}$.

The maximum concentration of $MnSO_4$ that was soluble in the experimental medium was 0·064% (w/v). The highest specific activity recorded in the presence of manganese was at an initial concentration of 0·014% (w/v) $MnSO_4$ (Fig. 3). At this concentration the specific activity of pediocin PD-1 reached a peak of 2560 AU after 32 h of fermentation. Concentrations of 0·004% (w/v) and 0·014% (w/v) $MnSO_4$ yielded specific activity levels of 1172 and 1800 AU $ml^{-1}/O.D._{600}$, respectively, during the same growth period. The cultures reached similar optical density readings after 32 h of fermentation. After 55 h of fermentation in the presence of 0·014% (w/v) $MnSO_4$ the specific activity decreased to approx. 2000 AU $ml^{-1}/O.D._{max}$, which is similar to the maximum specific activity recorded when cultured in the presence of 0·024, 0·044 and 0·064 g l^{-1} $MnSO_4$.

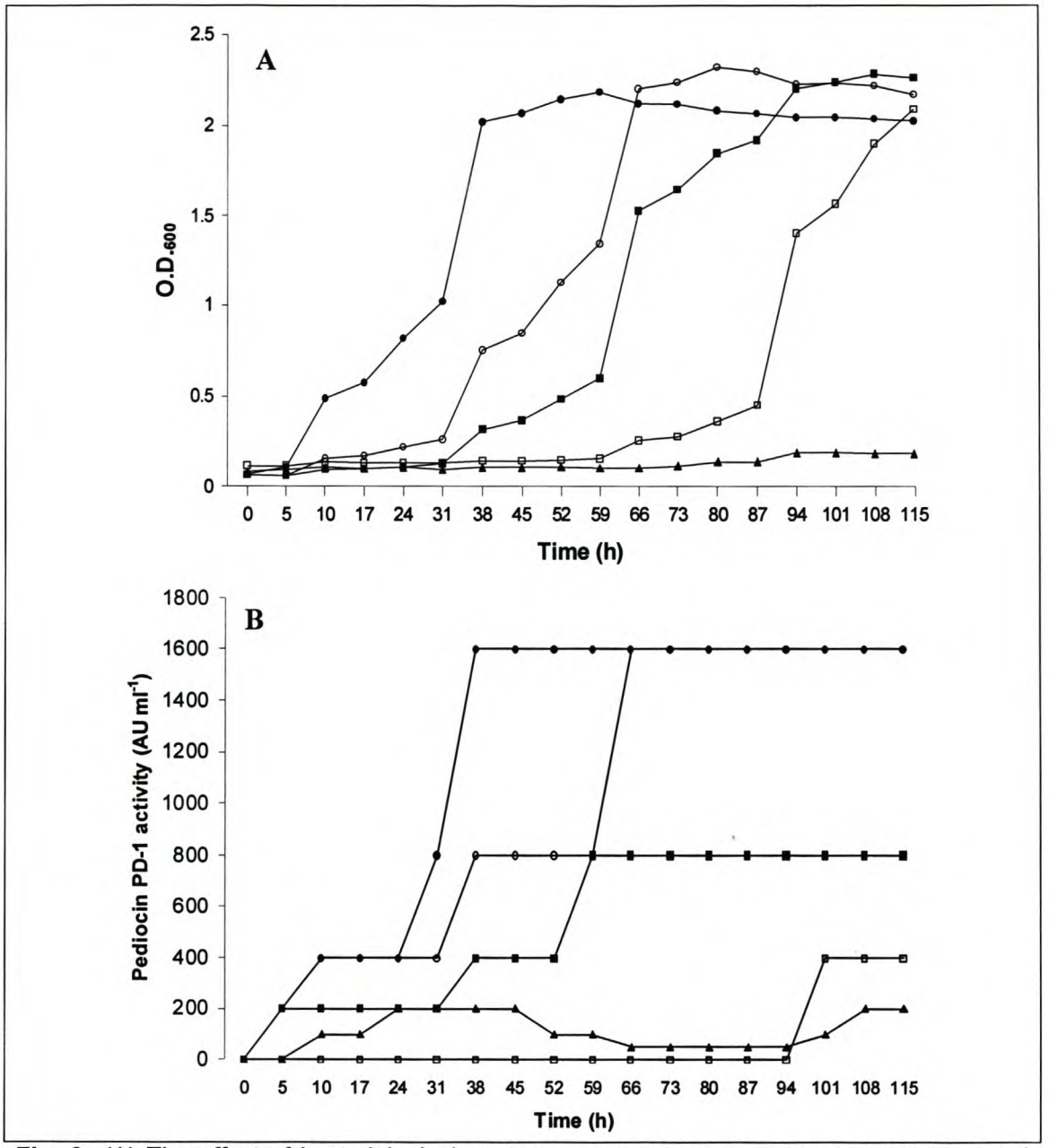

Fig. 2 (A) The effect of bacteriological peptone, meat extract, tryptone, yeast extract and casamino acids on the growth of *Ped. damnosus* NCFB 1832. (B) The effect of bacteriological peptone, meat extract, tryptone, yeast extract and casamino acids on the production of pediocin PD-1. Symbols: (A, B) ●, bacteriological peptone; ○, meat extract; ■, tryptone; □, yeast extract; σ, casamino acids.

The addition of $MgSO_4$ (0·05%, w/v) resulted in a very slight increase of growth, which was similar to that recorded with $MnSO_4$, but did not result in an increase in the specific production of pediocin PD-1 (data not shown).

Increased concentrations of Tween 80 stimulated the production of pediocin PD-1 (Fig. 4). The maximum concentration of Tween 80 that was soluble in the experimental medium was 3% (v/v). At this concentration the specific activity increased to 4000 AU $ml^{-1}/O.D._{.max}$ after 55 hours of fermentation. Results recorded when cells were grown in the presence of 0·5, 1·0 and 2·0% (v/v) Tween 80 were

similar. Lower concentrations of Tween 80 resulted in no drastic increase of pediocin PD-1 activity.

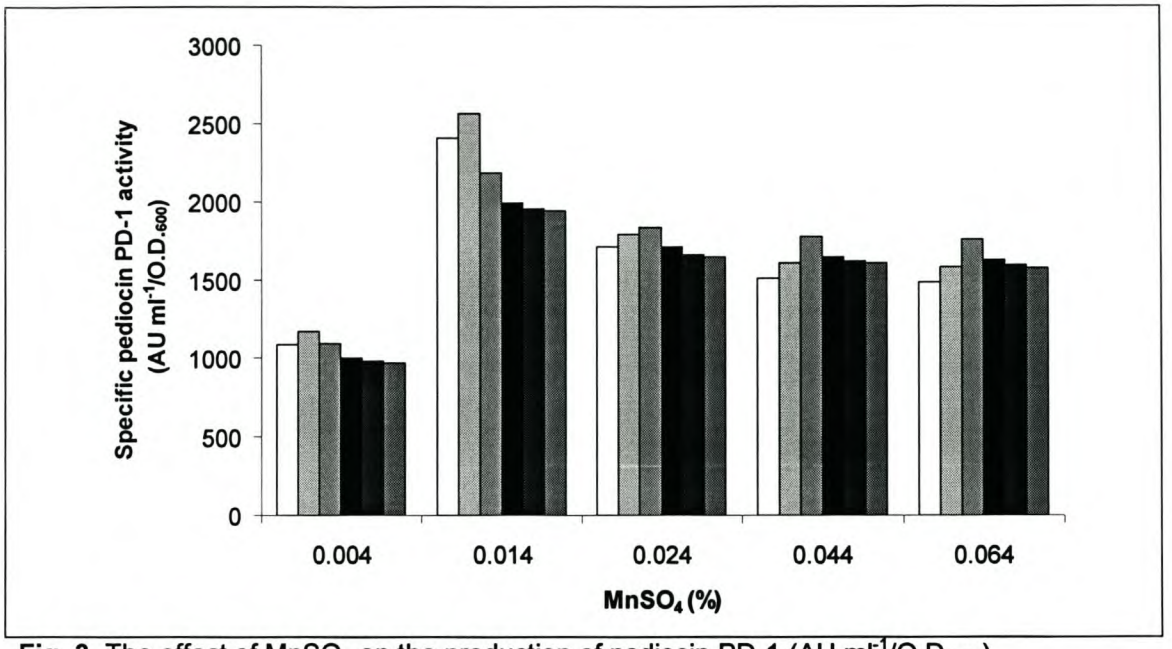

Fig. 3 The effect of $MnSO_4$ on the production of pediocin PD-1 (AU $ml^{-1}/O.D._{600}$). Symbols: □, activity at 27 h; ▨, activity at 32 h; ▩, activity at 48 h; ■, activity at 55 h; ▧, activity at 78 h; ▩, activity at 96 h.

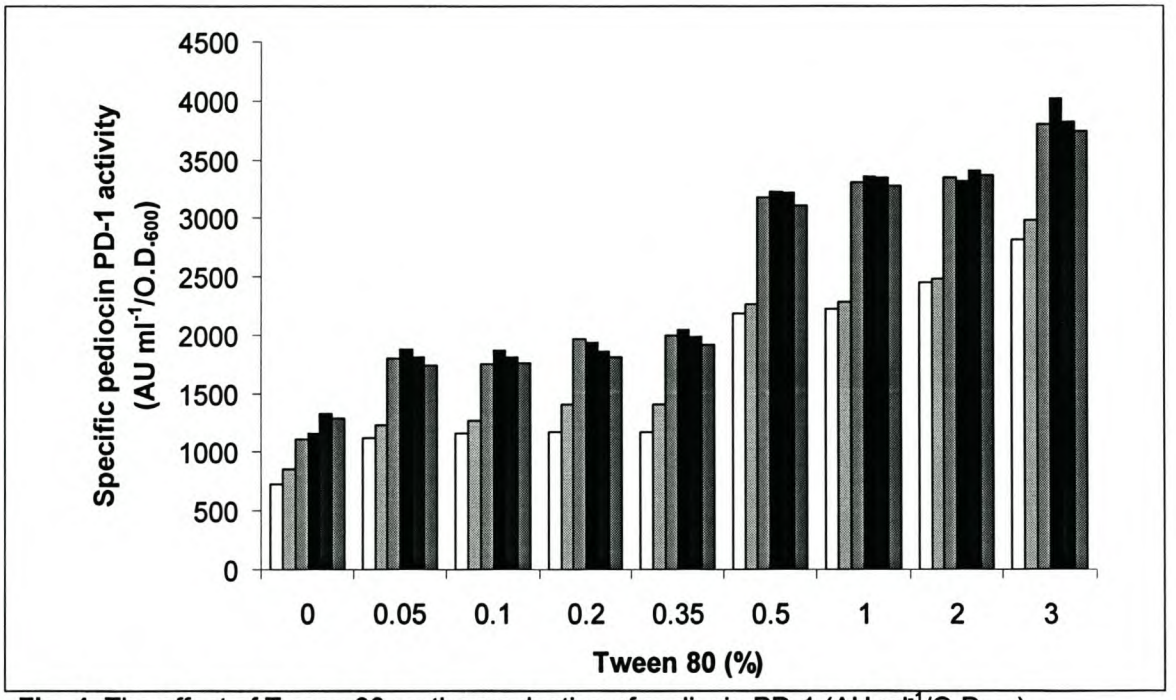

Fig. 4 The effect of Tween 80 on the production of pediocin PD-1 (AU $ml^{-1}/O.D._{600}$). Symbols: □, activity at 27 h; ▨, activity at 32 h; ▩, activity at 48 h; ■, activity at 55 h; ▧, activity at 72 h; ▩, activity at 96 h.

A1.3.5 OPTIMIZATION OF GROWTH MEDIUM AND PEDIOCIN PD-1 PRODUCTION

The effect on the production of pediocin PD-1 in 1 l MRS media supplemented with 1·7% (w/v) bacteriological peptone, 0·014 (w/v) $MnSO_4$ and 3% (v/v) Tween 80 is shown in Fig. 5. No significant difference with regard to maximum cell growth ($O.D._{max}$) and growth rate was observed between the optimized and control media (data not shown). However, specific bacteriocin activity increased by a factor of approx. 6 after 55 h of fermentation (Fig. 5 and Table 2).

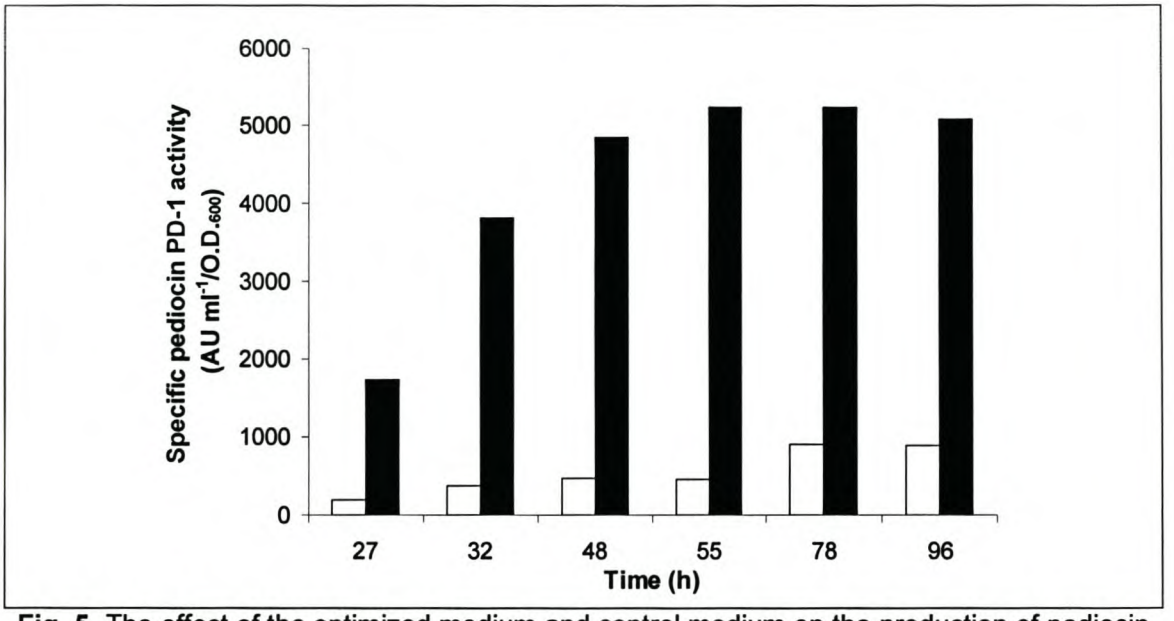

Fig. 5 The effect of the optimized medium and control medium on the production of pediocin PD-1 (AU $ml^{-1}/O.D._{600}$). Symbols: ■ , optimized medium; □ , control medium.

Table 2 Effect of initial pH on the growth of *Ped. damnosus* NCFB 1832 and the production of pediocin PD-1 under optimized and control conditions*

	Control	Optimized medium
Initial pH	6.2	6.7
Final pH	3.55	3.58
Δ pH	2.65	3.12
$O.D._{max}$	1.803	1.851
Maximum bacteriocin production (AU ml^{-1})	1600	9400
Specific bacteriocin production (AU $ml^{-1}/O.D._{max}$)	887	5078

*The values represent the average of two experiments.

A1.4 DISCUSSION

Pediocin PD-1 production occurred throughout logarithmic growth but stopped as the cells entered the stationary growth phase. A decrease in bacteriocin production after the cessation of growth is frequently observed for other bacteriocins (Parente and Ricciardi 1999). However, in our fermentation under controlled and uncontrolled pH no reduction in activity levels of pediocin PD-1 occurred during stationary growth phase. Thus, even though production of pediocin PD-1 is growth associated, conditions leading to higher maximum cell growth ($O.D._{max}$) did not necessarily result in higher levels of pediocin PD-1 production.

Many studies indicated that temperatures and pH levels lower than the optimal for growth resulted in higher levels of bacteriocin production. This was observed for lactococcin A (Parente *et al.* 1994), enterocin 1146 (Parente and Ricciardi 1994), lactocin S (Mørtvedt-Abildgaard *et al.* 1995), amylovorin 1471 (De Vuyst *et al.* 1996), nisin Z (Matsusaki *et al.* 1996) and mesenterocin (Kim *et al.* 1997). A few bacteriocins, including pediocin AcH and plantaricin C, are produced only at a pH below 5·0 (Biswas *et al.* 1991; Yang and Ray 1994; Bárcena *et al.* 1998). Pediocin AcH production at a low pH was attributed to posttranslational processing of the bacteriocin (Biswas *et al.* 1991). However, this effect is strain or species-dependent, since pediocin AcH is produced at pH 6·0 by *Lb. plantarum* WHE2 (Ennahar *et al.* 1996). In our case, a controlled pH of 6·7 resulted in a significantly lower pediocin PD-1 yield than at an uncontrolled initial pH of 6·7. In the latter case, the pH dropped dramatically to reach levels of approximately 3·5 in the stationary phase and allowed for the highest level of pediocin PD-1 production recorded. These results suggest that the production of pediocin PD-1 may increase when the pH level drops during the growth phase of the organism. A possible explanation for this observation may be found in less stringent binding of the peptide to the cell wall of *Ped. damnosus* NCFB 1832 as fermentation continues and the pH decreases. A similar phenomenon has been described for nisin, produced by *Lactococcus lactis* subsp. *lactis* (Hurst and Dring 1968). 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 (Hurst and Dring 1968).

Bacteriocin production is strongly dependent on the composition and concentration of complex nutrients. Bacteriological peptone supported the fastest growth rate and resulted in the highest specific activity of pediocin PD-1. Despite the lower growth rate observed on meat extract, the level of pediocin PD-1 produced was similar to the level recorded in medium supplemented with bacteriological peptone. Otherwise, a more-or-less linear correlation was obtained between the rate of bacteriocin production and growth rate. All the nitrogen sources tested, except casamino acids, supported growth.

Both anions and cations affect bacteriocin production, but their influence may be strain specific (Parente and Ricciardi 1999). Mg^{2+} has been shown to increase pediocin AcH production (Biswas *et al.* 1991). The highest specific activity of

pediocin PD-1 was achieved at an initial concentration of 0·014% (w/v) $MnSO_4$, approximately two times higher than recorded at a concentration of 0·004% (w/v). A further increase in $MnSO_4$ concentration did not result in an increase in specific activity, but a slight decrease despite similar growth kinetics.

Increase of Tween 80 concentration resulted in the increase of specific pediocin PD-1 production up to the highest concentration tested (3%, v/v), despite a slight decrease in growth where a concentration of more than 1% (v/v) was added to the medium. Tween 80 appears to stimulate the production of some bacteriocins (Parente and Hill 1992; Daba *et al.* 1993; Matsusaki *et al.* 1996; Verellen *et al.* 1998). However, it may simply have the effect of preventing bacteriocin adsorption on polypropylene and glass surfaces (Joosten and Nuñez 1995), thus increasing apparent bacteriocin titres.

Conclusions from this study are that pediocin PD-1 production is not only stimulated by the presence of specific growth factors (e.g. bacteriological peptone, $MnSO_4$ or Tween 80), but may also be stimulated by the lowering in pH during growth (highest ΔpH), and thus also the amount of organic acids produced.

A1.5 ACKNOWLEDGEMENTS

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APPENDIX II

The effect of bacteriocins pediocin PD-1, plantaricin 423 and nisin on biofilms of *Oenococcus oeni* on a stainless steel surface

APPENDIX II

The Effect of Bacteriocins Pediocin PD-1, Plantaricin 423 and Nisin on Biofilms of *Oenococcus oeni* on a Stainless Steel Surface

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ABSTRACT

The effect of pediocin PD-1, plantaricin 423 and nisin was tested against established biofilms formed by a commercial starter culture of *Oenococus oeni* on stainless steel slides and planktonic (free-living) cells. The percentage viable and non-viable cells were determined by using the Baclight™ viability probe and an epifluorescent microscope with image analysis. Pediocin PD-1 (3000 AU/mL) removed all cells in a biofilm of *O. oeni* which formed in acidic grape medium after 5 hr. Plantaricin 423 (3000 AU/mL) and nisin (3000 AU/mL) killed all viable cells in the biofilm. However, approximately 42% and 49% of the original cells on the stainless steel surface remained attached with the majority staining non-viable when treated with the latter two peptides, respectively. In a modified Chardonnay must, all cells (viable and non-viable) of *O. oeni* in the biofilm were removed after 1 hr when treated with pediocin PD-1 (3000 AU/mL). In similar experiments, treatment with plantaricin 423 and nisin resulted in undetectable numbers of viable cells in the biofilm. Approximately 36% and 43% of the original cells on the stainless steel surface remained attached with the majority staining non-viable after 5 hr of treatment with the latter two peptides, respectively. After 5 hr of treatment with the respective antimicrobial peptides (3000 AU/mL), the planktonic cell numbers of *O. oeni* decreased from 1.3×10^{10} cfu/mL to undetectable numbers of viable cells in acidic grape medium. The same experiment performed in modified Chardonnay must yielded no detectable cells of *O. oeni*. To our knowledge, this is the first report on the application of bacteriocins in the destruction of malolactic biofilms on a stainless steel surface.

A2.1 INTRODUCTION

Malolactic bacteria occur throughout the winemaking process, but normally only commence growth after primary (alcoholic) fermentation [28]. Malolactic fermentation (MLF) is considered important for the deacidification, flavor modification and microbial stability of wine, especially in wines produced from grapes grown in cool

climates which often have a high acid (tartrate plus malate) content. MLF is, however, also desired in some white and red wines produced from grapes grown in warmer climates, since it often introduces favourable organoleptic compounds [16]. Since MLF is a delicate process, it is important to control the presence of natural occurring malolactic bacteria.

Many wines, including Chardonnay, are fermented in stainless steel tanks prior to maturation in oak vats. Apart from this, the must and wine is pumped through stainless steel pipelines and valves. Adherence of malolactic bacteria to stainless steel surfaces may have a pronounced affect on the ability of malolactic bacteria to survive during the primary (alcoholic) fermentation and conduct MLF. Furthermore, if biofilms of malolactic bacteria are not effectively destroyed, they may contaminate wine in which MLF is undesirable or even lead to the development of bacteriophages which may cause stuck or sluggish MLF in wines dependent on a secondary fermentation.

Numerous papers have been published regarding biofilm formation by food-borne pathogens and bacteria that, after adhesion to stainless steel surfaces, are a source of contamination for food products (reviewed by Kumar and Anand [26] and Zottola and Sasahara [42]). Previously, most of the research in this field focused on the adhesion [2, 3, 27], growth and development [12, 41], and chemical removal [10, 24] of bacteria from stainless steel surfaces. Although a few reports mentioned the effect of antimicrobial peptides and antibacterial agents on biofilms [1, 23], they focused primarily on the effect these compounds have on biofilms of medical importance. Jayaraman *et al.* [21], however, reported on the effect of *in situ* production of gramicidin S by an established biofilm of *Bacillus brevis* prior to the colonization of the sulfate-reducing bacterium *Desulfovovibrio vulgaris*, as a method to inhibit the growth of the latter bacterium on stainless steel surfaces. Bower *et al.* [5] reported on the antimicrobial activity of surface-adsorbed nisin to food contact surfaces to control pathogenic organisms [20].

Little is known regarding the effect bacteriocins have on stainless steel-adhered malolactic biofilm communities, hence nothing has been reported regarding the use of bacteriocins to control these biofilms in the wine industry. This study was aimed at determining if a commercial starter culture of *O. oeni* is able to form biofilms on stainless steel and if so, if bacteriocins could be used to prevent biofilm formation.

A2.2 MATERIALS AND METHODS

A2.2.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

Pediococcus damnosus NCFB 1832 was grown in De Man Rogosa and Sharpe (MRS) broth (Oxoid, Hampshire, England) for 55 hr at 30°C. The production of pediocin PD-1 was optimized by supplementing MRS broth (Oxoid) with

bacteriological peptone (1.7%, w/v), $MnSO_4$ (0.014%, w/v) and Tween 80 (3%, v/v) and adjusting of the pH to 6.7 with 2N NaOH.

Lactobacillus plantarum 423 was cultured in MRS broth (Oxoid) for 15 hr at 30°C. Optimal plantaricin 423 production was obtained in the latter medium, supplemented with tryptone, $MnSO_4$ and Tween 80 and adjusted to pH 5.8, as described by Verellen *et al.* [39]. A strain of *Oenococcus oeni,* isolated from a commercial starter pack (Lallemand, Saint-Simon, France) was cultured in acidic grape broth [9] at 26°C.

A2.2.2 PREPARATION OF BACTERIOCINS

Cell-free supernatants containing pediocin PD-1 and plantaricin 423 were obtained by centrifuging 2 L of each culture at 9800 × g for 10 min at 8°C. These cell-free supernatants and a 1.3% (w/v) suspension of Nisin (1×10^6 I.U./g, Aplin & Barrett Ltd., Trowbrige, Wilts, England) were tested for antibacterial activity against metabolically active cells of *O. oeni* embedded in acidic grape soft agar (0.75% w/v agar), according to the spot-on-lawn technique [29], and expressed as AU (activity units)/mL. Based on the activity levels obtained, the supernatants of the three bacteriocins were diluted with sterile distilled water to represent equal activity levels (AU/mL). These supernatants were then individually freeze-dried and the lyophilisate of each re-dissolved in 10 mL sterile distilled water.

A2.2.3 THE EFFECT OF PEDIOCIN PD-1, PLANTARICIN 423 AND NISIN ON BIOFILMS FORMED BY *O. OENI*

Stainless steel (type 304, grade 2B finish), was cut into 75 × 25 × 2 mm slides. The slides were wiped with 96% (v/v) ethanol, rinsed five consecutive times in distilled water, submerged in distilled water and autoclaved. The slides were then aseptically transferred to a 250 mL screw-cap bottle containing 240 mL acidic grape medium and inoculated with *O. oeni* (1%, v/v, OD_{600} = 0.6). After nine days of static incubation at 26°C the slides were aseptically removed from the bottle, rinsed for five seconds with sterile distilled water to remove non-adherent bacteria, stained with 300 µL of the Live/Dead *Bac*Light viability probe (Molecular Probes Inc., Eugene, Oregon, USA) and left for 15 min in the dark at room temperature. Care was taken not to disturb the slides.

In a separate, but similar experiment, pediocin PD-1, plantaricin 423 and nisin suspensions were added to a 9-d-old culture of *O. oeni* to obtain final activity levels of 100, 500, 1000, 2000 and 3000 AU/mL, respectively. After periods of 1 hr, 3 hr and 5 hr of incubation at 26°C, the stainless steel slides were aseptically removed and studied under the microscope. Slides incubated in the absence of bacteriocins served as control.

Images of the biofilms on the slides were captured using a high performance CCD camera (Cohu) mounted on a Nikon Eclipse E400 epi-fluorescence microscope, equipped with an ×60/1.4 Dic H oil objective and filters. A minimum of 20 images was selected at random on each disk and the percentage viable and non-viable cells

per optical field calculated. Corrections were made for spectral overlap and background fluorescence and the images analyzed with Scion Image software (U.S. National Institutes of Health; http:// rsb. info. nih. gov/nih-image).

A2.2.4 THE EFFECT OF PEDIOCIN PD-1, PLANTARICIN 423 AND NISIN ON PLANKTONIC CELLS OF *O. OENI*

Cells of *O. oeni* were grown for 9 days at 26°C to an optical density of approx. 1.1 (at 600 nm) in acidic grape broth (240 mL in a 250 mL screw-cap bottle), containing sterile stainless steel slides, as described before. The three bacteriocins were then added to the respective cultures to represent final concentrations of 100, 500, 1000, 2000 and 3000 AU/mL, respectively. Cultures to which no bacteriocins were added served as control. Stirring was kept to the minimum to maximize contact of the bacteriocin to the planktonic cells. By doing this, care was taken to avoid disturbance of biofilm formation on the slides. After 10 min, 30 min, 1 hr, 3 hr and 5 hr, respectively, 100 μL was withdrawn from each culture, diluted in sterile saline, plated out onto acid grape agar, incubated at 26°C and the number of viable cells determined after 14 days at 26°C.

A2.2.5 THE EFFECT OF THE THREE BACTERIOCINS ON BIOFILM FORMATION AND PLANKTONIC CELLS OF *O. OENI* IN A MODIFIED CHARDONNAY GRAPE MUST MEDIUM

Chardonnay must (2 L) was centrifuged at 9800 × g for 10 min at 8°C and the supernatant supplemented with 5% (w/v) yeast extract (Biolab) to stimulate bacterial growth. The must was then divided into 240 mL volumes and dispensed into 250 mL screw-capped bottles. The pH was adjusted to 4.5 with 2N NaOH and heated to boiling point. Sterile stainless steel slides were added to each of the three volumes and inoculated with *O. oeni* and incubated at 26°C for nine days. The 9-day-old cultures then each received 3000 AU/mL of a specific bacteriocin. The effect of the bacteriocins on biofilm formation and the planktonic cells was recorded as described before.

A2.2.6 REPLICATION OF EXPERIMENTS

All experiments were done in duplicate with a minimum of three repeats for each fermentation. Viable and non-viable cell counts obtained in the biofilms and planktonic cultures for all three repeats of each fermentation did not vary by more than 10%.

A2.3 RESULTS AND DISCUSSION

A2.3.1 GROWTH AND ATTACHMENT OF *O. OENI* TO STAINLESS STEEL SLIDES

The planktonic cell density of *O. oeni* increased from 0.6 ($O.D._{600}$) to approximately 1.25 after nine days at 26°C. During this time the cells gradually adhered to the stainless steel surfaces to form mature and evenly dispersed biofilms. Approximately 75% and 86% of the surface areas of the slides submerged in acidic grape medium and in Chardonnay must, respectively, were covered with cells. Cells which emitted a green fluorescence had intact cell membranes and were viable, whereas cells which fluoresced red were in a bactericidal or bacteriostatic state. Based on these observations, only a small percentage of the cells in the biofilms (5 to 7% of the slide surface areas), whether generated in acidic grape broth or Chardonnay must, were non-viable.

A2.3.2 EFFECT OF THE BACTERIOCINS ON MATURE BIOFILMS

The addition of pediocin PD-1 to a 9-d-old biofilm of *O. oeni* resulted in a drastic decrease in viable cell numbers, as depicted in the differences recorded between the viable cell numbers in the control biofilm (no pediocin PD-1 added) versus the viable cell numbers recorded on the slides in the presence of the bacteriocin (Table 1, Fig. 1A). In general the number of viable cells decreased as incubation in the presence of pediocin PD-1 increased from 1 to 5 hr. After 1 hr in the presence of 100 AU mL^{-1} pediocin PD-1, less than 3% viable cells of the total number of cells in the biofilm were detected and after a further 4 hr of incubation, less than 0.1% viable cells of the total number of cells in the biofilm were detected. The effect of pediocin PD-1 on the viable cells in the biofilm was more pronounced as the concentration increased to 500 AU/mL and above.

The non-viable cells in the biofilm increased from approximately 5% (control biofilm with no pediocin PD-1) to approximately 52% after 1 hr in the presence of 100 AU/mL pediocin PD-1 (Table 1, Fig. 1A). The number of non-viable cells remained more-or-less the same after 5 hr in the presence of 100 Au/mL pediocin PD-1. However, at activity levels of 500 AU/mL and above, pediocin PD-1 resulted in a more efficient removal of the cells in the biofilm (from approx. 52% cell coverage at 100 AU/mL to no adherent cells detected after 5 hr at 3000 AU/mL).

In the presence of plantaricin 423 the number of viable cells in the biofilm decreased from approximately 70% coverage (control) to approximately 9% coverage after 1 hr and approximately 4% coverage after 5 hr at 100 AU/mL (Table 1, Fig. 1B). As expected, very few viable cells were recorded after 1 hr in the presence of 2000 AU/mL plantaricin 423 and very low levels of viable cells detected after longer incubation at this concentration or at higher levels of the bacteriocin. No drastic changes, i.e. less than 4% coverage of cells based on an average of three repeats in

duplicate experiments, were recorded in the number of non-viable cells after 5 hr in the presence of 100 AU/mL plantaricin 423 (Table 1, Fig. 1B). However, a slight but consistent decrease in the number of cells were observed at higher levels of plantaricin 423 or when the biofilm remained for longer in contact with the same concentration of the bacteriocin. After 5 hr of treatment with plantaricin 423 (3000 AU/mL) approximately 42% of the original cells remained in the biofilm with the majority staining non-viable.

Table 1. Percent coverage of *Oenococcus oeni* cells on stainless steel slides submerged in acidic grape broth and treated with bacteriocins[a]

Bacteriocin concentration (AU/mL)	Contact time (hr)	Viability[b]	Pediocin PD-1	Plantaricin 423	*Nisin*
100	1	V	2.33	8.90	5.47
	1	NV	52.17	65.06	60.54
	3	V	1.00	5.02	2.63
	3	NV	53.37	66.25	63.99
	5	V	0.10	3.98	0.54
	5	NV	52.79	68.65	67.98
500	1	V	1.01	5.04	1.90
	1	NV	35.85	66.35	66.70
	3	V	0.76	2.69	1.26
	3	NV	28.11	64.55	68.15
	5	V	0.05	1.65	0.50
	5	NV	21.36	62.47	70.33
1000	1	V	0.34	2.00	0.48
	1	NV	15.02	57.65	68.48
	3	V	0.12	0.81	0.32
	3	NV	12.55	55.52	67.35
	5	V	0.08	0.14	0.30
	5	NV	9.98	53.99	65.05
2000	1	V	0.08	0.54	0.13
	1	NV	4.11	52.39	62.13
	3	V	0.01	0.09	0.05
	3	NV	2.57	48.00	59.65
	5	V	0	0	0.02
	5	NV	1.01	46.83	54.65
3000	1	V	0	0	0.01
	1	NV	0.42	44.65	55.66
	3	V	0	0	0
	3	NV	0.04	42.66	53.10
	5	V	0	0	0
	5	NV	0	41.98	49.45
control viable			69.52	69.52	69.52
control non-viable			5.57	5.57	5.57

[a] Each value represents an average cell count of 20 microscopic fields per treatment, with a minimum of three repeats per treatment.
[b] V = viable; NV = nonviable.

The biofilm treated with nisin (Table 1, Fig. 1C) produced more-or-less the same pattern of viable cell decrease than recorded for pediocin PD-1 (Table 1, Fig. 1A) and plantaricin 423 (Table 1, Fig. 1B). However, the percent coverage of non-viable cells in the biofilm increased at 100 and 500 AU/mL nisin as the contact time increased from 1 to 5 hr, respectively (Table 1, Fig. 1C). A decrease in cells were observed only at higher activity levels of nisin (1000 AU/mL and above). After 5 hr of treatment with nisin (3000 AU/mL) approximately 49% of the original cells remained in the biofilm with the majority staining non-viable.

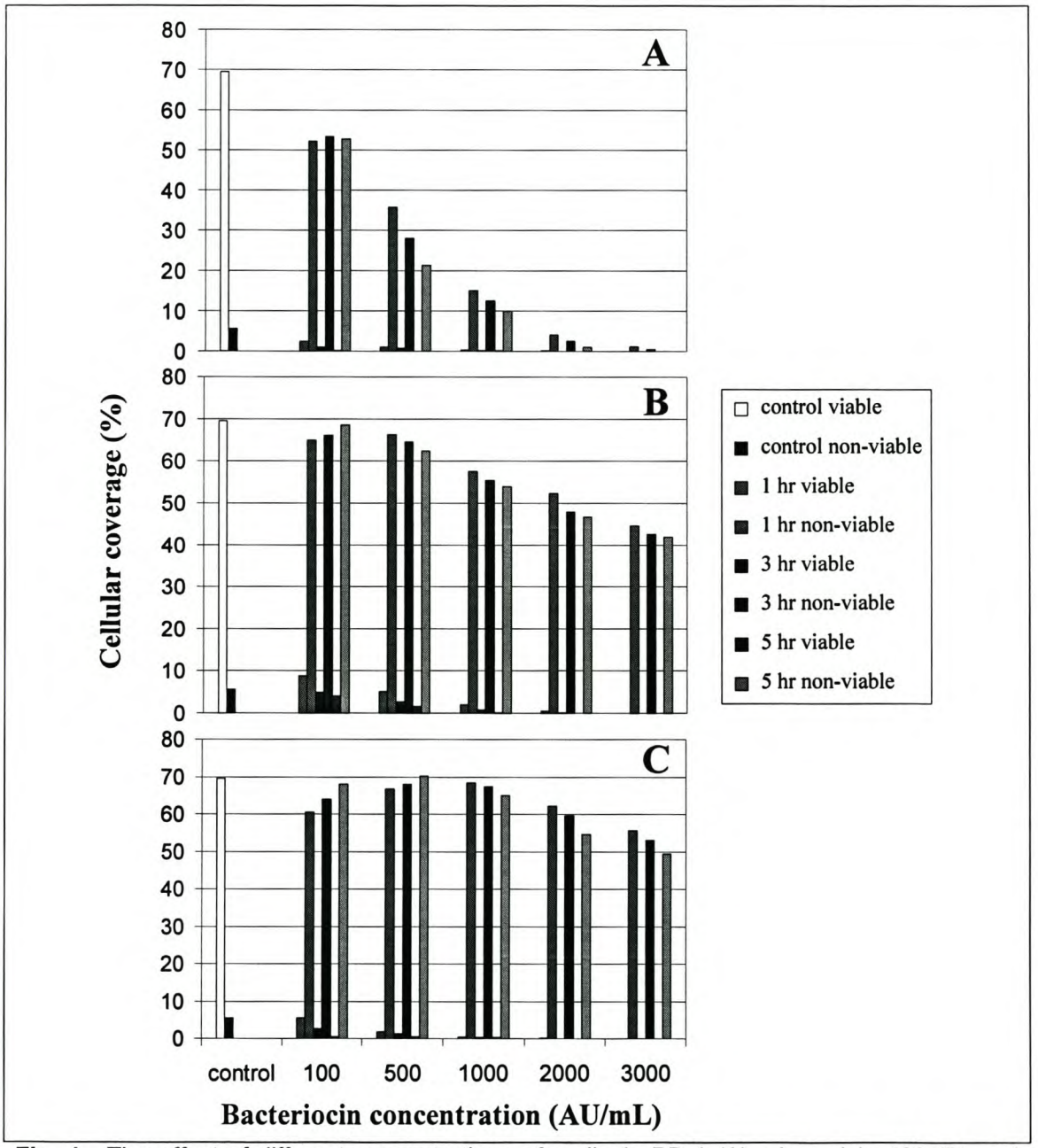

Fig. 1. The effect of different concentrations of pediocin PD-1 (A), plantaricin 423 (B) and Nisin (C) on an established biofilm of *O. oeni* in acidic grape medium at specific time intervals.

A2.3.3 EFFECT OF BACTERIOCINS ON PLANKTONIC CELLS

After 9 days of incubation at 26°C the planktonic cells of *O. oeni* in the control medium, i.e. in the absence of bacteriocins, reached 1.4×10^{10} cfu/mL. An increase in the activity levels and contact time of each bacteriocin resulted in a more-or-less linear reduction in planktonic cell counts (Fig. 2A-C). Pediocin PD-1 (3000 AU/mL) reduced the planktonic cell numbers from 1.4×10^{10} cfu/mL to 1.8×10^{2} cfu/mL after 5 hr of contact (Fig. 2A). This correlates to a reduction of eight log cycles (i.e approximately 80%) in viable cell numbers. Similar results were recorded after 3 hr with 3000 AU/mL plantaricin 423 (Fig. 2B) and 5 hr with 1000 AU/mL Nisin (Fig. 2C).

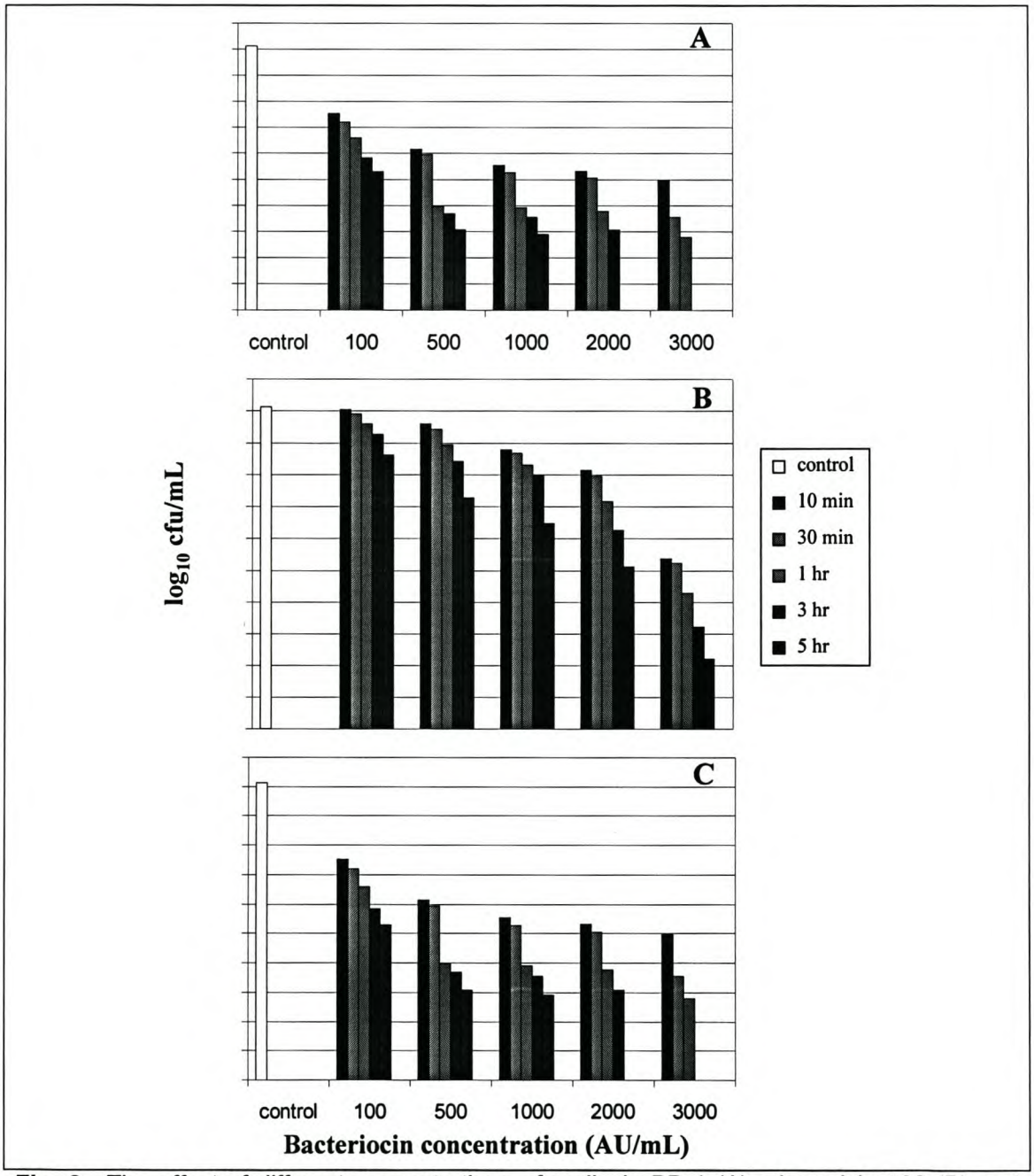

Fig. 2. The effect of different concentrations of pediocin PD-1 (A), plantaricin 423 (B) and Nisin (C) on planktonic cells of *O. oeni* at specific time intervals.

A2.3.4 EFFECT OF PEDIOCIN PD-1, PLANTARICIN 423 AND NISIN ON BIOFILMS AND PLANKTONIC CELLS OF *O. OENI* IN CHARDONNAY MUST

The coverage of viable cells decreased from approximately 80% (control) to no adherent viable cells detected after 1 hr of incubation in the presence of 3000 AU/mL pediocin PD-1, plantaricin 423 and nisin, respectively (Table 2, Fig. 3). In the presence of pediocin PD-1 no adherent non-viable cells were detected within 1 hr (Table 2, Fig. 3). However, afte 1 hr in the presence of plantaricin 423 and nisin, the numbers of adherent non-viable cells detected were approximately 38% and 48%, respectively, followed by a slight reduction after 3 and 5 hr (Table 2, Fig. 3).

Table 2. Percent coverage of *Oenococcus oeni* cells on stainless steel slides submerged in modified grape must and treated with bacteriocins[a]

Bacteriocin concentration (AU/mL)	Contact time (hr)	Viability[b]	Pediocin PD-1	Plantaricin 423	Nisin
3000	**1**	V	0	0	0
	1	NV	0.01	37.54	47.98
	3	V	0	0	0
	3	NV	0	36.09	45.79
	5	V	0	0	0
	5	NV	0	35.68	42.79
Control viable			78.78	78.78	78.78
Control non-viable			7.04	7.04	7.04

[a] Each value represents an average cell count of 20 microscopic fields per treatment, with a minimum of three repeats per treatment.
[b] V = viable; NV = nonviable.

The planktonic cells in the control medium, i.e. in the absence of bacteriocins, reached 1.3×10^{10} cfu/mL (Fig. 4). Pediocin PD-1 reduced the planktonic cell numbers to undetectable cfu after 5 hr of contact, whilst plantaricin 423 and nisin yielded the same result after 3 hr and 1 hr, respectively (Fig. 4).

Concluded from the results obtained in the present study, the three bacteriocins effectively removed cells of *O. oeni* from a mature biofilm. Pediocin PD-1 proved to be the most effective, whether tested on biofilms formed in acidic grape broth or in Chardonnay must. Microbial cells in biofilms are known to be as much as 1000 times more resistant to biocides [7, 25, 32, 40]. In cases of extensive biofouling, thick biofilms are formed which may include many metabolically dormant cells with altered growth rates and physiology, resulting in increased resistance to antimicrobial agents [11, 14, 31].

It is well known that biofilms on food-contact surfaces show increased resistance when treated with conventional sanitizers such as acid anionic biocides and quaternary ammonium compounds [13, 17, 26]. The reduced efficacy of antimicrobial agents is likely due to the ineffective penetration of biofilms [19] or

variation in environmental conditions on the contact surface [26]. In some studies where disinfectants proved to be effective, a rapid re-formation of biofilms were reported [22].

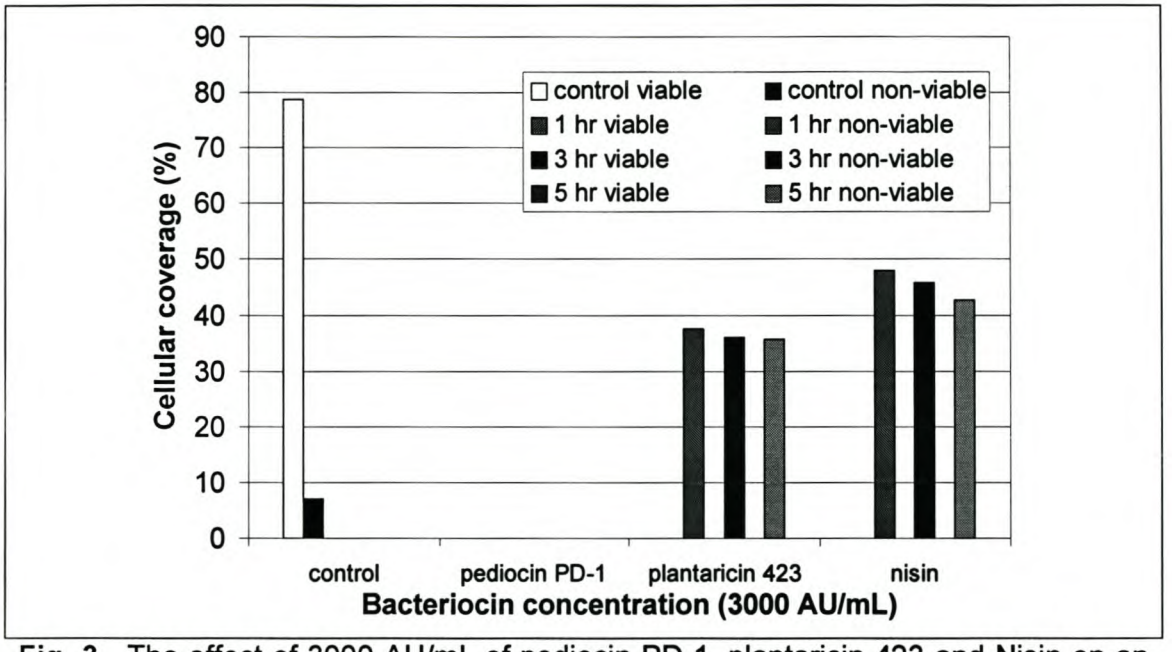

Fig. 3. The effect of 3000 AU/mL of pediocin PD-1, plantaricin 423 and Nisin on an established biofilm of *O. oeni* in a modified Chardonnay must medium at specific time intervals.

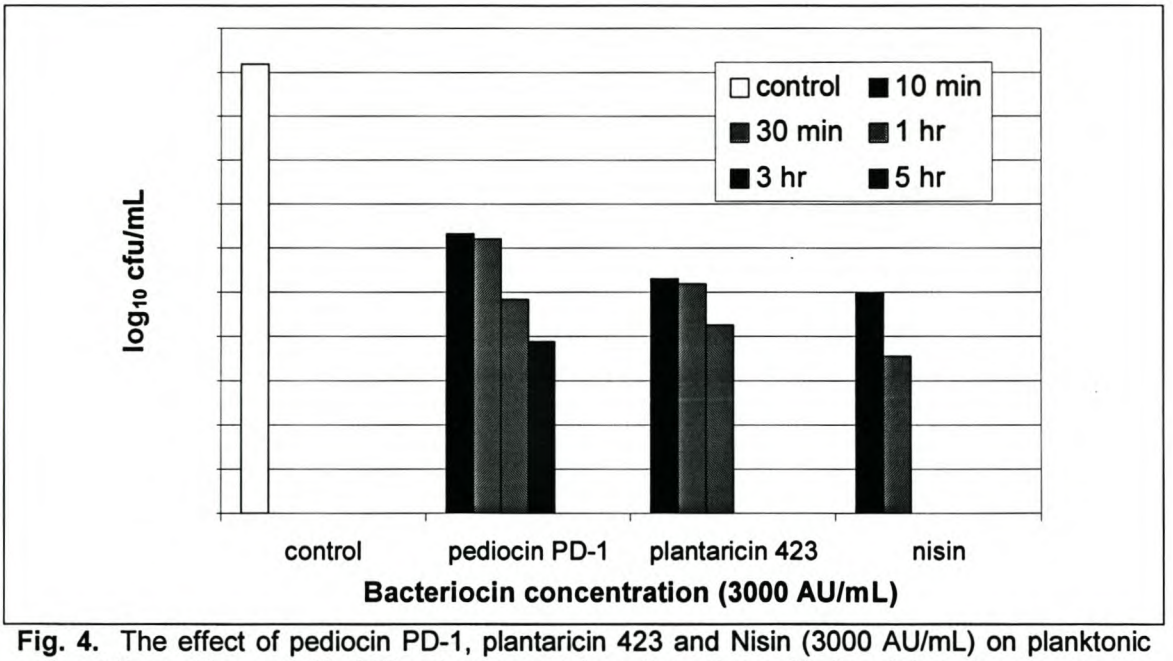

Fig. 4. The effect of pediocin PD-1, plantaricin 423 and Nisin (3000 AU/mL) on planktonic cells of *O. oeni* in a modified Chardonnay must medium at specific time intervals.

Resistance of biofilms to antimicrobial agents is attributed to the combined mechanisms and varied properties associated with the biofilm, including reduced diffusion, physiological changes due to reduced growth rates, the production of

enzymes degrading antimicrobial substances and often exopolysaccharide (EPS) matrixes [10, 24, 26]. Although EPS may act as a diffusion barrier, molecular sieve and adsorbent [6], the resistance to antimicrobial compounds is lost as soon as the three-dimensional structure of the biofilm is disrupted [18, 36]. Since many strains of *O. oeni* are known to produce EPS [37], this is an important hurdle to take into consideration when selecting an antimicrobial agent to prevent biofilm formation.

Plantricin 423 is homologous to the class IIA pediocins PA1/AcH [38]. Pediocins PA-1/AcH and the lantibiotic nisin form ion-permeable channels in the cytoplasmic membrane of susceptible cells, resulting in an increase in membrane permeability, disturbing the membrane potential and causing an efflux of intracellular low-molecular-mass compounds [34]. Ultimately, the biosynthesis of macromolecules and energy production is inhibited resulting in cell death. In some cases cell lysis has been observed for pediocin AcH [4]. Cells treated with nisin over an extended period often undergo autolysis [30]. However, the inhibition of cell wall biosynthesis is a comparatively slow process. Thus, pore formation is considered the primary mode of action of nisin and pediocins PA-1/AcH (33, 35).

The mechanism of action of pediocin PD-1 remains to be elucidated and is the subject of current investigation. Preliminary results reveal that a low concentration of pediocin PD-1 induces complete lysis of sensitive cells of *O. oeni*. Cell lysis is only observed at high concentrations of nisin and plantaricin 423. Dislodgement of cells of *O. oeni* in a biofilm, when treated with pediocin PD-1, may be due to cell lysis. This may explain why pediocin PD-1 superseeded the other two bacteriocins tested.

The bacteriocins included in this study are also active against other lactic acid- and malolactic bacteria, including certain food-spoilage bacteria [8, 15, 39]. The use of antimicrobial peptides to control the formation of biofilms offers a promising alternative to conventional treatment strategies, especially in the wine industry where the implementation of chemical disinfectants, including SO_2, is becoming more restricted. Similar studies have to be conducted to determine the effect of pediocin PD-1, plantaricin 423 and nisin in other food- and beverage environments.

A2.4 CONCLUSIONS

The three bacteriocins included in this study, pediocin PD-1, plantaricin 423 and nisin, successfully killed all viable cells in an established biofilm of *O. oeni* which formed in acidic grape medium. Apart from being the most effective against viable cells of *O. oeni* in the biofilm, pediocin PD-1 also proved to be the most effective in the removal of non-viable cells from the stainless steel surfaces. Similar results were obtained in Chardonnay must. Based on results obtained in this study, it is safe to assume that all cells of *O. oeni* (viable and non-viable) will be removed from these stainless steel surfaces after 5 hr at a concentration of 3000 AU/mL pediocin PD-1. These results also suggest that, of the three bacteriocins evaluated, pediocin PD-1 would be the best choice to prevent the potential re-formation of malolactic biofilms

on stainless steel surfaces. The application of bacteriocins in the control of bacterial biofilm formation could be one of the answers to a safer and environmentally friendlier method of sanitation.

A2.5 ACKNOWLEDGMENTS

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