Investigation of bacteriocins from lactic acid bacteria and their impact in winemaking

by

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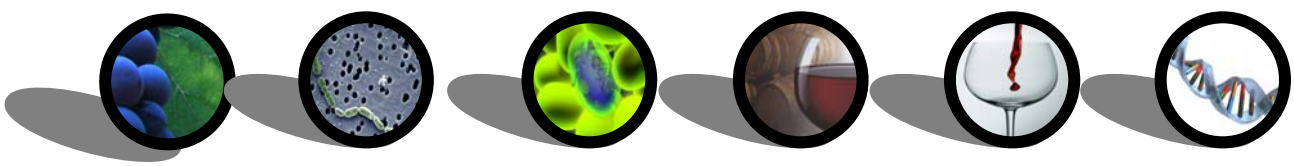

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis 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.

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Caroline Knoll **Date**

SUMMARY

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria and are active against other bacteria, either in the same species (narrow spectrum) or across genera (broad spectrum). The application of bacteriocins during the vinification process might help to prevent the production of undesired compounds by inhibiting the indigenous bacterial microflora and allowing malolactic fermentation to be conducted by a selected bacterial strain. Furthermore, the use of bacteriocins might allow reducing the total sulphur dioxide amount in wine.

The purpose of this study was the selection of lactic acid bacteria (LAB) belonging to the genera *Oenococcus*, *Lactobacillus* and *Pediococcus* with the ability to produce bacteriocins, with respective biological activity against undesired indigenous wine LAB and the capability to complete malolactic fermentation.

The first objective of this study was the screening of LAB isolated from South African red wines for the production of bacteriocins. Only 27 strains out of 330 wine isolates, belonging to the species *Lb. plantaru*m, *Lb. paracasei*, *Lb. hilgardii* and *O. oeni*, showed activity towards various wine-related and non wine-related indicator strains with the colony-overlay method. It is the first time that bacteriocin activity is reported in *O. oeni*.

The second objective was the detection and identification of known structural bacteriocin genes of *Lb. plantarum* wine strains. Furthermore, the web server BAGEL was used to *in silico* analyse putative bacteriocin-encoding genes in the genome of *O. oeni* and primers were designed to amplify four possible bacteriocin-encoding genes. A PCR-based screening revealed the presence of the plantaricin encoding genes *plnA*, *plnEF*, *plnJ* and *plnK* in five selected *Lb. plantarum* strains. Moreover, PCR analysis rendered positive results with all four chosen putative bacteriocin-encoding genes in the eight tested *O. oeni* strains with antimicrobial activity. The latter genes of *O. oeni* were heterologously expressed in different *Escherichia coli* host strains, but no antimicrobial activity could be detected.

The third objective of this study was the transformation and expression of the heterologous bacteriocin genes nisin A and pediocin PA-1 in two selected *Lb. plantarum* strains. To enhance their antimicrobial activity a plasmid containing the nisin A gene was successfully cloned into the two strains. Indeed, an enhanced antimicrobial activity could be detected, but the transformed plasmid was not stable.

The fourth objective in this project was the evaluation of bacteriocin production in liquid media. A co-culture experiment with a plantaricin producing *Lb. plantarum* strain and an *Enterococcus faecalis* strain as indicator was performed. A complete inhibition of cell growth of *Ent. faecalis* was observed within 72 hours.

The last objective was the evaluation of the impacts of phenolic compounds on the activity of nisin and pediocin. The short term influence of two phenolic acids, two flavan-3-ols, grape tannins and oak tannins on the activity of nisin and pediocin PA-1 was investigated. No influence on the activity was detected. Furthermore, synergistic effects on bacterial growth inhibition were observed.

This study confirms the potential use of either bacteriocin additives or bacteriocin-producing LAB in order to control the bacterial microflora during the vinification process.

OPSOMMING

Bakteriosiene is ribosomale gesintetiseerde antimikrobiese peptiede wat deur bakterieë geproduseer word en aktief is teen ander bakterieë, òf in dieselfde spesie (nou spektrum) òf in die genera (breë spektrum). Die toepassing van bakteriosiene gedurende die wynmaakproses kan help om die produksie van ongewenste komponente te voorkom deur die inheemse bakteriese mikroflora te inhibeer en toe te laat dat appelmelksuurgisting deur ‘n geselekteerde bakteriese ras uitgevoer word. Verder kan die gebruik van bakteriosiene ook die toevoeging van swaeldioksied tot wyn verminder.

Die doel van hierdie studie was om melksuurbakterieë (MSB) van die genera *Oenococcus*, *Lactobacillus* en *Pediococcus* te selekteer vir hul vermoë om bakteriosiene, wat biologies aktief teen ongewenste wyn MSB is, te produseer en wat ook daartoe instaat is om appelmelksuurgisting te kan voltooi.

Die eerste objektief van hierdie studie was om MSB, geïsoleer uit Suid-Afrikaanse rooiwyn, te toets vir die produksie van bakteriosiene. Slegs 27 rasse van 330 wyn isolate, van die spesies *Lb. plantarum*, *Lb. paracasei*, *Lb. hilgardii* en *O. oeni*, het aktiwiteit teen verskeie wynverwante en nie-wynverwante indikator rasse getoon.

Die tweede objektief was die deteksie en identifikasie van bekende strukturele bakteriosien gene van *Lb. plantarum* wynrasse. Verder was die web bediener BAGEL gebruik om putatiewe bakteriosien-enkoderende gene in die genoom van *O. oeni in silico* te analiseer en inleiers was ontwerp om vier moontlike bakteriosien-enkoderende gene te amplifiseer. ‘n PKR gebaseerde sifting het die teenwoordigheid van die plantarisien enkoderende gene *plnA*, *plnEF*, *plnJ* en *plnK*, in vyf geselekteerde *Lb. plantarum* rasse getoon. Bowendien het die PKR analises ook positiewe resultate met al vier gekose putatiewe bakteriosien-enkoderende gene in die ag getoetsde *O. oeni* rasse gegee. Die laasgenoemde gene van *O. oeni* was heteroloog in verskillende *E. coli* gasheerrasse uitgedruk, maar geen antimikrobiese aktiwiteit kon gewaar word nie.

Die derde objektief van hierdie studie was die transformasie en uitdrukking van die hetereloë bakteriosien gene nisien A en pediosien PA-1 in twee geselekteerde *Lb. plantarum* rasse. Om hul antimikrobiese aktiwiteit te verbeter, is ‘n plasmied wat die nisien A geen bevat suksesvol in die twee rasse ingekloneer. ‘n Verbeterde antimikrobiese aktiwitiet is waargeneem, maar die getransformeerde geen was nie stabiel nie.

Die vierde objektief van hierdie projek was die evaluering van bakteriosien produksie in vloeibare medium. ‘n Ko-kultuur eksperiment met ‘n plantarisien produserende

Lb. plantarum ras en ‘n *Ent. faecalis* ras, as indikator, was uitgevoer. ‘n Algehele inhibering in die selgroei van *Ent. faecalis* was binne 72 ure geobserveer.

Die laaste objektief was die evaluering van die effek van fenoliese komponente op die aktiwiteit van nisien en pediosien. Die kortermyn invloed van twee fenoliese sure, twee flavan-3-ole, druif tanniene en eiktanniene op die aktiwiteit van nisien en pediosien PA-1 was bestudeer. Geen invloed was op die aktiwiteit gewaar nie. Verder was sinergistiese effekte op die inhibering van bakteriese groei geobserveer.

Hierdie studie bevestig die potensiële gebruik van òf bakteriosiene òf bakteriosien-produserende MSB om die bakteriese mikroflora gedurende die wynmaakproses te beheer.

This thesis is dedicated to my grandmothers Gertrud Knoll and Margarete Nagel

Hierdie tesis is opgedra aan beide my oumas Gertrud Knoll en Margarete Nagel

Diese Masterarbeit ist meinen Großmüttern Gertrud Knoll und Margarete Nagel gewidmet.

BIOGRAPHICAL SKETCH

Caroline Knoll was born in Hamburg, Germany on the 6th of April 1981. She obtained her 'Abitur' at commercial high school Feldbergschule in Oberursel, Germany in 2000.

Caroline obtained a Bachelor of Applied Science and Honours in Beverage Technology at the University of Applied Sciences in Geisenheim, Germany in 2005. In 2006, she enrolled for an MSc degree at the Institute for Wine Biotechnology, Stellenbosch University.

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PREFACE

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the International Journal of Food Microbiology to which Chapter 3 will be submitted for publication, except Chapter 4 which was submitted to the American Journal of Enology and Viticulture.

CONTENTS

Chapter 1

INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Malolactic fermentation (MLF) is a secondary fermentation that usually takes place at the end of alcoholic fermentation and is carried out by one or more species of lactic acid bacteria (LAB). Depending on the wine style, MLF can be beneficial or detrimental. It contributes to the stabilization of wine by deacidification and removal of residual nutrients (Fleet, 2001). Moreover, the organoleptic profile and quality of the final product are changed via secondary metabolic reactions.

Four genera were identified as the principal organisms involved in the MLF: *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* (Lonvaud-Funel, 1999). Of all the species of LAB, *O. oeni* is probably the best adapted to overcome the harsh environmental wine conditions. Although some *Pediococcus* and *Lactobacillus* strains can survive in wine, it is more likely that these two genera produce undesirable by-products than positive sensory attributes.

From a winemaker's perspective it is necessary to control the MLF to either enhance positive characteristics or to reduce possible negative impacts (Mills, Rawsthorne, Parker, Tamir and Makarova, 2005). Spontaneous MLF is often unpredictable since it can take place any time during or several months after the end of alcoholic fermentation. A more precise application and better control can be achieved by inoculating bacterial starter cultures in order to perform MLF.

Several strategies can be used to control MLF. It can be promoted through (a) strain selection; (b) starter culture development; (c) development of malolactic reactors with free or immobilized bacteria or enzymes; or (d) the construction of recombinant wine yeast strains performing alcoholic and malolactic fermentation (Bauer and Dicks, 2004). Moreover, to prevent MLF, antimicrobial compounds (e.g. lysozyme) as wine preservatives or genetically modified yeast and bacteria strains with the ability to produce lysozyme or bacteriocins, for instance, might be applicable.

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria and are active against other bacteria, either in the same species (narrow spectrum) or across genera (broad spectrum) (Klaenhammer, 1988). Bacteriocins of LAB seem to mostly inhibit other LAB, which are likely to be competitors in the same (acidic) ecological niche (Eijsink, Axelsson, Diep Dzung, Håvarstein, Holo and Nes, 2002).

Bacteriocins are characteristically chosen for evaluation and application as specific antagonists against spoilage bacteria and food pathogens. However, their efficiency in foods may be limited or compromised for several reasons, such as food related factors: pH, storage temperature, bacteriocin adsorption to food components, or microbial diversity and sensitivity. Furthermore, the costs impedes the use of bacteriocins as food additives (Chen and Hoover, 2003).

Consumers are increasingly health conscious and prefer minimally processed products that contain little or no chemical preservatives. Several bacteriocins, such as nisin, are known to have enhanced or synergistic effects when used in combination with other antimicrobial compounds such as chemical preservatives (e.g. sulfur dioxide), natural phenolic compounds (e.g. carboxylic acids) or other antimicrobial proteins (e.g. lysozyme) (Chung and Hancock, 2000; Grande et al., 2007; Rojo-Bezares, Saenz, Zarazaga, Torres and Ruiz-Larrea, 2007). The application of bacteriocins during the vinification process might help to prevent the production of undesired compounds by inhibiting the indigenous LAB microflora and allowing the MLF to be conducted by selected bacterial strains. Moreover, the use of bacteriocins is a promising alternative to meet the consumer demands for lower sulfur dioxide amounts in the final product.

Thus, there is a continuous demand for new and more effective bacteriocins. Moreover, research on existing bacteriocins is being done to solve both biological and economical problems.

1.2 PROJECT AIMS

This study is part of a research project funded by Lallemand Inc. to unravel the ability of wine indigenous LAB and commercial starter cultures to produce bacteriocins.

The goal of this study was the selection of LAB belonging to the genera *Oenococcus*, *Lactobacillus* and *Pediococcus* with the capability of producing bacteriocins, with biological activity against potential wine spoilage LAB and the ability to successfully complete MLF. Furthermore, selected LAB strains were genetically modified in order to achieve an enhanced bacteriocin activity and a broader inhibition spectrum. Moreover, to investigate concerns regarding limited bacteriocin activity in wine, the impact of other wine components on their activity was studied.

The specific aims were the following:

(i) Screening and selection of wine LAB producing bacteriocins;

(ii) Detection and identification of known and novel structural bacteriocin genes and *in silico* sequence analysis;

(iii) Transformation and expression of heterologous known bacteriocin genes in commercial starter cultures;

(iv) Evaluation of the production of the bacteriocin, plantaricin, in liquid media;

(v) Evaluation of the influence of phenolic compounds on the activity of nisin and pediocin.

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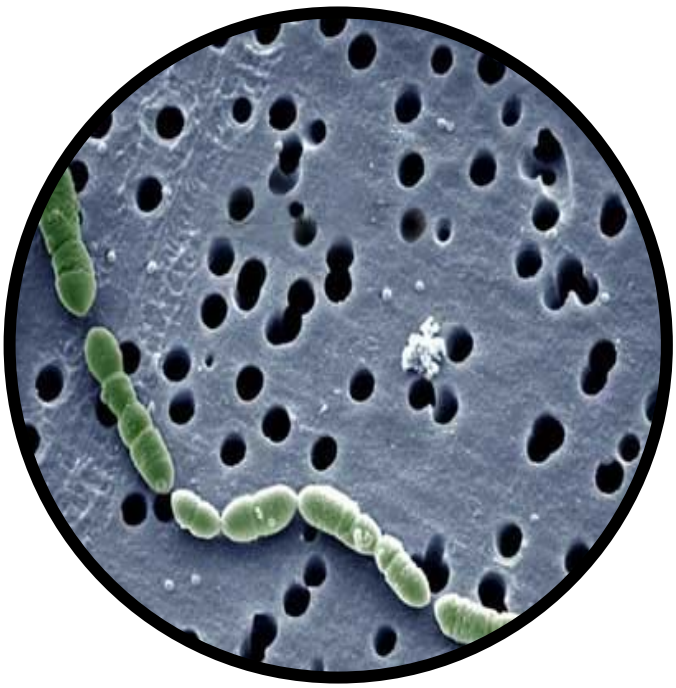
Chapter 2



LITERATURE REVIEW

Lactic acid bacteria: Their Genetics, Bacteriocins and Biotechnology

2. LITERATURE REVIEW

2.1 GENERAL INTRODUCTION

The must and wine environment harbours numerous microorganisms which have both positive and negative impacts on the final product. Moreover, these microorganisms interact with each other in both stimulating and inhibiting ways. Better understanding of the wine microbial ecology, population dynamics and interactions between microbes will assist in the control and preservation of wine microbes.

2.1.1 MICROBES OF WINE

The role of wine microbes is not only limited to the conversion of grape juice into wine through alcoholic fermentation (AF) which is mainly performed by *Saccharomyces cerevisiae* and the conversion of L-malic acid into L-lactic acid during malolactic fermentation (MLF) which is mainly performed by *Oenococcus oeni*. It also includes numerous other species/strains of yeast and lactic acid bacteria (LAB) which act on grape or wine compounds to produce metabolites important for wine aroma, flavour, texture and stability.

However, the bouquet and quality of a wine are influenced by several factors such as the grape quality, cultivar, the microorganisms involved during the vinification process and the wine style imposed by the winemaker. The winemaking process itself is a complex ecological niche where the biochemistry and interaction of yeasts, LAB, acetic acid bacteria (AAB) and fungi (e.g. *Botrytis*, *Aspergillus*, *Penicillium*, *Oidium* and *Cladosporum*) and their viruses play a crucial role in the end product. Therefore, it is important to identify and understand the ecological interactions that take place between the different microbial groups, species and strains (Fleet, 2003).

Species of yeasts, bacteria and fungi naturally occur on grapes, leaves and soil where their populations are mainly influenced by the amount of rainfall prior to harvest, physical damage of the berry, use of fungicides and time between harvest and fermentation. Depending on cellar hygiene, they are also found on equipment surfaces and can be wide spread in a cellar. Depending on the stage of maturity, the yeast genera *Aureobasidium*, *Metschnikowia*, *Hanseniaspora* (*Kloeckera*), *Cryptococcus* and *Rhodotorula* genera can mostly be found on the surface of healthy grapes. This microflora influences the growth of spoilage and mycotoxigenic fungi on grapes, yeast species and

strains that contribute to the AF, as well as the LAB that contribute to MLF (Fleet, 2003). Moreover, increased populations of LAB and AAB occur on damaged grapes which has an influence on yeast during AF.

The microbial ecology of wine including yeast, bacteria and filamentous fungi contributes to the wine production and chemical composition of wine, though yeast has the most important influence because of their role in performing the AF (Fleet, 1993). Various yeasts occur during the winemaking process. These include oxidative species (e.g. *Pichia* spp., *Candida* spp.), weakly fermentative species (e.g. *Hansenula anomala*, *Kloeckera apiculata*) and fermentative species (e.g. *Brettanomyces*/*Dekkera*, *Saccharomyces* spp., *Schizosaccharomyces pombe*) (Fugelsang, 1997). Two groups of bacteria are particularly significant in wine microbiology – LAB (e.g. *Lactobacillus* spp., *Pediococcus* spp. and *Oenococcus oeni*) and AAB (*Gluconobacter* spp., *Gluconacetobacter* spp. and *Acetobacter* spp.).

Various factors have an impact on the microbial ecology of wine, of which the chemical composition of the grape juice and the fermentation processes are the most important. In a complex microbial environment, it is most likely that the interaction between the different species and strains will determine the final ecology.

From the perspective of the vinification process, the relevant outcomes of these interactions are whether or not they enhance or inhibit the growth of any specific species or strains (Fleet, 2003). Due to their essential role during MLF and their ability to produce antimicrobial compounds which play a significant role in the bacterial ecology, this review will focus on LAB.

2.1.2 LACTIC ACID BACTERIA

The term LAB mainly refers to the characteristic feature of the basal metabolism of these bacteria, the fermentation of hexose sugars primarily yielding lactic acid (Makarova and Koonin, 2007). LAB are Gram-positive, anaerobic, non-sporulating, acid tolerant bacteria and include both homofermenters and heterofermenters. The homofermenters primarily produce lactic acid, while heterofermenters yield a variety of fermentation by-products, including lactic acid, acetic acid, ethanol, carbon dioxide and formic acid (Diep, Havarstein and Nes, 1996; Hugenholtz et al., 2002; Kleerebezem and Hugenholtz, 2003b).

2.1.2.1 The ecology of LAB in wine

The bacteria associated with spontaneous MLF belong to different genera of LAB. They are present in all grape musts and wines. Their ability to multiply depends on several factors such as the stage of the winemaking process, chemical and physical composition of wine and microbial interactions between the bacteria and other wine microorganisms. Four genera were identified as the principal organisms involved in the MLF: *Lactobacillus* (*Lb*.), *Leuconostoc* (*Lc.*), *Oenococcus* (*O*.) and *Pediococcus* (*P*.) (Lonvaud-Funel, 1999). These genera have the ability to tolerate low pH, high ethanol concentration and to grow in wine. *O. oeni*, *Lb. brevis*, *Lb. plantarum*, *Lb*. *hilgardii*, *Lc. mesenteroides*, *P. damnosus* and *P. pentosaceus* are the most common species associated with wine.

O. oeni was isolated, characterised and initially named *Leuconostoc oenos* in the mid 1960s (Garvie, 1967). With the introduction of molecular techniques, however, a new genus, *Oenococcus*, was described. *Lc. oenos* was reclassified as *O. oeni*, and was the sole species within this genus (Dicks, Dellaglio and Collins, 1995). Recently, Endo and Okada (2006) isolated from a composting distilled shochu residue a second species belonging to this genus, namely *O. kitaharae*, which is a non-malolactic fermenting species. *O. oeni* has the ability to adapt well to high ethanol concentrations (up to 15% v/v), low pH (as low as 2.9) and limited nutrient conditions. These characteristics enable *O. oeni* to out-compete other potential MLF bacteria during the later stages of vinification and thus dominate in wine after AF, until the end of MLF (Bartowsky, 2005). For these reasons and for its least association with off-flavours or other undesirable metabolites, *Oenococcus* starter cultures are most widely used for winemaking. Although species of *Lactobacillus* and to a lesser extent *Pediococcus* species are capable of performing MLF, it is more likely that these two genera produce undesirable by-products rather than positive sensory attributes in wine.

2.1.2.2 Impact of LAB on wine

Malolactic fermentation is an important step in the vinification process. The bacterial–driven decarboxylation of L-malic acid to L-lactic acid contributes to deacidification, flavour enhancement and complexity via secondary metabolic reactions. Moreover, it helps to increase subsequent microbiological stability of the wine by removing residual nutrients and producing bacteriocins (Fleet, 2001). Because of its deacidification function, MLF is favoured in high-acid wines produced in cool-climate regions and less desired in low-acid wines in warm-climate regions (Mills, Rawsthorne, Parker, Tamir and Makarova, 2005).

Organoleptic metabolically-derived compounds that are formed during MLF include diacetyl, acetaldehyde or acetoin. Moreover, MLF can also reduce bitterness, astringency and vegetal notes. Positive contribution descriptors after completion of malolactic fermentation are amongst others nutty, honey, buttery flavours and more body and roundness. Negative associated descriptors are sweaty, animal notes, wet leather or rancid aroma (Bartowsky and Henschke, 2004; Lonvaud-Funel, 1999; Palacios, Suárez, Krieger, Théodore, Otano and Pena, 2004). Other undesired product that are formed are biogenic amines which are generated by decarboxylation of amino acids, and might cause toxicological reactions to sensitive consumers (Lonvaud-Funel, 2001).

Furthermore, some LAB isolated from wine have been reported as being capable of producing bacteriocins and may be responsible for antibacterial effects observed amongst bacteria in wine (Holo, Jeknic, Daeschel, Stevanovic and Nes, 2001; Lonvaud-Funel and Joyeux, 1993; Navarro, Zarazaga, Saenz, Ruiz-Larrea and Torres, 2000; Rojo-Bezares, Saenz, Navarro, Zarazaga, Ruiz-Larrea and Torres, 2007a; Strasser de Saad and Manca de Nadra, 1993). The harsh physicochemical wine environment (such as high ethanol content, low oxygen concentration) causes a natural selective pressure on the inhabiting microorganisms. Moreover, these microorganisms might produce toxins such as killer toxins (by yeast) and bacteriocins (by bacteria) to either allow invasion into the established environment or to prevent an establishment of other yeast and bacteria species into an occupied niche. It is necessary to control MLF, permit precise application or prevention, to improve positive attributes, or to reduce possible negative impacts on the end product (Mills et al., 2005). Bacteriocins might play a future key role in regulating MLF.

2.2 BACTERIOCINS OF LACTIC ACID BACTERIA

Bacteriocins are antimicrobial peptides with narrow or broad host ranges produced by numerous bacteria. Many bacteriocins are produced by food-grade LAB and are odourless, colourless and non-toxic to humans. This fact presents an opportunity to direct or prevent the development of specific bacterial species in beverages and food without external addition of antibiotics. This might be valuable in preservation or food safety applications and also might have implications for the development of a desired flora in wine-associated fermentations.

2.2.1 NATURE OF BACTERIOCINS

LAB are known to produce various metabolic products with and without antagonistic activity, such as diacetyl, hydrogen peroxide, acetoin, other organic acids, reuterin and bacteriocins. Some of these end products act as bio-preservatives by changing the original food properties which eventually results in inhibition of spoilage organisms (**Fig. 2.1**).

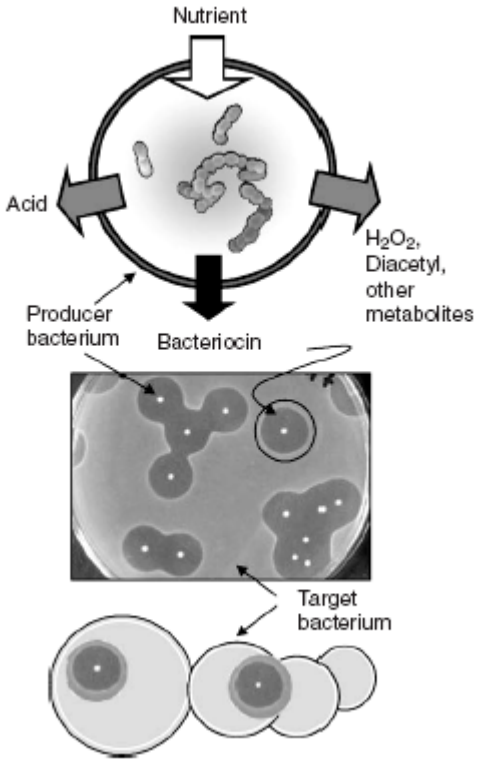


Fig. 2.1. LAB are able to produce various antimicrobial substances of which bacteriocins are often the most effective inhibitor of related bacteria (Deegan, Cotter, Hill and Ross, 2006).

The ability to produce bacteriocins is a highly advantageous characteristic of LAB. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by LAB. They are active against other bacteria, either in the same species (narrow spectrum), or across genera (broad spectrum) (Klaenhammer, 1988). Producer organisms are immune to their own bacteriocin(s), a property that is mediated by specific immunity proteins (Cotter, Colin and Ross, 2005). Both Gram-negative and Gram-positive bacteria produce small heat-stable bacteriocins, but so far they are found less frequently in Gram-negative bacteria (Diep and Nes, 2002), while within the Gram-positive bacteria group LAB seem to produce a large variety of these compounds (Drider, Fimland, Hechard, McMullen and Prévost, 2006; Nes and Johnsborg, 2004). Most of the bacteriocin-producing LAB are isolated from endogenous fermented food. It appears that the preservative effect of many LAB is partly due to their bacteriocin production, which is considered to give the producers an advantage in competing with other bacteria sharing the same ecological niches (Diep et al., 2002).

Bacteriocins produced by LAB can be categorized into three different classes according to their biochemical and genetic properties (**Table 2.1**). The present review focuses on class II bacteriocins, since all bacteriocins produced by LAB isolated from wine are classified as class II.

Table 2.1
Classification scheme for bacteriocins

Class	Characteristics	Subclass	Description	Examples		Reference
				Bacteriocin	Producer	
Class I	Lantibiotics (containing lanthionine and β-lanthionine)	A (1)	Elongated, cationic, membrane active, slight + or - net charge	Nisin A	*Lactococcus lactis*	Buchmann and Banerjee (1988)
				Nisin Z	*Lactococcus lactis*	Mulders et al. (1991)
		A (2)	Elongated, cationic, membrane active, highly - net charge	Lacticin 481	*Lactococcus lactis*	Piard et al. (1992)
		B	Globular, inhibit enzyme activity	Mersacidin	*Bacillus* spp. strain HIL Y-85,54728	Niu and Neu (1991)
Class II	Small (<10kDa), moderate (100°C) to high (121°) heat-stable, non-lanthionine-containing membrane active peptides	II a	antilisterial pediocin-like bacteriocins	Pediocin PA-1	*Pediococcus acidilactici* PAC 1.0	Marugg et al. (1992)
				Leucocin A	*Leuconostoc gelidum* UAL 187	Hastings et al. (1991)
				Plantaricin 423	*Lactobacillus plantarum* 423	van Reenen et al. (1998)
		II b	Two-peptide bacteriocins	Plantaricin EF	*Lactobacillus plantarum* C11	Moll et al. (1999)
		II c	Other peptide bacteriocins	Lactococcin 972	*Lactococcus lactis* IPLA 972	Martinez et al. (1999)
Class III	Large (>30kDa) heat-labile proteins			Helveticin J	*Lactobacillus helveticus* 481	Joerger and Klaenhammer (1990)

Source: Adapted from Drider et al. (2006)

Class II bacteriocins can be divided into three subclasses. Class IIa is the largest group. It has a conserved N-terminal amino acid sequence (YGNGVXC) and displays a high specific activity against the food pathogen *Listeria monocytogenes* (Hechard and Sahl, 2002). A large variety of LAB belonging to the genera *Lactobacillus, Enterococcus, Pediococcus, Carnobacterium,* and *Leuconostoc* is producing subclass IIa bacteriocins. Subclass IIb includes bacteriocins with two peptides which require the combined activity of both peptides and show very low, if any, bacteriocin activity when tested individually. Moreover, no sequence similarities appear between these complementary peptides. Subclass IIc bacteriocins are grouped on the basis that their N- and C-termini are covalently linked, resulting in a cyclic structure (Maqueda et al., 2004).

Class II bacteriocins are small, heat-stable, cationic and hydrophobic peptides. They are generally very stable at acidic pH and as pH increases, their heat stability decreases. Moreover, bacteriocins are usually sensitive to proteolytic enzymes, such as trypsin, proteinase K and protease (Chen and Hoover, 2003). Unlike lantibiotics, class II bacteriocins are not subject to extensive post-translational modification, but synthesized as precursor molecules mostly containing a leader peptide of the so-called double glycine type (Cotter et al., 2005; Håvarstein, Diep and Nes, 1995). This leader peptide is recognized and cleaved by a dedicated ABC transporter which results in translocation of the active bacteriocins into the medium (Håvarstein et al., 1995).

The ability of numerous LAB to produce one or more bacteriocin displays an important skill sustained over many generations. Bacteriocin production is advantageous, since these peptides inhibit the growth of bacteria competing for the same ecological niche

and the same resources. This is supported by the fact that their inhibition spectrum is mostly narrow and most likely to be effective against related bacteria competing for the same nutrients (Drider et al., 2006). It appears that, by producing several bacteriocins belonging to different classes with different inhibitory spectra, LAB compensate their narrow spectrum. *Lactobacillus plantarum* C11 for example, produces two types of bacteriocins which have different target cell specificities (Anderssen, Diep, Nes, Eijsink and Nissen-Meyer, 1998). Moll et al. (1999) demonstrated that plantaricin EF shows high conductivity for monovalent cations, while plantaricin JK is more selective for anions. Consequently, having opposite ion selectivity, plantaricin EF forms pores with cation selectivity and plantaricin JK with anion selectivity. This may also help to overcome the development of resistance mechanisms in target organisms (Eijsink, Axelsson, Diep Dzung, Håvarstein, Holo and Nes, 2002).

2.2.2 ECOLOGY OF BACTERIOCINS

Bacteriocin research has been mostly focused on genetics, mode of action or on uncovering novel bacteriocins. Recently the bacterial ecology and evolution of these antimicrobial peptides were examined in greater detail, since little is known about their roles in bacterial communities. Theoretical and experimental studies of bacterial ecology have been reviewed by Riley and Wertz (2002) and Riley, Goldstone, Wertz and Gordon (2003). The cell-cell communications of food-related bacteria have been summarized by Gobetti, De Angelis, Di Cagno, Minervini and Limitone (2007).

Bacteriocins might be exploited as anti-competitors permitting invasion of a strain into an established microbial environment. They might also be used as a defence mechanism to prohibit an establishment of other strains or species into an occupied niche (Riley et al., 2002). Furthermore, an additional role has been proposed, in which bacteriocins mediate quorum sensing (Eijsink et al., 2002; Kleerebezem, Quadri, Kuipers and de Vos, 1997b). Quorum sensing describes a mechanism of cell-cell communication, in which bacteria produce, release, detect and respond to signalling-molecules accumulated. This results in a cascade of events when a ‘quorum’ (e.g. a certain threshold concentration) is reached (Eijsink et al., 2002; Gobbetti et al., 2007). The capacity to synthesize bacteriocins and the ability to behave collectively as a group has obvious advantages. Bacteriocins play a fundamental role in the ecology of microbial populations,

but little is known about the comprehensive interactions at an ecological and evolutionary level in diverse populations (such as biofilms) (Chen et al., 2003).

2.2.3 ORGANISATION OF THE GENE CLUSTERS AND BIOSYNTHESIS OF CLASS II BACTERIOCINS

Production and export of class II bacteriocins require several genes: bacteriocin structural genes, genetic determinants involved in immunity, transport (ABC-transporter) and modifications (for lantibiotics). The relevant bacteriocin genes are mostly plasmid encoded, but they can also be located on the chromosome or on transposons. The gene clusters are most often arranged in operons and can be located in one operon or be spread over several operons, where one operon carries the structural and immunity gene, a second operon carries the gene coding for secretion and a third operon carries genes involved in regulation of bacteriocin production (Chen et al., 2003; Ennahar, Sashihara, Sonomoto and Ishizaki, 2000).

The bacteriocin synthesis operon consists of a structural gene followed by a gene encoding the immunity protein. The bacteriocin structural gene encodes a precursor peptide which is secreted and processed by either dedicated machinery or via the *sec*-dependant pathway. In the case of class IIb bacteriocins, two structural genes can follow each other as in the case of *plnEF* and *plnJK.* For most non-lantibiotics the immunity genes often follow immediately after the structural genes, apparently to secure protection of the producers from their own bacteriocins (Diep et al., 2002). A second operon encoding the machinery necessary for the processing, transport, and secretion of the bacteriocins is generally located in the vicinity of the bacteriocin genes. This operon is composed of two or more genes encoding an ABC transporter and its accessory protein (**Fig. 2.2 A**) (Eijsink et al., 2002).

Production of numerous class II bacteriocins is regulated by a so-called three component regulatory system (Eijsink et al., 2002; Gobbetti et al., 2007; Kleerebezem et al., 1997b). In this case a regulatory operon is involved in bacteriocin production. This operon contains a gene encoding for an induction factor (IF) or secreted bacteriocin-like peptide pheromone (Pph) followed by two genes encoding for a histidine kinase sensor protein (HPK) and a response regulator (RR). The IF serves as an indicator of the cell-density. The secreted pheromone binds to the HK which results in activation of the RR which then triggers the expression of all operons needed for bacteriocin synthesis,

transport, and regulation (**Fig. 2.2 B**) (Maldonado, Jimenez-Diaz and Ruiz-Barba, 2004). Only if the external pheromone has reached a certain threshold level, bacteriocin production is switched on. This level depends on transcriptional activity in the producing cell as well as on the number of cells present. This sensing of its own growth, which is possibly similar to that of competing species, allows the producing bacterium to commence bacteriocin production when conditions are likely to become more severe as for instance the competition for nutrients. Furthermore, the cell-cell communication makes the synchronization of bacteriocin production possible (Eijsink et al., 2002). The production of several class II bacteriocins, such as plantaricins EF / JK by *Lactobacillus plantarum* C11 (Diep, Havarstein and Nes, 1995; Diep, Myhre, Johnsborg, Aakra and Nes, 2003), plantaricin NC8 by *Lactobacillus plantarum* NC8 (Maldonado et al., 2004; Maldonado, Ruiz-Barba and Jimenez-Diaz, 2003) is regulated via this quorum-sensing or autoinduction mechanism mediated by inducer peptides. The released peptide pheromones are often not enough to either set off or sustain bacteriocin production (Kleerebezem et al., 1997b).

Environmental aspects and growth conditions such as temperature, pH, ethanol concentration, competing microorganisms, as well as medium composition and structure seem to play an essential role in the regulation of bacteriocin production (Diep et al., 1995; Kleerebezem et al., 1997b). However, to which extend these interactions play a role is still poorly understood. A similar killer and killer-sensitive phenomena is known within yeast interactions. There is evidence that the production of killer toxins may determine species and strain evolution during fermentation. But also here, several winemaking variables influence the expression of these toxins (Fleet, 2003).

2.2.4 MODE OF ACTION

Although the mode of activity of bacteriocins can differ, the cell envelope is commonly their target. The majority is active by inducing membrane permeabilisation. This is reflected by the fact that Class II bacteriocins have an amphiphilic helical structure, which allows them to insert into the membrane of the target cell, leading to depolarisation and death (**Fig. 2.3**) (Cotter et al., 2005). To form the core of the pores, this structure is believed to face with the polar side towards the centre of the channel, while the non-polar side faces the hydrophobic phase of the phospholipid bilayer (Diep et al., 2002). This creation of pores in the membrane of their target cells result in dissipation of the proton motive force,

intracellular ATP depletion and leakage of nutrients and metabolites (Deegan et al., 2006). Moreover, to form a pore, interactions with the cytoplasmic membrane of the target cell are necessary. Initial electrostatic interactions between the positively charged peptide and anionic lipids, which are in large quantities present in the membranes of Gram-positive bacteria, play a role to some extent in this mode of action.

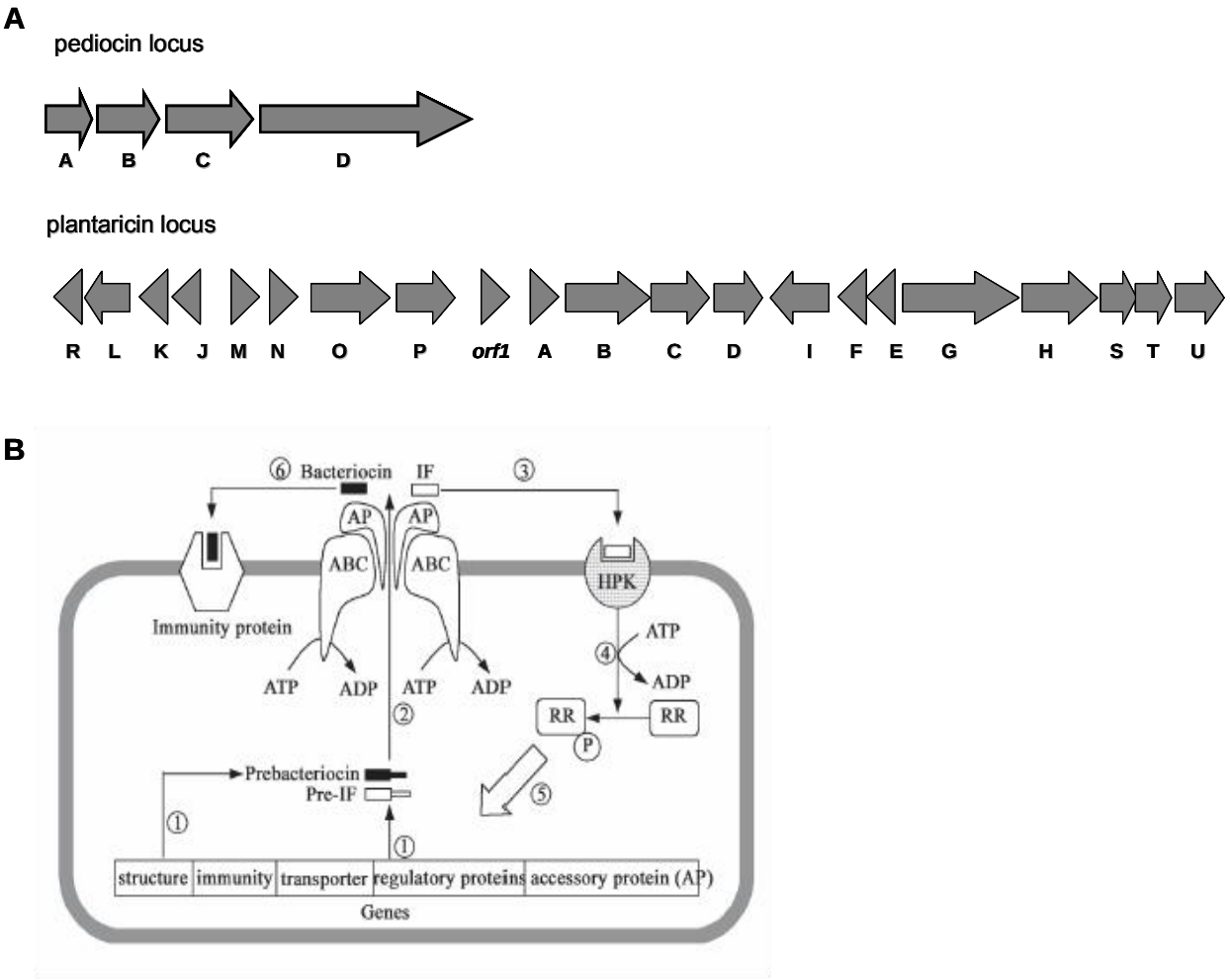


Fig. 2.2. A Examples of genetic loci of a class IIa bacteriocin: pediocin PA-1 and a class IIb bacteriocin: plantaricin. Pediocin locus: *ped*A, pediocin PA-1 precursor (62 aa); *ped*B, immunity gene (112 aa); *ped*C and *ped*D, genes for transport and secretion of the active peptide (174 aa, 724 aa) (Horn et al., 1998). Plantaricin locus: *pln*ABCD, regulatory operon (3.2kb): A, peptide pheromone; B, histidine protein kinase; C and D, antagonizing response regulators, C as an activator and D as a negative regulator; *pln*GHSTUV, transport operon (2.6kb); *pln*JKLR, bacteriocin and immunity genes (1.3kb); *pln*EFI, bacteriocin and immunity gene (1.3kb); *pln*MNOP, unknown functions (2.8kb)(Diep et al., 1996; 2003). **B** A schematic diagram of the biosynthesis of class II bacteriocins, according to Chen and Hoover (2003): 1, Formation of prebacteriocin and prepedpide of induction factor (IF); 2, The prebacteriocin and pre-IF are processed and translocated by the ABC-transporter, resulting in the release of mature bacteriocin and IF; 3, Histidine protein kinase (HPK) senses the presence of IF and autophosphorylates; 4, The phosphoryl group (P) is then transferred to the response regulator (RR); 5, RR activates transcription of the regulated genes; 6, producer immunity.

Thus, the sensitivity to bacteriocins depends partly on the physiological state of the cell (Eijsink et al., 2002). Up to this stage, it is not entirely clear whether bacteriocins act through receptors in the target cell membrane or if there is specificity in possible receptors.

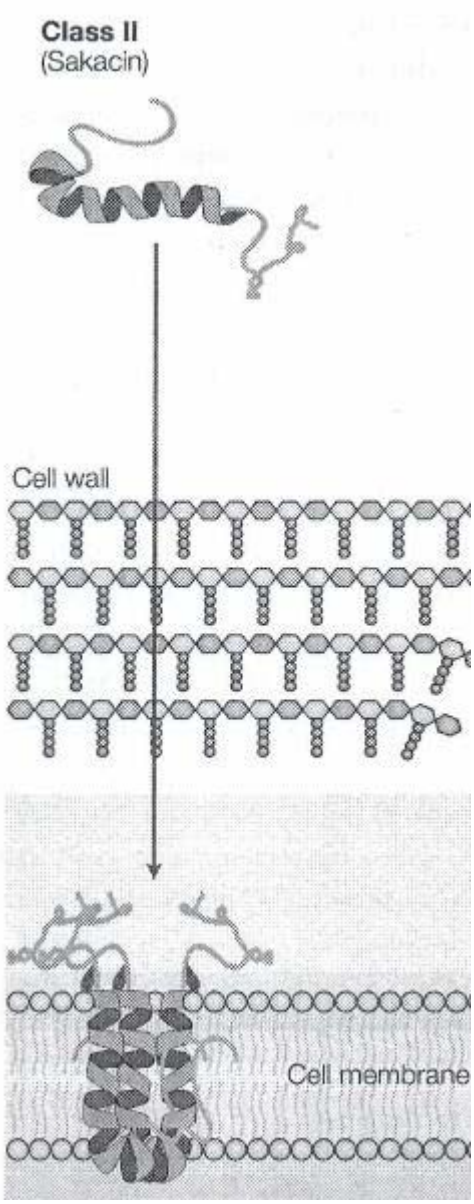


Fig. 2.3. Mode of action of class II bacteriocins, according to Cotter et al. (2005).

2.2.5 APPLICATION OF BACTERIOCINS IN FOOD

Most bacteriocin-producing LAB are indigenous food isolates and due to their great potential in food preservation, bacteriocins have been subject to extensive research during the last years. They have been shown to have great potential in biopreservation, for example in dairy products, canned food and alcoholic beverages. Although numerous methods other than bacteriocins are available for the preservation of food and beverages, an increasingly health conscious public are looking for foods that have not undergone extensive processing and contain no chemical preservatives. Bacteriocins are often promoted as potential biopreservatives, but it is generally suggested that these antimicrobial peptides should not primarily be used to prevent the growth of spoilage microorganisms. They rather should be used in addition to decrease the possibility of spoilage (Deegan et al., 2006).

Bacteriocins can be introduced into food to improve its safety in the following ways: (i) in fermented food where bacteriocins can be produced *in situ* by bacterial cultures which can replace either all or part of a starter culture; (ii) purified or semi-purified bacteriocins can alternatively be added directly as an additive; or (iii) an additive based on a fermentate of a bacteriocin-producing strain (Cotter et al., 2005). Incorporating purified bacteriocins might not always be attractive to the food and beverage industry, since in this

form bacteriocins may have to be labelled as an additive like other preservatives and regulatory approval might be necessary (Deegan et al., 2006).

Several important factors must be considered when screening for a bacteriocin-producing strain with potential in food application: the bacteriocin should have a broad spectrum of inhibition and be highly active; it should also be heat-stable, have no associated health risks and it should bring beneficial effects such as improved safety, quality and flavour (Cotter et al., 2005). The physical and chemical properties of the food or beverage can also influence the efficiency and stability of a certain bacteriocin and have to be considered (Deegan et al., 2006). In case of purified bacteriocins, optimization of yield and kinetics during production must be taken into account in order to make commercial use of bacteriocins cost-effectively.

The most broadly studied and commercially available bacteriocin is nisin. It was approved for use as an antimicrobial in food by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1969 which is an international scientific expert committee that is administratively joint by the Food and Agriculture Organisation of the United Nations-FAO and the World Health Organisation-WHO. Moreover, nisin has been given the food additive number E234 (EEC, 1983 EEC commission directive83/463/EEC) and is currently permitted for use in over 50 countries (Delves-Broughton, 2005).

Other bacteriocins, such as pediocins and lacticins, have found applications in various food systems which were reviewed recently by Chen et al. (2003) and Deegan et al. (2006). Bacteriocins have shown to be effective either added as an ingredient or produced by bacteriocin-producing bacteria strains in the food system (Deegan et al., 2006). While nisin is mostly used in canned food or dairy products, pediocin has the ability to protect fresh and fermented meat. Lacticins have been tested as biopreservatives in natural yoghurt, cottage cheese and infant milk formula. The use of a plantaricin producing starter culture has been demonstrated for the fermentation and preservation of olives (Ruiz-Barba, Cathcart, Warner and Jimenez-Diaz, 1994).

2.2.6 APPLICATION OF BACTERIOCINS IN WINE

In the wine industry, controlling the growth of microorganisms at critical stages during the winemaking process is vital. Sulphur dioxide (SO_2) is mostly used to control or inhibit microbial growth, since it has a broad inhibition spectrum and is active over a long term.

Moreover, besides being an antiseptic, SO_2 is also an antioxidant and antioxidasic and therefore required during vinification (Ribéreau-Gayon, Glories, Maujean and Dubourdieu, 2000). However, concerns regarding health risks, as well as wine labelling requirements on SO_2 levels in the bottle, have led to the search for alternative compounds to reduce the amount of SO_2 added in wine.

Lysozyme, which is an antimicrobial compound isolated from egg whites, is one of the alternatives. It is only active against Gram-positive bacteria and has no effect on yeast (Gerbaux, Villa, Monamy and Bertrand, 1997). Moreover, it was observed that bacteria belonging to the genera *Pediococcus* and *Lactobacillus* are more resistant than *Oenococcus* (Delfini et al., 2004). In addition, high costs and issues regarding protein instability in red wines exist (Tirelli and De Noni, 2007). This reflects that lysozyme is not perfectly adapted for general use in the wine environment. Bacteriocins are therefore an attractive option that could provide at least part of the solution.

2.2.6.1 Use of bacteriocins in wine

In situ bacteriocin production can be a promising application during the vinification process. Nonetheless, the use of bacteriocin-producing bacteria entails a careful selection of strains that are well adapted to the harsh wine environment. These strains must be able to grow under fermentation conditions and produce large amounts of bacteriocin to inhibit the growth of undesired bacteria. A bacteriocin-producing strain can be utilized either as co-culture with the MLF starter culture or directly as starter culture. If used as a co-culture, the bacteriocinogenic strain should be compatible with the starter cultures required for MLF. When used as a starter culture, the strain must be able to carry out MLF optimally besides being able to produce enough bacteriocin amounts for protection against spoilage bacteria (Deegan et al., 2006; Grande et al., 2007).

The possibility of controlling bacterial growth during winemaking and preservation by bacteriocins such as nisin, pediocin PA-1, pediocin N5p and plantaricin 423 has been investigated in several studies (Bauer, Nel and Dicks, 2003; Daeschel, Jung and Watson, 1991; Radler, 1990a; 1990b; Strasser de Saad et al., 1993; Yurdugül and Bozoglu, 2002). A bactericidal mode of action has been shown against a number of LAB, including *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*. It was demonstrated that these peptides are stable under winemaking conditions and do not affect yeast growth. However, Daeschel and Bower (1991-1992) observed a decrease in nisin activity in Pinot Noir over a 4-month storage period, while little decrease was observed in Chardonnay.

Moreover, Nel, Bauer, Wolfaardt and Dicks (2002) 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 when compared with nisin and plantaricin 423. Rojo-Bezares, Saenz, Zarazaga, Torres and Ruiz-Larrea (2007b) observed that suitable combinations of nisin and SO_2 can control the growth of spoilage bacteria in wine which consequently allows a decrease in the levels of SO_2. Chung and Hancock (2000) reported a synergistic influence between nisin and lysozyme and proposed the benefits of using combinations of both to prevent spoilage.

Bacteriocins can also be used to promote quality, rather than only to prevent spoilage (Cotter et al., 2005). In wine, bacteriocins can be used to control the indigenous LAB microflora thus preventing the production of undesired compounds (Daeschel et al., 1991; Radler, 1990a). However, as some LAB occurring in some red wines might improve the flavour, the complete inhibition of LAB is not always advantageous. The natural LAB microflora in wine might be capable of producing bacteriocins, but might not be perfectly adapted to the harsh wine environment and therefore not be able to complete malolactic fermentation. Furthermore, Bauer et al. (2003) have shown that grape must does not contain the required factors needed for the production of pediocin PD-1.

2.2.6.2 Bacteriocins and wine biotechnology

The addition of nisin to beer has been approved for example in Australia and New Zealand (Delves-Broughton, 2005). Nonetheless, nisin and other bacteriocins are not yet authorised as additives in wine in any of the wine producing countries. Cost considerations may have a negative impact on the acceptance of peptide-based wine preservation methods. Therefore, having a MLF bacteria starter culture, preferably *O. oeni* or *Lb. plantarum*, conducting MLF as well as producing bacteriocins to inhibit spoilage bacteria, will offer a great benefit.

Since bacteriocins are encoded by genes, the genetic modification of bacteria and yeast strains to produce antimicrobial compounds provide a promising alternative. Strain properties and produced bacteriocin amounts could be enhanced by heterologous expression of bacteriocin genes. The timing of bacteriocin production can also be manipulated by using inducible production systems (Rodriguez, Martinez, Horn and Dodd, 2003; Zhou, Li, Ma and Pan, 2006).

This approach to incorporate bacteriocins into vinification by transforming traditional yeast starter cultures (S. cerevisiae) with the required genetic material has been

demonstrated with pediocin PA-1 and plantaricin 423 (Schoeman, Vivier, du Toit, Dicks and Pretorius, 1999; Van Reenen, Chikindas, Van Zyl and Dicks, 2003). Yeast strains expressing a bacteriocin would be applicable in wines where MLF is undesired or in combination with resistant MLF starter cultures.

2.2.6.3 Concept of hurdle technology

Several bacteriocins show synergistic or additive effects when used in combination with other antimicrobial compounds, such as chemical preservatives, natural phenolic compounds and other antimicrobial proteins. In order to overcome limitations of bacteriocins asa result to various factors which influence the efficacy of bacteriocins in food systems and their mostly narrow activity spectra, the concept of hurdle technology was introduced to improve shelf life and enhance food safety. Since bacteriocins can be combined with selected hurdles in order to increase the microbial stability (**Fig. 2.4**), this application of bacteriocins as part of the hurdle technologies has recently raised great interest (Carrete, Vidal, Bordons and Constanti, 2002; Deegan et al., 2006; Grande et al., 2007; Ross, Griffiths, Mittal and Deeth, 2003). Nevertheless, considerations regarding type of food, microbial composition, as well as legal preservation techniques must be taken in account when combining and applying different hurdles (Grande et al., 2007). In wine most of the hurdle technologies cannot be applied due to the legal regulations. However, methods such as the combination of bacteriocins with potassium metabisulphite (SO_2 active molecule), lysozyme, phenolic compounds, pH adjustments and possibly heat treatments could be taken into consideration.

Nisin has been shown to have synergistic effects in combination with SO_2 and growth inhibition of wine bacteria were observed (Rojo-Bezares et al., 2007b). As previously mentioned, when nisin was used to control the microflora in wine, Daeschel et al. (1991) observed a decrease in nisin activity in Pinot Noir over a 4-month storage period to less than 90%, while little decrease was observed in Chardonnay. These authors suggested that nisin may be interacting with polyphenolic compounds present in red wine, but absent in white wines. Later on, Daeschel et al. (1991-1992) verified that tannins caused an immediate decrease of nisin levels when tested in a wine model system. Nevertheless, Grande et al. (2007) observed that the antimicrobial activity of enterocin AS-48 increased in combination with the phenolic compounds carvacrol, geraniol, eugenol, terpineol, caffeic acid, p-coumaric acid, citral and hydrocinnamic acid. Moreover, nisin and lysozyme have been shown to act synergistically against Gram-positive bacteria (Chung et al., 2000).

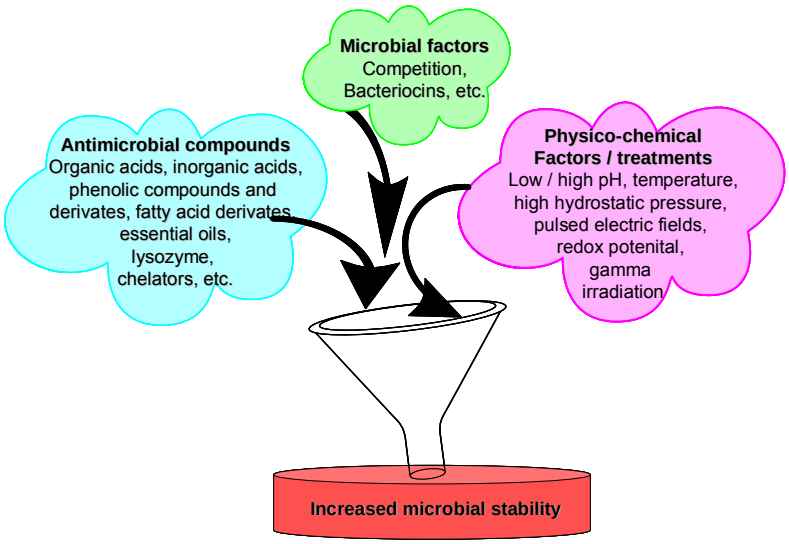


Fig. 2.4. Application of bacteriocins as part of hurdle technology (adapted from Gálvez et al., 2007).

2.3 GENETICS AND BIOTECHNOLOGY OF LACTIC ACID BACTERIA

LAB are an important group of bacteria, several of which are used for food and beverage fermentations and preservation. In addition, LAB are a priceless source of antimicrobial peptides: the bacteriocins (Cotter et al., 2005). Because of their growing importance in our daily lives, research exploiting molecular genetics and manipulations of these organisms are increasing progressively and the genetic investigation of LAB strains is expanding to fully assess their potential.

2.3.1 GENOMIC DISCOVERY

The “Lactic Acid Bacteria Genome Consortium” (LABGC) is a working unit which was initiated to generate publicly accessible genome sequences of food-grade LAB (Klaenhammer et al., 2002; Mills, 2004). In 2002, the LABGC started a collaboration with the Joint Genome Institute (JGI), a high throughput sequencing facility run by the United States Department of Energy, to generate draft sequences of eleven bacterial genomes, five of which (*Lb. casei, Lb. brevis, Lc. mesenteroides, P. pentosaceus* and *O. oeni*) can be readily isolated from wines or musts (Mills et al., 2005). The first *Lactobacillus* genome

to be sequenced was that of *Lb. plantarum* WCFS1 (Kleerebezem et al., 2003a). At the time of writing (August 2007), the genome sequences of 12 different species which can potentially be found in wine, have been completed or are being sequenced (see **Table 2.2**).

Table 2.2
Sequenced genomes of some wine related LAB

Genus	Species	Strain	Size (Mbp)	G+C (%)	Status	Reference / Institution
Oenococcus	*oeni*	PSU-1	1.8	38	C	Mills et al. (2005)
	oeni	ATCCBAA331	1.8	37.5	IP	JGI
	oeni	IOEB84.13	1.8	37.9	IP	Siezen et al. (2004)
Lactobacillus	*plantarum*	WCFS1	3.3	44.5	C	Kleerebezem et al. (2003a)
	johnsonii	NCC533	2	34.6	C	Pridmore et al. (2004)
	casei	ATCC334	2.5	41.1	IP	JGI
	casei	BL23	2.6	4.6	IP	Siezen et al. (2004)
	casei	DN-114001	3.14	46.3	NP	Danone Vitapole & INRA[a], France
	casei	Shirota	3.04	46.3	NP	Yakult, Japan
	rhamnosus	HN001	2.4	46.4	IP	Klaenhammer et al.(2005)
	helveticus	CNRZ32	2.4	37.1	IP	Klaenhammer et al.(2005)
	helveticus	CM4	2	37	C	Klaenhammer et al.(2005)
	helveticus	DPC4571	2.05	37.8	IP	Teagasc & Univ. College Cork, Ireland
	sakei	23K	1.9	41.2	C	Siezen et al. (2004)
	delbrueckii ssp. *bulgaricus*	ATCCBAA365	2.3	45.7	IP	JGI
	delbrueckii ssp. *bulgaricus*	ATCC11842	2.3	50	IP	Siezen et al. (2004)
	delbrueckii ssp. *bulgaricus*	DN-1001007	2.1	NA	IP	Siezen et al. (2004)
	reuteri	ATCC55730	~ 2.0	NA	NP	Swedish Univ. of Agricultural Science & BioGaia, Sweden
	reuteri	100-23	NA	38.6	IP	Univ. of Otago, NZ & JGI, USA
	reuteri	JCM1112	NA	38.8	IP	Univ. of Otago, NZ & JGI, USA
	brevis	ATCC367	2	43.1	IP	JGI
Leuconostoc	*mesenteroides*	ATCC8293	2	37.4	IP	JGI
Pediococcus	*pentosaceus*	ATCC257445	2	37	IP	JGI

Adapted from Klaenhammer et al. (2005) and Claesson et al. (2007).
C, complete; IP, in progress; NP, not public.
[a] WCFS, Wageningen Centre for Food Science.
JGI, Joint Genome Institute.

The fast progress of genome sequencing, detailed genome analysis, data mining and comparison of numerous LAB on genus, species and strain level, provide great knowledge about their diversity and evolution. Moreover, this information helps to understand the mechanisms that control and regulate bacterial growth, signalling, survival, stress response and fermentation processes (Klaenhammer et al., 2002; Siezen, van Enckevort, Kleerebezem and Teusink, 2004). Furthermore, genome analysis allows comprehensive studies about phylogenetic relationships and makes the establishment of patterns which analyse general trends of evolution for different sets of species possible (Makarova et al., 2006; 2007). Comparative-genomic analysis significantly assists not only the functional annotation of LAB genomes (Makarova et al., 2007), but also revealed that genes that are functionally related, are often organized into clusters or operons (Van Kranenburg et al., 2002). This insight into bacterial genomes may facilitate screening for genes encoding specific enzymes or proteins and enable the identification of flavour-forming capacity of LAB strains. It may also lead to the prediction of fermentation

performance under specific environmental conditions (pH, temperature) (Siezen et al., 2004; Van Kranenburg et al., 2002).

A comparative genomic approach is beneficial to predict new bacteriocins produced by LAB. Bacteriocins are small proteins with highly diverged sequences; identification by amino acid conservation is therefore problematic. Makarova et al. (2006) identified clustered genes for putative bacteriocins and associated proteins in seven *Lactobacillales* genomes based on genome context analysis. Two prebacteriocin families have been identified within these regions. The first family consists of precursor of the known bacteriocin, pediocin from *P. pentosaceus*, homologous to what is present in *Lc. mesenteroides* and *Lb. casei*. The second family included putative bacteriocin precursors distantly related to divercin V41 (Metivier et al., 1998) and were present in *P. pentosaceus* and *Lb. johnsonii.* Respective phylogenetic trees indicated that bacteriocin encoding genes are amongst those that are often transferred horizontally (Makarova et al., 2006).

Recently, BAGEL, a bacteriocin genome mining online-server was introduced to identify bacteriocins and their biosynthetic clusters through a knowledge-based database (De Jong, van Hijum, Bijlsma, Kok and Kuipers, 2006). When BAGEL was used for a brief screening process of several LAB species which can be found in wine, various loci were identified as putative bacteriocin-encoding genes (see **Table 2.3**). A number of motifs specifically described for bacteriocins have been identified in the genome of four LAB species. In the genome of *Lb. casei* a class II bacteriocin and a lantibiotic motif have been found. Similarities to the lantibiotic motifs have also been found in the genomes of *Lb. reuteri* and *Lc. mesenteroides*. In the genome of *P. pentosaceus,* a class IIa bacteriocin motif which has been reported previously by Makarova et al. (2006) was identified. Moreover, several other genes show similarities to klebicin C from *Klebsiella pneumoniae* and klebicin D from *Klebsiella oxytoca*, as well as to lincocin M18 from *Rhodospirillum rubrum* ATCC 111. Most of the displayed open reading frames (ORF's) were identified as putative bacteriocins which contain a number of bacteriocin-like features such as ABC transporter and histidine kinases (**Table 2.3**).

A combination of whole genome context analysis and the web-based bacteriocin genome mining tool might provide an important tool for identifying new bacteriocin-encoding genes in LAB, which might not be detected with classic screening methods such as 'spot on lawn' assays. However, in some cases, bacteriocin-related genes have been identified in the genomes of non-producers, either as incomplete set of genes or

containing mutated genes (Bolotin et al., 2001; Chaillou et al., 2005; Moretro et al., 2005). In the case *of P. pentosaceus* ATCC 25745 an incomplete locus of genes which encodes products resembling those involved in a regulated pediocin-like bacteriocin production was identified. This incomplete locus makes this bacterium a poor bacteriocin producer (Diep, Godager, Brede and Nes, 2006).

Table 2.3
Summary of results obtained by the application of BAGEL on various genomes

Organism	gene/locus	product	size[a]	ABC[b]	HK[b]	C39[b]	immunity[b]	motif	Name of gene found in database
Lb. brevis ATCC367	LVIS 1273	hypothetical protein	74						bacteriocin
	LVIS 0779	nucleoid DNA-binding protein	91	+4					COG1659: uncharacterized protein
	LVIS 1869	Phosphotransferase system, HPr	87	-6					prophage MuMc02, bacteriocin protein
Lb. casei ATCC334	LSEI_0954	hypothetical protein	49	-2/+3	-3				
	LSEI_0016	hypothetical protein	71	+6				GWAXGXXXG	
	LSEI_0146	hypothetical protein	98						Klebicin D activity protein
	LSEI_0547	hypothetical protein	82						Propionicin F
	LSEI_2140	hypothetical protein	69	-8/+5					
	LSEI_0062	hypothetical protein	86	-5			-7		
	LSEI_2239	Co-chaperonin GroES (HSP10)	93	+7	-5				
	LSEI_1287	Small membrane protein	86	+4					Linocin M18 bacteriocin protein
	LSEI_2390	hypothetical protein	65			-6			Linocin M18 bacteriocin protein
	LSEI_1161	F0F1-type ATP synthase, subunit	70					AXXXAAXGA| AXXXAAXGA	
	LSEI_0943	hypothetical protein	89	-3	-8/+8				
Lb. delbrueckii ssp. *bulgaricus ATCC 11842*	Ldb1618/ GroES	10 kDa chaperonin GroES	94	+2	-3				
	Ldb1285	hypothetical protein	98	+4					Klebicin C activity
	Ldb2150	hypothetical protein	100	+8					1912296A pyocin AP41:subunit=I
Lb. delbrueckii ssp. *bulgaricus* ATCC BAA365	LBUL_0878	hypothetical protein	96	-8/+5	-5				
	LBUL_1898	hypothetical protein	87	-3	-6				
	LBUL_1497	Co-chaperonin GroES (HSP10)	94	+3	-3				
	LBUL_1987	hypothetical protein	100	-8					1912296A pyocin AP41:subunit=I
	LBUL_0876	hypothetical protein	37	-6/+7	-3				
	LBUL_1499	hypothetical protein	35	+1	-5		-2		
Lb. johnsonii NCC533	LJ0769	bacteriocin lactacin F, subunit	75	-2/+6			-2/+6		bacteriocin lactacin F, subunit
	LJ0769b	bacteriocin lactacin F, subunit	62	-3/+5			-3/+5		Lactacin F, subunit lafX precursor
	LJ0779	hypothetical protein	72	-6/+8			6		
	LJ1515	30S ribosomal protein S16	90						COG1659: uncharacterized protein
	LJ0771	hypothetical protein	71	-5/+3			-5/+3		
	LJ0763b	lactacin F two-component system	50	+4			+4		
	LJ0775b	hypothetical protein	64	-2			-2		
	LJ0558	hypothetical protein	82	-3	-6				
	LJ1789	hypothetical protein	95						
	LJ0780	hypothetical protein	76	-7/+7			-7		
Lb. reuteri F275	Lreu_1871	cytochrome b5	81						bacteriocin
	Lreu_0728	ribosomal protein S21	63	-2					bacteriocin 28b
	Lreu_0462	ATP synthase F0, C subunit	72					AXXXAAXGA| AXXXAAXGA	
Lb. sakei 23K	LSA0560_b	Putative bacteriocin inducing	44	-6					putative bacteriocin inducing
	LSA1397/ rpmI	50S ribosomal protein L35	66						1912296A pyocin AP41:SUBUNIT=I
	LSA1365	hypothetical protein	72	-3/+1	8				
Lc. mesenteroides ATCC8283	LEUM_0069	hypothetical protein	56	+4			-6		
	LEUM_1043	hypothetical protein	98	+6					Linocin M18
	LEUM_0089	hypothetical protein	59	+3			-7		
	LEUM_0070	hypothetical protein	65	-1		-1			
	LEUM_0085	hypothetical protein	58	-2		-2			
	LEUM_0086	hypothetical protein	53	-4		-4			
	LEUM_0088	hypothetical protein	45	-5		-5			
	LEUM_0090	hypothetical protein	58	+5			-5		
	LEUM_0068	Transcriptional regulator, xre	87	-6		-6			
	LEUM_1360	Ribosomal protein S14	89				-3		klebicin C activity
	LEUM_1970	Ribosomal protein S6	98	+3					S06218 colicin E1 - Shigella s
	LEUM_1874	F0F1-type ATP synthase, subunit	75					AXXXAAXGA| AXXXAAXGA	
O. oeni PSU-1	OEOE_0110	hypothetical protein	58		-4				
	OEOE_1548	hypothetical protein	84	-8					
	OEOE_0116	hypothetical protein	59		4				
	OEOE_0306	hypothetical protein	81				-5		
P. pentosaceus ATCC25745	PEPE_0082	Prebacteriocin	60	+3		3		YGNGVXC| YGNGXXCXXXXC	pediocin
	PEPE_0796	hypothetical protein	92						bacteriocin BCN5
	PEPE_0847	Ribosomal protein S16	90						Linocin M18

ABC, ABC transporters; HK, Histidine kinase; C39, protease; immunity, immunity gene.

[a] Number of amino acids.

[b] Distance of gene, present upstream (+) / downstream (-) in the genomic context of the putative bacteriocin, in number of ORFs.

2.3.2 BACTERIOCINOGENIC LAB ISOLATED FROM WINE

Although a variety of LAB of oenological origin are known to produce bacteriocins (Holo et al., 2001; Navarro et al., 2000; Rojo-Bezares et al., 2007a; Strasser de Saad et al., 1993; Yurdugül et al., 2002), few studies have been conducted to investigate the production of bacteriocins by LAB in wine and its presence in finished wine.

Increasing research in molecular microbial ecology, molecular biology and vast progress in genomic studies offer new valuable tools to study microbial populations in food environments (Grande et al., 2007). Advanced genomic studies will allow the identification of novel bacteriocins, independent of the influence of various factors such as environmental conditions, inducible character of bacteriocins or producing capacity of the strain (Cotter et al., 2005; Nes et al., 2004). The use of molecular microbial ecology approaches such as Real Time-PCR (polymerase chain reaction) (Grattepanche, Lacroix, Audet and Lapointe, 2005; Neeley, Phister and Mills, 2005), DGGE (denaturing gradient gel electrophoresis) (Renouf, Claisse and Lonvaud-Funel, 2006) and TTGE (temporal temperature gradient gel electrophoresis) (Ogier, Son, Gruss, Tailliez and Delacroix-Buchet, 2002) may help to understand the complex interactions of the microbial flora in wine that lead to inhibition, survival and adaptation to environmental stress. Furthermore, these approaches will also contribute to the better understanding of the biology of bacteriocins and microbial populations at cellular and molecular levels (Grande et al., 2007).

Although the application of bioengineered/modified bacteriocins or microorganisms is not yet approved in wine, using such techniques might help to improve the stability and production of bacteriocins so that they may be more suitable for the vinification process and other food systems.

2.3.3 GENETIC ENGINEERING SYSTEMS

Important advances in the genetic study of LAB resulted in the development of numerous genetic techniques, transformation protocols, vectors and integration systems, as well as safe food-grade selection systems. For further commercial and scientific development of LAB, modified and controlled expression and secretion of existing or novel genes and their products is crucial. Since there is almost no overlap between the energy (carbon)

metabolism and the biosynthesis (nitrogen) metabolism in LAB, they are ideal objects for metabolic engineering (Hugenholtz and Kleerebezem, 1999). *Lactococcus lactis* is still by far the most comprehensively studied species among LAB and several examples of successful genetic engineering are available (Kleerebezem et al., 2003b).

2.3.3.1 Transformation methods

Three methods can be used to transfer genetic material (DNA) into bacterial cells: transformation, conjugation and transduction. Bacterial transformation is the transfer of DNA. To achieve the uptake of extracellular DNA, the bacterial cells have to be 'competent', which is usually induced chemically (Bartowsky, 2005). Electroporation has become the preferred option for transforming LAB (Mills, 1999). Electroporation makes use of high voltage, short duration electronic pulses to permeablize bacterial cell membranes and thus assists the passage of DNA (Luchansky, Muriana and Klaenhammer, 1988).

An alternative to transformation is the use of conjugative systems to promote genetic transfer of plasmids between bacteria. This technique firstly entails the enzymatic preparation of the plasmid DNA prior to replicative transfer and secondly the formation of the mating channel through which DNA is transferred into a recipient cell (Lanka and Wilkins, 1995). The third method of transferring DNA is transduction, which involves a virus acting as a vector to transfer the desired genes to a target cell (Bartowsky, 2005).

2.3.3.2 Gene expression systems

Advances in genetic and molecular biology of LAB have led to the construction of constitutive gene expression cassettes, inducible gene expression systems and specific protein targeting systems described by several authors (De Vos, 1999a; Ruhdal Jensen and Hammer, 1998).

A nisin-controlled expression (NICE) system was developed, which allows regulated overproduction of numerous proteins by several Gram-positive bacteria, especially *Lact. lactis*. (Kleerebezem, Beerthuyzen, Vaughan, de Vos and Kuipers, 1997a; Pavan et al., 2000; Zhou et al., 2006). The crucial elements for system construction, its application and further improvements were discussed by Zhou et al. (2006). Moreover, Bron et al. (2002) have shown that this system can also be used for the construction of nisin-controlled conditional knockout mutations in essential genes.

A new approach was developed by Jensen and Hammer (1998) to meet the growing demand for the precise tuning of gene expression. They constructed a library of synthetic promoters of various strengths by introducing slight changes in the sequences flanking the -35 and -10 consensus sequences of bacterial promoters. Their use has been greatly simplified through the creation of promoter libraries in a single PCR step (Solem and Jensen, 2002). Furthermore, the latter authors developed a system that enables the efficient chromosomal insertion in *Lact. lactis* of the chosen 'synthetic' promoter upstream of a gene of choice, permitting endogenous gene expression at a pre-designated level. Since then, several libraries have been constructed and adapted for use in several organisms, including yeast (Hammer, Mijakovic and Jensen, 2006; Jeppsson, Johansson, Ruhdal Jensen, Hahn-Hägerdal and Gorwa-Grauslund, 2003; Rud, Jensen, Naterstad and Axelsson, 2006; Ruhdal Jensen et al., 1998). Moreover, Mijakovic, Petranovic and Jensen (2005) reviewed the possibilities of these synthetic promoter libraries and their applications in individual and simultaneous regulation of multiple genes. Gene expression activation at a certain stage of fermentation can be advantageous, when a gene product (e.g. bacteriocin) inhibits the growth of either the host cell or other bacteria invading the wine environment. For this strategy, a regulatory mechanism with a promoter that is activated by an environmental signal such as change of pH, temperature or growth phase, for instance, might be useful (Ruhdal Jensen et al., 1998).

Several heterologous systems have been developed for the production of LAB bacteriocins. Food-grade overexpression of the genes encoding the secretion and processing machinery based on the inducible P*nisA* (De Ruyter, Kuipers, Beerthuyzen, van Alen-Boerrigter and de Vos, 1996; Kleerebezem et al., 1997a) or the salt-inducible P*gad* (Sanders, Venema and Kok, 1997) promoters offers another approach for achieving high level heterologous bacteriocin expression. Kempermann et al. (1999) enhanced the secretion of pediocin PA-1 in a *Lact. lactis* strain with an induced overexpression of *pedC* behind the P*gad* promoter. Moreover, Venema et al. (1995) had observed that overexpression of *pedD* led to increased pediocin PA-1 production in pediococci.

The design of systems for the heterologous production of bacteriocins, such as ABC-transporters and secretory pathway systems, may help to overcome problems that bacteriocin-producing LAB often face in food processes (e.g. poor adaptability, low bacteriocin production or genetic instability) (Rodriguez et al., 2003).

2.3.3.3 Development of vector systems

Several attempts have been made to develop efficient vector systems for LAB. Numerous vectors have been modified by introducing promoter, terminator and selection marker. This plasmid based system depends on a selection marker, but strains for food-grade applications should not contain antibiotic selection markers or inducible gene expression systems (Singh, Ahmed and Pandey, 2006). Thus, markers based on natural properties of LAB are ideal for food related applications. Different food-grade cloning and vector systems were reviewed by De Vos (1999b), Shareck, Choi, Lee and Miguez (2004) and Takala (2005).

Food-grade cloning markers can be divided into two classes based on their selection: dominant and complementary markers (De Vos, 1999b). Dominant markers are comparable to antibiotic selection markers; they can be transferred and selected virtually into any host sensitive to the selective agent (e.g., bacteriocin, heavy metal resistance), or lacking the ability to metabolize the agent used in selection (e.g. carbohydrate utilization). Complementary markers need host strains specifically engineered to lack a certain essential property, which is then complemented by the food-grade marker (e.g. carbohydrate utilization). However, there are also other systems, which do not meet with these criteria. Integrative transformation systems, for example, do not necessarily require food-grade markers for a final construct showing food-grade status (Takala, 2005).

One approach to replace antibiotic markers is the use of dominant food-grade bacteriocin-resistant markers. One of the first food-grade systems based on bacteriocin resistance were the plasmids pVS40, pFM011 and pFK012 containing the nisin resistance marker *nsr* from *Lact. lactis* (Froseth and McKay, 1991; Hughes and McKay, 1991; Von Wright, Wessels, Tynkkynen and Saarela, 1990). Takala and Saris (2002) constructed the plasmid pLEB590, a food-grade vector containing the nisin immunity gene, *nisI*, as a selection marker and which showed potential for overexpression of valuable genes in dairy starter culture.

Another alternative approach is the utilisation of a two plasmid system. Emond et al. (2001) made use of a system through separation of the antibiotic marker (on a $RepB^-$-plasmid) from the $RepB^+$ vector into which the genes of interest were cloned. Both plasmids are involved in the gene transfer step, but the marker plasmid can be subsequently removed by curing. Cotter et al. (2003) developed a system using the $RepA^+$ temperature-sensitive helper plasmid and a $RepA^-$ cloning vector. Thus, native plasmids can be precisely engineered while providing the extra advantages of allowing multiple

alterations and maintaining the food-grade status of the plasmid, as well as facilitating the transfer of such plasmids between hosts.

2.3.3.4 Bacterial host systems for heterologous protein production

Although a variety of bacterial host systems are available, it is generally difficult to choose an adequate host and promoter system for heterologous protein production, since it often depends on the target protein itself. Moreover, many bacterial systems are not able to modify proteins post-translationally (e.g. glycosylation) (Terpe, 2006). Terpe (2006) gave an overview of the most commonly used host and promoter systems and host including *Bacillus brevis*, *Bacillus megaterium*, *Bacillus subtilis*, *Caulobacter crescentus* and *Escherichia coli*. To date *Lact. lactis* is by far the most extensively studied species amongst LAB and numerous examples of metabolic engineering in this species are available (Kleerebezem et al., 2003b; Singh et al., 2006; Takala et al., 2002). However, interest in other LAB such as *Lb. plantarum*, *Lb. casei, Lb. helveticus* and *Lb. sakei* is increasing considerably and gene expression systems are expanding (Diep et al., 2006; Mathiesen, Sørvig, Blatny, Naterstad, Axelsson and Eijsink, 2004; Pavan et al., 2000; Sørvig, Grönqvist, Naterstad, Mathiesen, Eijsink and Axelsson, 2003; Sørvig, Mathiesen, Naterstad, Eijsink and Axelsson, 2005). Many LAB species or strains are food-grade organisms, offering great opportunities for the heterologous production of commercially important proteins or peptides (Billman-Jacobe, 1996). Rodriguez et al. (2003) reviewed and compared different experimental strategies that have been used to produce bacteriocins heterologously by LAB using an ABC-dedicated transport system or a general secretory pathway.

Some systems may not be suitable for certain species or strains. Therefore, it is necessary to develop systems that are optimally suited for specific conditions or applications. Moreover, the use of genetically modified organisms (GMOs) for the *in situ* production of bacteriocins or other interesting peptides, such as flavour peptides, still faces opposition from industries and consumers, unless they can be convinced of their advantages (Rodriguez et al., 2003).

2.3.4 POTENTIAL OF GENETICALLY IMPROVED LAB

Currently, there is no application of genetically modified (GM) bacteria, yeasts or plants in wine production. Nevertheless, molecular biology has helped to gain valuable knowledge

about microbial genetics, biochemistry and physiology (Bartowsky, 2005). This knowledge has already been used to manipulate and develop commercial LAB starter cultures with novel metabolic capabilities, thus ensuring consistent fermentations and improved flavours. Moreover, whole genome sequencing progress and subsequent technologies will have an influence on metabolic engineering (Kleerebezem et al., 2003b).

2.3.4.1 Genetic modification of *Lb. plantarum* strains involved in food fermentations

Lb. plantarum is another wine related LAB species of interest, because of its ability to conduct MLF and to produce antibacterial activity towards many LAB species.

The dominant microorganism in a certain food fermentation controls the outcome of the fermentation. As the main microorganism, *Lb. plantarum* will produce highly acidified products containing mostly lactic acid, but also other compounds such as acetic acid, acetaldehyde, ethanol and diacetyl which can affect the flavour outcome (Hugenholtz et al., 1999). LAB have been investigated for their production of lactic acid, B-vitamins and low-calorie sugar alcohols (Hugenholtz et al., 1999; 2002). Several metabolic engineering approaches in *Lb. plantarum* have been reported and the following examples will be mostly focused on the level of the main carbon metabolism. Ferain et al. (1998) constructed a strain of *Lb. plantarum* deficient for both D- and L-lactate dehydrogenase (*ldh*) activities by gene inactivation. This study showed that a major rerouting of both glucose and glucose/citrate metabolism leads to the production of end-products such as acetoin, ethanol, acetate, mannitol and succinate. Moreover, the additional cloning of the ethanol genes, *adh* and *pdc* from *Zymomonas mobilis*, resulted in an increased ethanol production. A similar approach was demonstrated by Liu, Nichols, Dien and Cotta (2006a). The latter authors tried to increase the ethanol production by introducing a pyruvate decarobxylase gene from the Gram-positive bacterium, *Sarcina ventriculi* (Spdc), into an LDH-deficient *Lb. plantarum* strain. Furthermore, Hugenholtz et al. (1999) suggested that a much higher ethanol production could be achieved by using the same approach with more ethanol-tolerant LAB strains. Ladero et al. (2007) reported a metabolic engineering approach to achieve a high-level sorbitol production by reversing the catabolic pathway in a *Lb. plantarum* strain deficient for LDH activities. To eliminate undesired fermentation products of *Lb. plantarum*, Liu (2006b), introduced additional mutations on the chromosome of *Lb. plantarum* TF103, by inactivating two of the chromosomal L-*ldh* and D-*ldh* genes were inactivated. They targeted the acetolactate synthase gene (*als*) that

converts pyruvate to acetolactate in order to eliminate the production of acetoin and 2,3-butanodial.

Metabolic engineering in LAB deals with rerouting the major metabolic flux in these bacteria or through altered expression of relevant heterologous genes (Hugenholtz et al., 2002). Similar approaches could be used to eliminate undesired aroma compounds (volatile phenols) or health hazard compounds (biogenic amines) produced by *Lb. plantarum* during MLF in wine. Moreover, the antibacterial activity of many *Lb. plantarum* strains could be further exploited by enhancing the production of homologous bacteriocins (such as plantaricin) or introducing and expressing heterologous bacteriocin genes. Heterologous production of bacteriocins by strains of *Lb. plantarum* has already been achieved for the bacteriocin, acidocin, from *Lb. acidophilus* and lactocin S, from *Lb. sakei* (Leer, Van der Vossen, Van Giezen, Van Noort and Pouwels, 1995; Skaugen, Christie and Nes, 1999).

Genetic tools of critical importance in these strategies, such as new vectors for inducible gene expression in *Lb. plantarum*, as well as a synthetic promoter library have been developed and improved (Rud et al., 2006; Sørvig et al., 2003; 2005). Moreover, the NICE system (nisin controlled expression system) which has been proven to be very valuable for controlled gene expression has been adapted for utilization in the latter species (Pavan et al., 2000).

2.3.4.2 Targets for genetic improvement of *O. oeni* strains

LAB are not naturally optimized for maximal production rates of wine sensory important flavour compounds. Aroma compounds such as diacetyl or aldehydes are often secondary metabolites and are produced in smaller amounts. Genetic engineering approaches to modify and improve malolactic starter cultures such as *O. oeni* are of great interest for the wine industry. The completion of the *O. oeni* genome sequencing enables a better insight into the physiology, genetic diversity and performance of malolactic starter cultures (Mills et al., 2005).

Improved or newly implemented characteristics such as increased efficiency of malic acid degradation, optimized production of wine aroma attributes (esters, diacetyl) and reduced production of biogenic amines and ethyl carbamate would be beneficial features during malolactic fermentation. Moreover, the inhibition of spoilage bacteria which can possibly produce off-flavours and unwanted biogenic amines could be desired feature during the winemaking process. One possible approach could be the application of

bacteriocinogenic bacteria strains. The use of bacteriocin-producing LAB strains has been exploited in other food-industries such as the dairy industry (Deegan et al., 2006). A pediocin-producing *Lb. plantarum* strain was shown to efficiently inhibit *Listeria monocytogenes* (Loessner, Guenther, Steffan and Scherer, 2003). Schoeman et al. (1999) and van Reenen et al. (2003) expressed pediocin PA-1 from *P. acidilactici* and plantaricin 423 from *Lb. plantarum* 423, respectively, in *S. cerevisiae*. A similar strategy could be applied by expressing bacteriocins such as pediocin, plantaricin and nisin in *O. oeni*. **Table 2.4** shows potential genetic modifications of *O. oeni* to enhance malolactic fermentation, as reviewed by Pretorius et al. (2006).

Table 2.4
Targets for the genetic improvement of *O. oeni*

Desirable Properties	Focus Area	Examples of Potential Target Genes
Improved Fermentation Performance		
Increased ethanol, low pH, non-optimal temperature resistance	Protease associated with ATPase complex, heat shock proteins and stress proteins	*hsp 18*, *clpX*, *trxA*, *fstH*
Efficient malic acid degradation	Malolactic fermentation and increased strain viability	*mleA*, *mleR*, *mleP*, and genes involved in oleic acid assimilation
Improved tolerance to antibacterial compounds	Resistance against bacteriocins, sulfur dioxide, agrochemicals	Desired bacteriocins resistance gene
Improved tolerance against growth-inhibitory compounds such as tannins, fatty acids	Protease associated with ATPase complex	*ftsH*
Improved Processing Effciency		
Improved protein clarification	Proteases	Target genes not yet identified
Improved polysaccharide clarification	Glucanases, pectinases, arabinofuranoidases	Target genes not yet identified
Improved Wine Flavour or Other Sensory Attributes		
Optimized production of glycerol	Glycerol metabolisms	Glycerol dehydratase enzyme
Enhanced liberation of monoterpenes	β-glycosidases, glucanases, arabinofuranoidases	bgl (β-glycosidases) and other genes required for sequential release of monoterpenes from glycoside
Optimized levels of aldehydes	Enzymes involved in acetaldehyde metabolism and catabolism	Target genes not yet identified
Optimized levels of phenolics	Phenolic acid metabolism	Modified expression of pdc *alsS*, *alsD* (α-acetolactate synthase and decarboxylase), diacetyl reductase, citrate permease (*citP*), citrate lyase
Optimized levels of esters	Lipases and esterases	Increase in acyl transferases and decrease in esterase activities
Improved Wine Wholesomeness		
Reduced formation of biogenic amines	Amino acid decarboxylases	Deletion of *hdc* and *tdc* genes
Reduced formation of ethyl carbamate	Arginine degradation	Deletion of the arginine deiminase and ornithine carbamyl transferase
Reduced formation of glyoxal and methylglyoxal levels	Metabolism of glyoxal and methylglyoxal	Target genes not yet identified

Source: Pretorius et al. (2006)

2.3.4.3 Genetic engineering approaches with *O. oeni*

O. oeni is the most desired bacteria species for MLF during the vinification process, because of its ability to grow in the harsh wine environment and, among the wine-related LAB, it is the least associated with spoilage flavours or other unwanted metabolites (Mills et al., 2005). For these reasons, *O. oeni* has been the focus of much recent research.

However, *O. oeni* is difficult to work with when applying molecular tools for genetic manipulation. Only one successful transformation via electroporation has been reported by Dicks (Dicks, 1994), although the described procedure has not been successfully repeated since then. Two studies made use of conjugation systems and set up techniques and parameters to achieve reproducible conjugal frequencies (Zúñiga, Pardo and Ferrer, 1996b; 2003). Nonetheless, these methods could only be used in particular cases.

Plasmids of the family pAMß1/pIP501 were successfully transferred between *Lact. lactis* and *O. oeni*, but the plasmids caused structural instability and were too big for easy genetic manipulation (Zúñiga et al., 2003). The transfer of the transposons (Tn916 and Tn925) from *Enterococcus faecalis* to *O. oeni* showed that the transposons were not integrated randomly and were able to be stably maintained (Zúñiga et al., 1996b). Beltramo et al. (2004) constructed a new vector (pGID052) especially for conjugative transfer into *O. oeni* and performed successful conjugal experiments from *Lact. lactis* to *O. oeni* via mobilisation. Moreover, pGID052 seemed to be segregationally stable and might have potential as an expression vector. However, there has been no further progress since this development.

A different approach of developing a cloning vector was the characterisation of the extrachromosomal DNA of *O. oeni*. Several studies showed that *O. oeni* does harbour plasmids which have also been sequenced and could potentially be modified to be used as vectors for transformation (Brito and Paveia, 1999; Brito, Vieira, Santos and Paveia, 1996; Janse, Wingfield, Pretorius and van Vuuren, 1987; Mesas, Rodriguez and Alegre, 2001; Prévost, Cavin, Lamoureux and Divies, 1995; Zúñiga, Pardo and Ferrer, 1996a). Furthermore, Mesas, Rodriguez and Alegre (2004) reported the ability of curing *O. oeni* strains of their plasmids which could provide a new promising tool for genetic manipulations.

Transduction is theoretically possible for *O. oeni* (Bartowsky, 2005). Several bacteriophages have been identified in fermenting must/wine (Davis, Silveira and Fleet, 1985; Henick-Kling, Lee and Nicholas, 1986) and several had their genomes sequenced (Gindreau, Torlois and Lonvaud-Funel, 1997; Parreira, Sao-Jose, Isidro, Domingues, Vieira and Santos, 1999; Poblet-Icart, Bordons and Lonvaud-Funel, 1998; Santos, São-José, Vieira, Paveia and Santos, 1998; São-José, Parreira, Vieira and Santos, 2000; 2004). Further investigations into their function of infection, genome organisation and recombination systems could help to develop a transduction system for *O. oeni* (Bartowsky, 2005).

2.4 CONCLUDING REMARKS

Various strains of LAB are used for food fermentations and for the preservation of fermented foods. In wine, species belonging to the genera *Oenococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* are mainly involved in the vinification process and have an impact on the organoleptic profile and quality of wines, especially those undergoing MLF.

LAB with the ability to produce bacteriocins present a beneficial opportunity to control or prevent the development of specific bacterial species in beverages. It might also have implications for the development of a desired microflora in wine fermentations.

Increasing research and advances in molecular genetics and manipulations, as well as genetic investigation of LAB have provided important knowledge about microbial genetics, biochemistry and physiology. Moreover, new valuable tools help to study microbial ecologies, to identify new genes and offer new possibilities in developing commercial LAB starter cultures with novel metabolic abilities.

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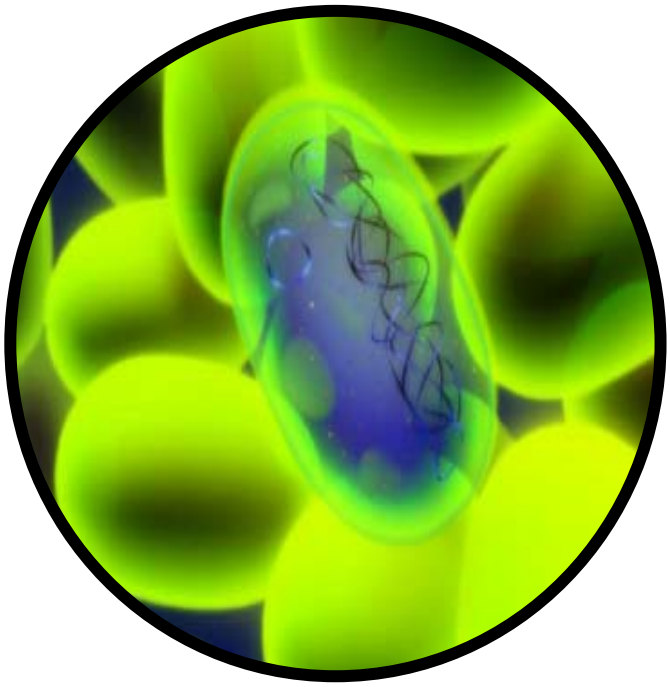
Chapter 3



RESEARCH RESULTS

Bacteriocin production by lactic acid bacteria of oenological origins

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3. RESEARCH RESULTS

Bacteriocin production by lactic acid bacteria of oenological origin

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Abstract

A total of 330 lactic acid bacteria isolated from South African red wines during alcoholic and malolactic fermentations and 9 commercial malolactic bacteria starter cultures were screened for antimicrobial activity. Of the entire screened isolates, 26 strains, belonging to the species *Lb. plantaru*m, *Lb. paracasei*, *Lb. hilgardii* and *O. oeni*, showed activity towards various wine-related and non wine-related indicator strains. A PCR-based screening revealed the presence of the plantaricin encoding genes *plnA*, *plnEF*, *plnJ*, *plnK* in five selected *Lb. plantarum* strains. Furthermore, a co-culture experiment with *Lb. plantarum* and *Ent. faecalis* in enriched MRS broth was performed. A complete inhibition of cell growth of *Ent. faecalis* was observed within 72 hours. To analyse the potential of four putative bacteriocin-encoding genes in the genome of *O. oeni,* the four genes were heterologously expressed in different *E. coli* host strains. No antimicrobial activity could be detected. To enhance the antimicrobial activity of *Lb. plantarum*, a plasmid containing the nisin A gene was cloned into two strains of *Lb. plantarum*.

3.1 INTRODUCTION

Bacteriocins produced by lactic acid bacteria (LAB) can be defined as ribosomally synthesized compounds that inhibit the growth of other bacteria (Klaenhammer, 1988). They can exhibit broad or narrow spectrum and seem to be primarily aimed at other LAB sharing the same ecological niche (Diep and Nes, 2002; Eijsink, Axelsson, Diep Dzung, Håvarstein, Holo and Nes, 2002; Jack, Tagg and Ray, 1995; Klaenhammer, 1988). Consequently the ability to produce bacteriocins gives the producers a great advantage in competing with other bacteria for the same nutrients (Diep et al., 2002; Dykes, 1995;

Eijsink et al., 2002). Moreover, bacteriocins may also play a protective role and act to prohibit the invasion of other strains or species into an occupied niche or limit the establishment of neighbouring cells (Riley and Wertz, 2002). Furthermore, quorum sensing regulation of class II bacteriocins has been recently proposed. This phenomenon enables the bacterium to only invest energy in bacteriocin production if there are sufficient other producers around (Eijsink et al., 2002; Kleerebezem, Quadri, Kuipers and de Vos, 1997). Bacteriocin production has been shown to be influenced by several factors (e.g. pH, temperature, medium composition). It seems that the production of bacteriocins change as components of the environment, both biotic and abiotic, change (Diep, Havarstein and Nes, 1995; Grande et al., 2007; Kleerebezem et al., 1997; Riley et al., 2002).

Bacteriocins can be divided into three major classes on the basis of biochemical and genetic characterization, as detailed by Cotter, Colin and Ross (2005). They are often cationic, amphilic and membrane-permeabilizing molecules. The genes coding for bacteriocin production are mostly organized in operon clusters comprising four genes, which may be located on the chromosome, on plasmids or transposons. These genes include a structural gene, an immunity gene, a gene encoding an ABC transporter and a gene encoding an assessory protein essential for externalization (Klaenhammer, 1993).

There are many techniques described in literature to screen for bacteriocin-producing strains. These methods can be subdivided into direct and indirect screening methods which are described and summarized by De Vuyst and Vandamme (1994). Many of these methods are based on the diffusion of bacteriocins through semi-solid or solid culture media to inhibit growth of a sensitive indicator microorganism (e.g. ‘spot on the lawn method’, ‘well-diffusion method’). De Jong, van Hijum, Bijlsma, Kok and Kuipers. (2006) presented a web-based software tool BAGEL, which allows the *in silico* identification of putative bacteriocins and their biosynthetic clusters using a knowledge-based database. Moreover, BAGEL enables an open reading frame (ORF) detection which prevents the possible oversight of small non-conserved ORFs.

The most broadly studied and commercially available bacteriocin is nisin, originally produced by a number of *Lactococcus lactis* subsp. *lactis*. Nonetheless, other bacteriocins such as pediocins and lacticins have found applications in food systems which were recently overviewed by Deegan, Cotter, Hill and Ross (2006). While nisin is mostly used in canned food or dairy products, pediocin has the ability to protect fresh and fermented meat. Lacticins have been tested as bio-preservatives in natural yoghurt, cottage cheese and infant milk formula. Moreover, the use of a plantaricin producing starter culture has

been applied in the fermentation and preservation of olives (Ruiz-Barba, Cathcart, Warner and Jimenez-Diaz, 1994).

Some LAB of oenological origin have been described to produce bacteriocins, e.g. *Lb. plantarum* J23 (Rojo-Bezares, Saenz, Navarro, Zarazaga, Ruiz-Larrea and Torres, 2007a), *Lb. plantarum* J51 (Navarro, Zarazaga, Saenz, Ruiz-Larrea and Torres, 2000), *P. pentosaceus* (Strasser de Saad and Manca de Nadra, 1993), *Lb. plantarum* LMG 2379 (Holo, Jeknic, Daeschel, Stevanovic and Nes, 2001), *Lb. delbrueckii* subsp. *delbrueckii, Lc. mesenteroides* subsp. *cremoris* and *Lb. fructivorans* (Yurdugül and Bozoglu, 2002). Within the species of *Lb. plantarum,* the production of different bacteriocins has been reported. These bacteriocins can be divided into the following classes: class I: plantaricin C and W (Holo et al., 2001; Turner et al., 1999), class IIa: plantaricin C19 and 423 (Atrih, Rekhif, Moir, Lebrihi and Lefebvre, 2001; Van Reenen, Dicks and Chikindas, 1998), class IIb: plantaricin EF, JK, S, NC8 (Anderssen, Diep, Nes, Eijsink and Nissen-Meyer, 1998; Maldonado, Ruiz-Barba and Jimenez-Diaz, 2003; Moll et al., 1999; Stephens et al., 1998) and class IId: plantaricin 1.25ß (Remiger, Eijsink, Ehrmann, Sletten, Nes and Vogel, 1999).

However, no *O. oeni* strains have been reported to produce a bacteriocin. This species has been described to be sensitive to bacteriocins such as nisin and pediocin. Moreover, it was demonstrated that nisin and pediocin inhibit LAB found in wine, that they are stable under winemaking conditions, and have no negative effect on yeast growth or the sensorial wine profile (Bauer, Nel and Dicks, 2003; Daeschel, Jung and Watson, 1991; Radler, 1990a; 1990b; Rojo-Bezares, Saenz, Zarazaga, Torres and Ruiz-Larrea, 2007b).

During alcoholic and malolactic fermentations in wine, microbial interactions may occur between yeasts, LAB and yeast-LAB (Alexandre, Costello, Remize, Guzzo and Guilloux-Benatier, 2004; Lonvaud-Funel and Joyeux, 1993). Various factors are involved in these interactions (e.g. pH, alcohol, organic acids) and bacteriocins may play a significant role in inhibiting bacterial growth.

Bacteriocin production constitutes a powerful adaptation advantage for strains to dominate in a medium such as wine and may therefore play an important role in the ecology of wine microflora. There is an increasing interest in bacteriocins as food bio-preservatives, since the majority of bacteriocin-producing LAB are indigenous food isolates. The possibility of controlling bacterial growth during vinification and preservation by bacteriocins is a promising alternative to meet the consumer demands and preferences for minimally processed products that contain less chemical preservatives such as sulfur

dioxide (SO_2). It has also been shown that bacteriocins used in combination with organic acids, phenolic compounds or chemical preservatives result in enhanced inhibition of bacteria (Deegan et al., 2006; Grande et al., 2007; Rojo-Bezares et al., 2007b). Inoculating a bacteriocin-producing LAB culture is an indirect way to incorporate bacteriocins in wine and the success depends on the capability of the strain to grow and produce the bacteriocin under the fermentation conditions. Moreover, it should be compatible with the starter cultures required for MLF. When used as a starter culture the bacteriocin-producing strain must be able to perform MLF optimally besides being able to produce enough bacteriocin amounts for protection against spoilage bacteria.

The purpose of this study was to screen and select bacteriocin-producing LAB isolates from South African red wines during alcoholic and malolactic fermentation and to evaluate the production of bacteriocins in liquid cultures. In addition, the presence of known bacteriocin-encoding genes in *Lb. plantarum* and putative bacteriocin genes in *O. oeni* was investigated and heterologous expression studies were performed. Moreover, the nisin A gene from *Lact. lactis* was cloned into two strains of *Lb. plantarum* and their activity was tested.

3.2 MATERIALS AND METHODS

3.2.1 STRAINS, MEDIA AND GROWTH CONDITIONS

Three hundred and thirty LAB strains isolated from South African red wines during alcoholic and malolactic fermentations (collection of the Institute for Wine Biotechnology, Stellenbosch University, South Africa), as well as nine commercial starter culture strains (**Table 3.1**) (Lallemand, Toulouse, France) were tested for antimicrobial activity. Nineteen wine related and two unrelated bacterial strains listed in **Table 3.1** were used as indicator strains.

All strains were grown in MRS medium (Biolab Diagnostics (PTY) Ltd, Wadeville, Gauteng, SA), except for *O. oeni* which was grown in modified MRS supplemented with 5 g/L fructose and 4 g/L malic acid and the pH was adjusted to 4.5. *Listeria monocytogenes* was grown in Brain Heart Infusion broth (BHI; Biolab Diagnostics). *Escherichia coli* strains were grown in Luria-Bertani broth (Biolab Diagnostics) at 37°C on a rotary wheel at 50 rpm.

Selective antibiotic concentrations were as follows: 100 µg/mL of ampicillin (Sigma Aldrich, Johannesburg, South Africa); chloramphenicol (Roche, Johannesburg, South Africa), 50 µg/mL for *E. coli* BL21-(DE3)pLysS and 34.7 µg/mL for *E. coli* Rosetta-gami-(DE3)pLysS; 15 µg/mL of kanamycin (Roche); 12.5 µg/mL of tetracycline (Sigma-Aldrich). When appropriate, 30 µg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma Aldrich) were added.

3.2.2 DETECTION OF ANTIMICROBIAL ACTIVITY

The 'colony-overlay' method (Schillinger and Lücke, 1989) was used to detect antimicrobial activity. The method was modified as follows: 50 mL of MRS broth were inoculated with 5 mL of an overnight culture and incubated with shaking (180 rpm) for six hours at 30°C. MRS and BSM agar plates (Tichaczek, Nissen-Meyer, Nes I.F., Vogel and Hammes, 1992) were spotted with 10 µL overnight culture and incubated overnight at 30°C; the spotted *O. oeni* strains were incubated in an anaerobic chamber. A layer of 10 mL MRS supplemented with 0.7% agar containing 200 µL of overnight culture of the indicator organism was poured over the plate (except for *O. oeni*). *O. oeni* indicator strains were grown in modified MRS medium for 48 h at 30°C in anaerobic conditions. A layer of 10 mL modified MRS supplemented with 0.8 % agar containing 200 µL of the *O. oeni* strain was poured over the plate. The plates were incubated at 30°C under anaerobic conditions for 24 – 48 h. Growth inhibition was detected by a clearing zone around the producer strain.

To detect antimicrobial activity of the *Lb. plantarum* transformants, the method was modified as follows: strains were grown overnight in 5 mL MRS broth supplemented with 15 µg/mL ampicillin. MRS agar plates with and without ampicillin were used for spotting.

3.2.3 EVALUATION OF BACTERIOCIN PRODUCTION BY *Lb. plantarum* R1122 IN LIQUID MEDIA

The plantaricin producing *Lb. plantarum* R1122 were pre-cultured in MRS, respectively, at 30°C for 24 h. The sensitive indicator strain, *Enterococcus faecalis* LMG 13566, was pre-cultured in MRS at 37°C for 48h. 60 mL of MRS broth were then inoculated with 2% of the producer strain R1122 and either 1% or 0.5% of the indicator strain. As negative controls

each bacteria strain was grown without co-culture. The cultures were incubated at 30°C for 72 – 81 h.

Table 3.1
Bacteria strains used in this study

Species	Strain	Source
Bacteriocin producer		
Lactobacillus plantarum	R1122	Lallemand, France
	45.3, 3.5, 67.5, 85.2, 81.9, 19.11, 21.11, 65.6, 71.10	IWBT collection
Lactobacillus paracasei	20.4, 74.9	IWBT collection
Lactobacillus hilgardii	21.12, 77.8, 71.12, 28.5, 24.1, 16.5	IWBT collection
Lactococcus lactis subsp *lactis*	20729	DSM
Potential bacteriocin producer and indicator bacteria		
Oenococcus oeni	R1098, R1101, R1105 R1106, R1108, R1118, R1123, R1124	Lallemand, France
Lactobacillus plantarum	116.4, 81.2, 130.1 74.9, 107.4, 131.1, 131, 120.1, 113.1 65.1	IWBT collection
Indicator bacteria		
Oenococcus oeni	48	IWBT collection
Lactobacillus plantarum	116.4	IWBT collection
Lactobacillus hilgardii	51	IWBT collection
Lactobacillus paracasei	105.8	IWBT collection
Lactobacillus brevis	111	IWBT collection
Lactobacillus pentosus	42	IWBT collection
Lactobacillus fermentum	9328	ATCC
Leuconstoc mesenteroides	5	IWBT collection
Pediococcus acidilactici	118	IWBT collection
Pediococcus damnosus	1832	NCFB
Pediococcus pentosaceus	813	NCDO
Listeria monocytogenes	127	BfE
Enterococcus faecalis	13566	LMG

ATCC American Type Culture Collection (Rockville, Md., USA)
BfE Bundesanstalt für Ernährung (Karlsruhe, Germany)
DSM Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany)
IWBT Institute for Wine Biotechnology (Stellenbosch, South Africa)
LMG Culture Collection of the Laboratory of Microbiology (University of Gent, Belgium)
NCDO National Collection of Dairy Organisms (Reading, UK)
NCFB National Collection of Food Bacteria (c/o NCIMB Ltd.)

Samples were taken at specific time intervals and plated out in serial dilutions on Difco™ m Enterococcus agar (BD, Sparks, MD, USA) plates and on modified Homohiochhi-medium agar plates (Krieger, 1989). The Homohiochhi-medium agar plates were incubated at 30°C in an anaerobic chamber for 24 - 72 hours and the Enterococcus agar plates at 37°C for 24 h. All experiments were carried out in triplicate.

3.2.4 DNA ISOLATION AND TRANSFORMATION PROCEDURES

Preparation of bacterial DNA was carried out with FTA Cards (Whatman®, Merck, Johannesburg, South Africa) for PCR analyses. The manufacturer's recommendations were modified as follows: 1 mL of an overnight culture was centrifuged, washed with water and resuspended in 20 µL of water. 5 µL of sample were applied onto the FTA card, and dried for at least one hour at room temperature. Using a Uni-Core Punch (1.2 mm) a sample disc was taken from the desired sample spot. The sample disc was placed in a PCR amplification tube. 200 µL of FTA Purification Reagent were added to the PCR tube and incubated for 5 min at room temperature with a moderate manual mixing. All FTA Purification Reagent was discarded, 200 µL of 1x TE-Buffer (pH 7.5) added and incubated for 5 min at room temperature without shaking. The TE-Buffer was then discarded and the disc dried at 56°C for 10 min. At this stage, the FTA disc was ready for use in PCR reactions.

Total bacterial genomic DNA was isolated as described by Lewington, Greenaway and Spillane (1987).

Plasmid DNA from *E. coli* DH5α was extracted with the QIAprep®Spin Miniprep Kit (Qiagen, Southern Cross, Cape Town, South Africa) according to the manufacturer's recommendations.

Transformation of *Lb. plantarum* was carried out by electroporation as follows: (i) between 100 and 200 ng of plasmid DNA was added to 50 µL of freshly prepared competent cells and transferred to a pre-chilled Gene Pulser disposable cuvette (interelectrode distance 0.2 cm); (ii) electroporation was done at 1250 V (time constant: 5 ms), 201 Ω and 25 µF; (iii) immediately after electroporation 1 mL of pre-warmed MRSSM (MRS, 0.5 M sucrose, 0.1 M $MgCl_2$) was added and incubated for 3 h at 30°C; (iiii) the cells were then plated on selective MRS agar plates containing 25 µg/mL ampicillin and incubated for 48 h. Electrocompetent cells of *Lb. plantarum* were prepared as follows:

100 mL of growth medium (MRS, 1 % glycine, 40 mM DL-Threonine) were inoculated with 2 mL of an overnight culture of *Lb. plantarum* and incubated at 30°C for 3-4 hours to an OD_{600nm} = 0.4. The cells were then pelleted by centrifugation at 5000 rpm for 10 min at 4°C; washed twice in 100 mL ice cold SM buffer (952 mM sucrose, 3.5 M $MgCl_2$) and finally resuspended in 1 mL of the same solution, aliquoted into pre-chilled 1.5 mL eppendorf tubes and stored on ice (no longer than 4 hours) until use.

3.2.5 PCR AMPLIFICATION AND DNA MANIPULATIONS

In all experiments, DNA was amplified in 50 µL reaction mixtures containing 10 Extaq Buffer (TaKaRa, Bio Inc., Shiga, Japan), 10 mM of each deoxynucleoside triphosphates (dNTPs), 20 mM of each of the primers, and 1 U of Extaq polymerase (TaKaRa). As template either 50 ng of the genomic DNA, a FTA disc or 100 ng of plasmid DNA was used. PCR were performed in the TRIO-Thermoblock (Biometra, Göttingen, Germany). PCR resulting fragments were separated by agarose gel electrophoresis, and when required purified with the QIAquick® Gel Extraction Kit (Qiagen, Southern Cross Biotechnology, Cape Town, South Africa) according to the protocol supplied by the manufacturer.
All cloning steps were conducted according to standard procedures as described in Sambrook and Russell (2001). T4 DNA ligase and all restriction enzymes were purchased from Roche (Mannheim, Germany). Primer were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA).

3.2.5.1 Screening of *Lb. plantarum*

Twenty chosen *Lb. plantarum* strains were screened for known structural genes of plantaricin by PCR amplification using the FTA card system. Specific bacteriocin PCR primers shown in **Table 3.2** were used. The PCR conditions described by Rojo-Bezares et al. (2007a) were modified to 35 cycles of denaturation, annealing and polymerization. In addition, the PCR amplification of the malolactic enzyme gene (MLE) was used as a positive PCR control. The following PCR program was used: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and polymerization at 72°C for 90 s. A final elongation step was performed at 72°C for 5 min.

3.2.5.2 Amplification of *nisA* for heterologous expression in *Lb. plantarum*

Purified genomic DNA of *Lact. lactis* subsp *lactis* DSM 20729 served as template to amplify Nisin A (encoded by *nisA*). To allow subsequent cloning, *Bam*HI and *Sal*I sites were introduced at the 5' and 3' ends of the *nisA* primers. Amplification included denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing (temperature in **Table 3.2**) for 30 s, and polymerization at 72°C for 1 min. An extra final elongation step was performed at 72°C for 5 min.

The amplified PCR product for Nisin A was cloned into the pGEM-T easy vector (Plasmid pGEM-T Easy System I, Promega, Cape Town, South Africa).

The *nisA* gene was then digested with the appropriate enzymes and subcloned into the pBR322 plasmid (Bolivar, Rodriguez, Greene, Betlach, Heyneker and Boyer, 1977), at the *Bam*HI and *Sal*I restriction enzyme sites. The resulting plasmid, designated pBRNisA, was used to transform *E. coli* DH5α cells (see **Table 3.3**). Plasmid DNA was then purified with the QIAprep® Spin Miniprep Kit, using the protocol supplied by the manufacturer and used for electroporation into *Lb. plantarum* strains R1122 and 116.4.

3.2.5.3 Amplification of putative bacteriocin genes in *O. oeni*

The web server BAGEL was used to design primers to assist in the identification of putative bacteriocin genes in *O. oeni* strains (see **Table 3.2**).

Purified genomic DNA of *O. oeni* served as template in the PCR reactions. Amplification included denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing temperature in **Table 3.2** for 30 s, and elongation at 72°C for 30 s. A final elongation step was performed at 72°C for 5 min.

In addition, as positive controls for the heterologous expression study, primers were designed for nisin A and pediocin without their native promoters (see **Table 3.2**). pSRQ220 (Marugg et al., 1992) containing the 5.6-kbp *Sal*I-*Eco*RI – pediocin PA-1 expression fragment, served as template to amplify *peda*, *pedb*, *pedc*, *pedd*. Purified genomic DNA of *Lact. lactis* subsp *lactis* DSM 20729 served as template to amplify *nisA*. Amplification included denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing (temperatures in **Table 3.2**) for 30 s, and polymerization at 72°C for 1 min for *nisA*, and 3 min 40 s for pediocin. An extra final elongation step was performed at 72°C for 5 min.

Table 3.2
Primers used in this study

PCR purpose	Species/ plasmid	Designation	Sequence (5' - 3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
Bacteriocin identification						
	Lb. plantarum	*plnA* - F	GTACAGTACTAATGGGAG	53	450	Remiger et al. (1996)
		plnA - R	CTTACGCCATCTATACG			
		plnEF - F	GGCATAGTTAAAATTCCCCCC	53.2	428	Rojo-Bezares et al. (2007a)
		plnEF - R	CAGGTTGCCGCAAAAAAAG			
		plnEF - 2R	ACATTGGTATTTGACGTTAAGAGAACG			This study
		plnJ - F	TAACGACGGATTGCTCTG	51	475	Rojo-Bezares et al. (2007a)
		plnJ - R	AATCAAGGAATTATCACATTAGTC			
		plnK - F	AATCGCAGTGACTTCCAGAAC	53.7	469	Rojo-Bezares et al. (2007a)
		plnK - R	AGAGCAATCCGTCGTTAATAAATG			
		plnC8 - F	GGTCTGCGTATAAGCATCGC	60	207	Maldonado et al. (2003)
		plnC8 - R	AAATTGAACATATGGGTGCTTTAAATTCC			
		plS - F	GCCTTACCAGCGTAATGCCC	60	320	Stephens et al. (1998)
		plS - R	CTGGTGATGCAATCGTTAGTTT			
		plW - F	TCACACGAAATATTCCA	55	165	Holo et al. (2001)
		plW - R	GGCAAGCGTAAGAAATAAATGAG			
	O. oeni	OEOE 0110 - F	CATATGTCTAATAAAAATTTAACTGGT AAGTC (*Nde* I)	47	174	This study
		OEOE 0110 - R	GGATCCTCATTTTCCATTAATTTT (*Bam* HI)			
		OEOE 0116 - F	CATATGGATTATCAATTAAATACAA TCG (*Nde* I)	47	177	This study
		OEOE 0116 - R	GGATCCCTAGTCTTTAATAACTTTATTAAG (*Bam* HI)			
		OEOE 0306 - F	CATATGGTAGATAAAAATAAAAAGATAATTA TTAATC (*Nde* I)	53	243	This study
		OEOE 0306 - R	GGATCCTTAGTGGTAGCGATGCTC (*Bam* HI)			
		OEOE 1548 - F	CATATGCCAGAAGCATTACAGAATATTAAA GATAATT (*Nde* I)	50	252	This study
		OEOE 1548 - R	GGATCCTTAGGCGGTTTTGACTTGT (*Bam* HI)			
	Lact. lactis	NisA-Nde - F	CATATGAGTACAAAAGATTTTAACTTGGATT TGG (*Nde* I)	55	175	This study
		NisA-BamH - R	GGATCCTTATTTACTTACGTGAATACTACAAT GACAAG (*Bam* HI)			
		NisA-Bam - F	GATGGATCCTAGGCACGTTCGGCAGTAAC (*Bam* HI)	47	1010	This study
		NisA-SalI - R	GCCGTCGACTTCAGTAAAACTCCGTTTATCG (*Sal* I)			
	pSRQ220	Ped-Nde-F	CATATGAAAAAAATTGAAAAATTAACTGAAA AAGAAATGG *(Nde* I*)*	55	3395	This study
		Ped-Nde-R	GGATCCTCAGGCTATTCTTGATTATGAATTA ACC *(Bam* HI*)*			
PCR control						
	Lb. plantarum	MLE 1	GCGATGACAAAAACTGCAAGTGA	53	1644	Mtshali (2007)
		MLE 2	CTATTTGCTGATGGCCCGGTA			
Species Identification						
	Lb. plantarum	planF	CCGTTTATGCGGAACACCTA	56	318	Torriani et al. (2001)
		pREV	TCGGGATTACCAAACATCAC			
	Lb. paracasei	Y2	CCCACTGCTGCCTCCCGTAGGAGT	45	290	Ward and Timmins (1999)
		para	CACCGAGATTCAACATGG			
	Lb. hilgardii	H2	ACTNATTTGACATTAAGA	40	2300 + ~ 1000	Sohier et al. (1999)
		8623	CTGGTTCACTATCGGTCTC			
		pA	AGAGTTTGATCCTGGCTCAG	56	1500	Edwards et al. (1989)
		pH	TAACACATGCAAGTCGAACG			

Underlined fragments indicate introduced restriction sites and the corresponding enzyme is mentioned in brackets after the sequences.

The corresponding amplified products were cloned into the pGEM-T Easy vector and transformed into *E. coli* DH5α. The resulting plasmids, were then digested with *Bam*HI and *Nde*I and subcloned into pET-14b (Studier, Rosenberg, Dunn, Dubendorff and David, 1990). These plasmids (**Table 3.3**) were transformed into *E. coli* DH5α, JM109, BL21-DE3, BL21-DE3(pLysS) and Rosetta-gami (DE3)pLysS (**Table 3.4**). The *E. coli* transformants were then assayed for bacteriocin activity.

3.2.5.4 Genus and species identification

To identify the bacteriocin-producing isolated LAB strains, various species-specific PCR primers listed in **Table 3.2** were used. PCR were performed using the primers and conditions previously described (Rodas, Ferrer and Pardo, 2003; Sohier, Coulon and Lonvaud-Funel, 1999; Torriani, Felis and Dellaglio, 2001; Ward and Timmins, 1999) were applied. For the amplified fragments of the primers pA and pH, a restriction digest was carried out overnight with the enzymes *Bfa*I and *Mse*I (Rodas et al., 2003). Restriction patterns were analysed in a 2% agarose gel.

Table 3.3
Plasmids used in this study

Plasmid	Size (kb)	Description	References
pSRQ220	9.29	pBR322 containing the 5.6-kb *Sal*I-*EcoR*I pediocin PA-1 fragment from *P. acidilactici* PAC 1.0; Ap^r Tc^r	Marugg et al. (1992)
pBR322	4.36	Ap^r Tc^r	Bolivar et al. (1977)
pET-14b	4.67	T7 promoter; Ap^r	Studier et al. (1990)
pGEM-T Easy	3.02	*lac* Z; Ap^r	Promega
GeneJet	3.13	Ap^r	Inqaba Biotechnical Industries (PTY) LTD
pBRNisA	5.08	pBR322 containing the 1,01-kb *nisA* fragment from *L. lactis* DSM 20729 including the native promoter; Ap^r	This study
pOE10	4.84	pET-14b containing the 174-bp OEOE0110 fragment from *O. oeni* R 1118 including the T7 promoter; Ap^r	This study
pOE16	4.84	pET-14b containing the 177-bp OEOE0116 fragment from *O. oeni* R 1123 including the T7 promoter; Ap^r	This study
pOE15	4.91	pET-14b containing the 252-bp OEOE1548 fragment from *O. oeni* R 1123 including the T7 promoter; Ap^r	This study
pOE30	4.91	pET-14b containing the 243-bp OEOE0306 fragment from *O. oeni* R 1123 including the T7 promoter; Ap^r	This study
pNisA	4.84	pET-14b containing the 175-bp NisA from *L. lactis* 20729 DSM fragment with the T7 promoter; Ap^r	This study
pPed	8.05	pET-14b containing the 3.39-kb PA-1 pediocin fragment from *P. acidilactici* PAC 1.0 including the T7 promoter; Ap^r	This study

Ap, ampillicin; r, resistance; Tc, tetracycline.

Table 3.4
Escherichia coli strains used for cloning in this study

Species	Strain	Genotype	Source
E. coli	DH5α	*End A1 hsd r17* ($r^- m_k^+$) *sup E44 thi-1λ⁻ recA1 gyrA relAΔ (lacZYA- arg F)* U169 (*ψ80lacZΔM15)*	Gibco BRL, Life Technologies, Rockville, MD
E. coli	BL21-(DE3)	*F⁻ ompT hsdSB(rB⁻ mB⁻) gal dcm* (DE3)	Novagen (Merck, Darmstadt, Germany)
E. coli	BL21-(DE3)pLysS	*F⁻ ompT hsdSB(rB⁻ mB⁻) gal dcm* (DE3) pLysS (CM^R)	Novagen (Merck, Darmstadt, Germany)
E. coli	Rosetta-gami (DE3)pLysS	Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL (DE3) F'[lac^+ $lacI^q$ pro] gor522::	Novagen (Merck, Darmstadt, Germany)
E. coli	JM109	*F´ traD36 proA⁺B⁺ lacI^q Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14⁻ gyrA96 recA1 relA1 endA1 thi hsdR17*	Gibco BRL, Life Technologies, Rockville, MD

3.2.7 DNA SEQUENCING

The QIAquick® Gel Extraction Kit was used to purify amplified DNA from agarose gels. The DNA fragments were cloned into the pGEM-T Easy (Promega) or GeneJET (GeneJet™ PCR Cloning Kit, Inqaba Biotechnical Industries (PTY) LTD, Pretoria, South Africa) vectors. DNA sequencing was performed by the Central Analytical Facility of Stellenbosch University, South Africa.

3.2.8 HETEROLOGOUS EXPRESSION STUDY OF *E. coli*

The *E. coli* DH5α, JM109, BL21-DE3, BL21-DE3(pLysS) and Rosetta-gami (DE3)pLysS transformants referred to in 3.2.5.3 were grown overnight in 5 mL Luria-Bertani (LB) broth supplemented with the required amounts of antibiotics. 50 mL LB broth containing 1 mM of IPTG and antibiotics were then inoculated with 1 mL overnight culture and incubated at 37°C for 2h with vigorous agitation. 10 µL of the cultures were then spotted onto LB agar plates supplemented with 1 mM, 2 mM and 4 mM of IPTG, respectively. The plates were incubated at 37°C for 7h and overlaid with the indicator strain *P. pentosaceus* NCDO 813. The plates were further incubated at 37°C for 18h under anaerobic conditions. Growth inhibition was detected by a zone of clearing around the producer strain.

3.2.9 PROTEIN EXTRACTION AND EVALUATION

The Rosetta-gami transformants referred to in 3.2.5.3 were grown in 400 ml of LB broth supplemented with the required antibiotics to an OD_{600nm} of 0.6 at 37°C with vigorous agitation. The cultures were then induced with 1 mM IPTG and grown for another 3-5 hours. The cells were harvested (5500 rpm, 4°C, 10 min) and resuspended in 5 mL 0.2 M sodium-phosphate-buffer. The cells were then disrupted by three cycles of freezing for 3 min and thawing for 5 min. Once the cells were disrupted, 5 mL of $MgCl_2$ (5 mM final concentration) and 1 µL DNAse (Roche; 10-50 x 10^3 U/mL) were added. The suspension was incubated at 37°C for 20 min, centrifuged at 13000 rpm for 3 min and the obtained supernatant was filtered through a 0.22-µm-pore size filter before spotting it onto an indicator lawn.

3.3. RESULTS

3.3.1 DETECTION OF ANTIMICROBIAL ACTIVITY

A total of 330 LAB isolated from musts and wines originating from the Stellenbosch wine region (South Africa) and nine commercial malolactic starter cultures (eight *O. oeni* strains and one *Lb. plantarum* strain) were evaluated for the production of bacteriocins or antimicrobial peptides. Seventeen LAB and two non-LAB species were used as indicator organisms for antimicrobial activity. A wide range of LAB of oenological origin was used as indicators to detect growth inhibitory effects of oenological importance. The results are shown in **Table 3.5**, which only represents strains with antagonistic activity. From the 339 screened bacteria strains, the 9 commercial bacteria strains, 17 isolates showed antimicrobial activity against the various tested indicator organisms in a 'colony-overlay' test. Examples of antimicrobial activity detected by a clearing zone are shown in **Fig. 3.1**. The 17 isolates were identified by species specific PCR as *Lb. plantarum*, *Lb. paracasei* and *Lb. hilgardii* (see 3.3.3.1). The *Lb. hilgardii* isolates were the only ones not displaying any antibacterial activity towards *P. acidilactici* 118. All the other isolates were active against all the indicators tested.

From all the positive strains that showed antimicrobial activity, only ten *Lb. plantarum* and eight *O. oeni* strains were chosen for further investigations in order to reveal the bacteriocin structural genes and to consider novel bacteriocin-producing strains for use in wine.

3.3.3.1 Identification of isolates with antimicrobial activity

Molecular methods based on DNA amplification were used to identify the unknown strains showing antimicrobial activity. Species-specific primers were used in PCR reactions using conditions as already described by other authors (Edwards, Rogall, Blocker, Emde and Bottger, 1989; Sohier et al., 1999; Torriani et al., 2001; Ward et al., 1999).

The amplification of *Lb. hilgardii*'s 16S and 23S rRNA genes was performed using primers H2 and 8623. PCR yielded two bands of about 2300 and 1000 bp for isolates 21.12, 77.8, 71.12, 28.5, 24.1 and 16.5 (results not shown). When the primers pA and pH were used, fragments of approximately 1500 bp corresponding to the 16S-rRNA genes were obtained for the same isolates. Restriction analysis of the amplified 16S-rRNA with *Bfa*I and *Mse*I showed the expected profiles for *Lb. hilgardii* (Sohier et al., 1999). Primers

specific for the V1 region of the 16S-rRNA gene of *Lb. paracasei* enabled amplification of a 290 bp fragment in strains 20.4 and 74.9. Using species-specific primers for *Lb. plantarum*, a 318 bp amplicon was amplified for strains 81.9 and 19.11.

Table 3.5
Antimicrobial activity of representative isolates

Bacteriocin producing strain	Indicator microorganisms																							
	Lb. hilgardii 51	*P. pentosaceus* NCDO 813	*O. oeni 48*	*Lb. plantarum* 116.4	*O. oeni* R1098	*O. oeni* R1101	*O. oeni* R1123	*O. oeni* R1105	*O. oeni* R1106	*O. oeni* R1108	*O. oeni* R1118	*O. oeni* R1124	*Lb. brevis* 111	*Lb. paracasei* 105.8	*Lb. pentosus* 42	*Lb. fermentum* ATCC 9328	*Lc. mesenteroides* 5	*P. acidilactici* 118	*P. damnosus* NCFB 1832	*Listeria monocygenes* 127	*Ent. faecalis* LMG 13566
Lb. plantarum																								
R1122[b]	+++	+	+	+	+	+	+	+	+	+	+	+	‡	+	+	+	‡	+	+	+	+	+	+	+++
45.3[a]	+++	+	‡	+	+	+	+	+	+	+	+	+	‡	+	+	+	‡	+	+	+	+	+	+	+++
3.5[a]	+++	+	‡	+	+	+	+	+	+	+	+	+	‡	+	+	+	‡	+	+	+	+	+	+	+++
67.5[a]	+++	+	+	+	+	+	+	‡	+	+	+	+	‡	+	+	+	‡	+	+	+	+	+	+	+++
85.2[a]	+++	+	+	+	+	+	+	+	+	+	+	+	‡	+	+	+	‡	+	+	+	+	+	+	+++
81.9[a], 19.11[a], 71.10[a]21.11[a], 65.6[a]	+++	++	++	+	ND	ND	ND	ND	ND	ND	ND	ND	+++	+++	‡	+	+++	+	+	++	+++	+	+	+++
O. oeni																								
R1098[b]	++	++	+++	‡	ND	ND	ND	ND	ND	ND	ND	ND	++	++	‡	++	‡	++	++	++	++	++	++	+++
R1101[b]	++	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	++	++	‡	++	‡	++	++	++	++	++	++	+++
R1124[b]	++	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	++	+++	‡	++	‡	++	++	++	++	++	++	+++
R1105[b]	++	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	++	++	‡	++	‡	++	++	++	++	++	++	+++
R1106[b]	++	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	++	++	‡	++	‡	++	++	++	++	++	++	+++
R1108[b]	++	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	++	++	‡	++	‡	++	++	++	++	++	++	+++
R1118[b]	++	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	++	+++	‡	++	‡	++	++	++	++	++	++	+++
R1123[b]	+	++	++	‡	ND	ND	ND	ND	ND	ND	ND	ND	++	++	‡	++	‡	++	++	++	++	++	++	+++
Lb. paracasei																								
20.4[a]	+++	++	++	+	ND	ND	ND	ND	ND	ND	ND	ND	+++	+++	‡	+	+++	+	+	+++	+++	+	+	+++
74.9[a]	+++	++	++	+	ND	ND	ND	ND	ND	ND	ND	ND	+++	+++	‡	+	+++	+	+	+++	+++	+	+	+++
Lb. hilgardii																								
21.12[a]	-	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	+++	++	+	+	++	+	-	+++	+++	-	+	+++
77.8[a]	-	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	+++	++	+	+	++	+	-	+++	+++	-	+	+++
71.12[a]	+	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	+++	++	+	+	++	+	-	+++	+++	-	+	+++
28.5[a]	+	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	+++	++	+	+	++	+	-	+++	+++	-	+	+++
24.1[a]	+	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	+++	++	-	+	++	-	-	+++	+++	-	+	+++
16.5[a]	+	++	+	+	ND	ND	ND	ND	ND	ND	ND	ND	+++	++	-	+	++	-	-	+++	+++	-	+	+++

All indicator strains were tested for inhibition of growth as determined by a zone of growth inhibition in the indicator lawn culture. Inhibiton of growth was expressed as a zone >5 mm inhibition zone (+++); 3-4mm inhibition zone (++); 1-2mm inhibition zone (+); no inhibition (-); not determined (ND).

[a] Strains from the IWBT.

[b] Commercial starter cultures.

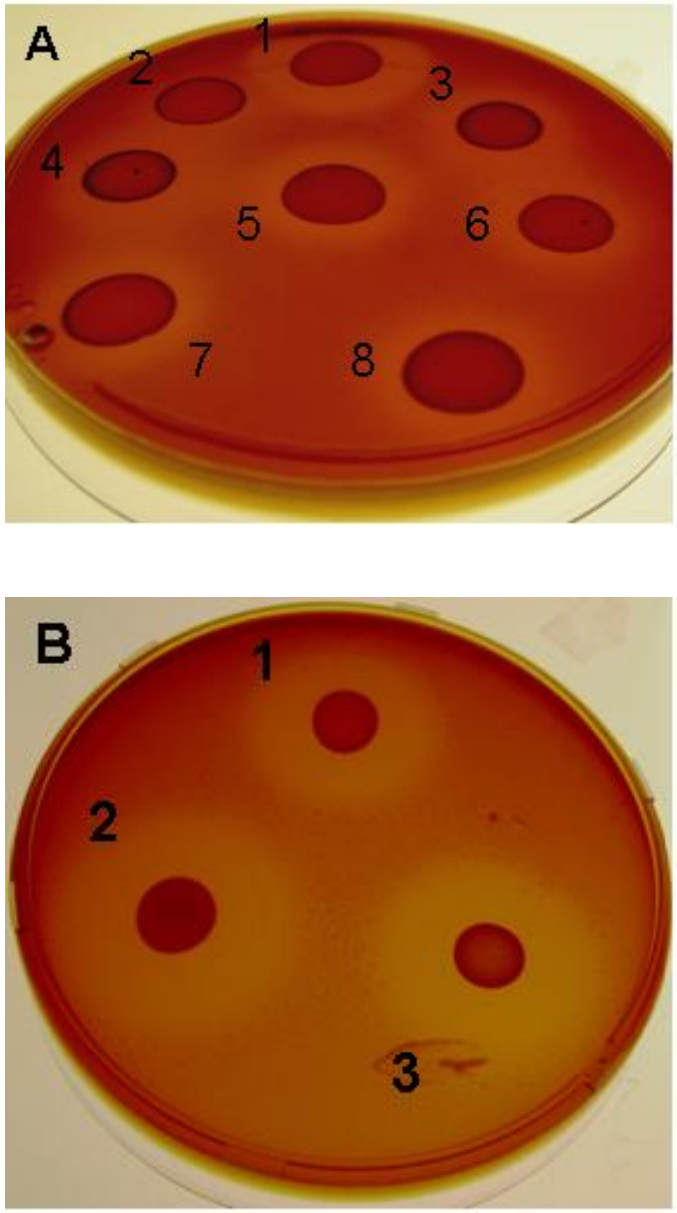

Fig. 3.1. Examples of LAB showing antimicrobial activity with the 'Colony-overlay' plate assay. (A) Bacteriocin-producing *Lb. plantarum* strains 67.5 (1), 81.9 (2), 19.11 (3), 21.11 (4), 65.6 (5), 71.10 (6), *Lb. paracasei* strains 20.4 (7) and 74.9 (8). *P. acidilactici* 118 was used as indicator organism; (B) *O. oeni* strains R1118 (1), R1101 (2) and R1124 (3) showing antimicrobial activity against *Lb. paracasei* 105.8.

3.3.2 EVALUATION OF *Lb. plantarum* R1122 FOR THE PRODUCTION OF BACTERIOCIN IN LIQUID MEDIA

The capability of *Lb. plantarum* R1122 to inhibit the growth of *Ent. faecalis* in liquid medium was tested in enriched MRS broth. The results are shown in **Fig. 3.2**. In order to be able to distinguish between the two strains, the culture samples were plated onto Enterococcus agar and modified Homohiochhi-medium agar. *Ent. faecalis* could be detected after 24 h on both media and displayed a dark red colour on the Enterococcus agar. The growth of *Ent. faecalis* and *Lb. plantarum* in single and in co-culture was monitored for 72 h. As shown in **Fig. 3.2**, *Ent. faecalis* on its own was able to grow from 10^7 CFU/mL to 10^9 CFU/mL within the monitored 72 h. In the co-culture different trends in the growth of *Ent. faecalis* were obtained. The cell growth reached 10^7 after 9 hours and remained constant for the first 48 h. After 48 h the growth started to drop to 10^5 CFU/mL and went further down to less than 10 CFU/mL between 54 h and 72 h. Meanwhile, *Lb. plantarum* was able to reach cell numbers of 10^9 after 9 hours in both single and co-cultures and remained constant for the rest of the monitored time.

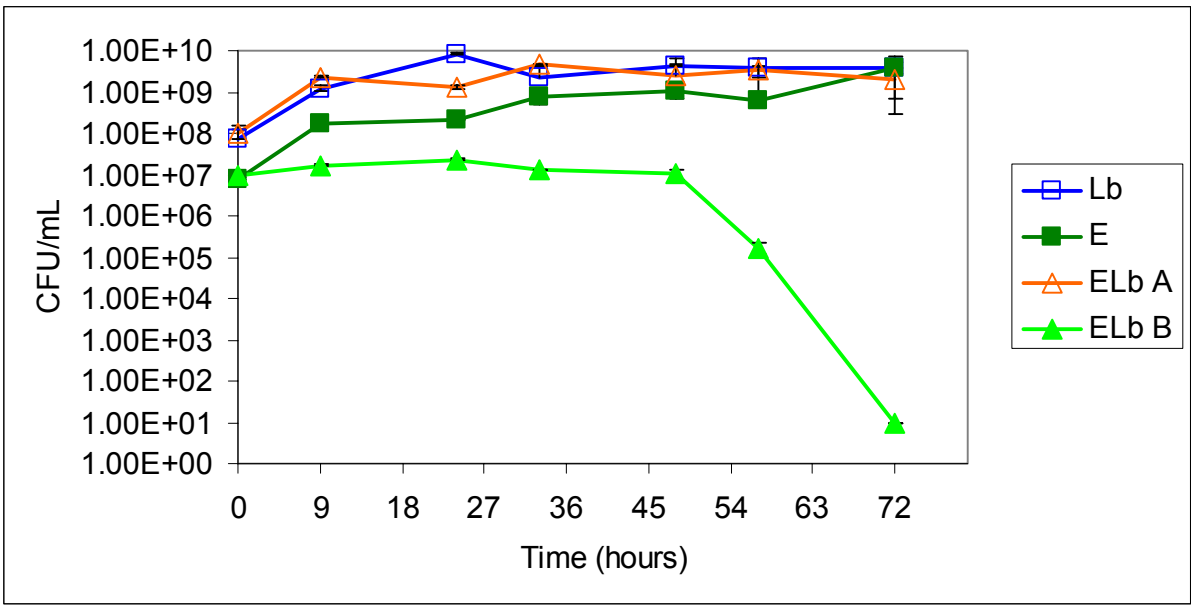

Fig. 3.2. Evaluation of bacteriocin production by *Lb. plantarum* R1122 in liquid medium in co-culture with *Ent. faecalis*. Lb (□), *Lb. plantarum* culture plated onto modified Homohiochhi-medium; E (■), *Ent. faecalis* culture plated onto Enterococcus agar; ELb A (Δ), mixed culture plated onto modified Homohiochhi-medium; ELb B (▲), mixed culture plated onto Enterococcus agar; only *Ent. faecalis* was enumerated. Error bars indicate standard deviation.

3.3.3 PCR DETECTION OF BACTERIOCIN STRUCTURAL GENES AND SEQUENCING

3.3.3.1 Screening of *Lb. plantarum*

PCR amplification was chosen to determine the existence of structural genes encoding described plantaricins and also to investigate the similarity of plantaricin genes between the different strains. In addition to the ten *Lb. plantarum* strains referred to in 3.3.1, ten *Lb. plantarum* strains were randomly chosen out of the IWBT culture collection.

The presence of the following genes related with bacteriocin production and regulation in the 20 selected *Lb. plantarum* strains was determined by colony PCR using specific primers described in the literature: *plnA*, *plnEF, plnJ, plnK*, and *plNC8* (Maldonado, Jimenez-Diaz and Ruiz-Barba, 2004; Remiger, Ehrmann and Vogel, 1996). Furthermore, the presence of *plS* and *plW* genes was investigated (Holo et al., 2001; Stephens et al., 1998). Results are shown in **Table 3.6**. PCR analysis rendered no results for the genes *plW* and *plNC8*. The strains R1122, 45.3, 3.5, 67.5 and 85.2 were chosen for further investigations and PCR on their genomic DNA was done to confirm the results (**Fig. 3.3**).

In order to verify that the obtained PCR products of the latter five strains indeed correspond to the expected bacteriocin-encoding genes, the fragments were cloned and sequenced. Although the genes *plnE* and *plnF* (previously referred to as *plnEF*) were simultaneously amplified, their sequences were independently analysed. The intergenic region was not considered. Analysis of the obtained nucleotide sequences revealed 100 % identity within the sequences of genes *plnA* (from strains 45.3, 67.5, 3.5), *plnE* (from strains 45.3, 67.5, 3.5, 85.2, R1122) and *plnK* (from strains 45.3, 67.5, 3.5, R1122). The *plnJ* gene from strain 67.5 showed 99 % homology with the sequences obtained for strains R1122 and 3.5. The *plnF* gene from strains 45.3, 67.5 and R1122 were 98 % identical to the *plnF* sequences from strains 3.5 and 85.2. The sequences were then translated *in silico* into amino acids and compared with reference strains to determine whether the sequences vary in the five strains tested. The reference strains were: *Lb. plantarum* WCFS1 (accession number NC004567), *Lb. plantarum* C11 (accession number X94434) and *Lb. plantarum* NC8 (accession number AF522077.2). The sequences for plnAp were 100 % identical to the plnAp peptide from the plantaricin locus of *Lb. plantarum* C11 and WCFS1 (**Fig. 3.4 A**). While the sequence for plnJp from strains 3.5 and R1122 showed 100% identity with the sequences of *Lb. plantarum* WCFS1, *Lb. plantarum* C11 and *Lb. plantarum* NC8, strain 67.5 showed 99% identity. At position 14, aspartic acid is

substituted with glycine (**Fig. 3.4 B**). PlnKp from all strains showed 100% identity to plnKp of *Lb. plantarum* C11 and NC8 and were highly homologous to plnKp from *Lb. plantarum* WCFS1 (**Fig. 3.4 C**). The sequences for reference plnEp with the primer pair plnEF-F and plnEF-R were incomplete, because the primers did not amplify the whole plnEp gene. A second reverse primer (plnEF-2R) was designed to amplify the whole gene. The amplified regions with the primer pair plnEF-F and plnEF-2R were 100% identical with the reference strains (**Fig. 3.4 D**). The sequences for plnFp from strains 3.5 and 85.2 were identical to the plnFp peptide from all three reference strains. Differences were found in the plnFp sequences from strains 45.3, 67.5 and R1122. Strains 45.3 and 67.5 showed the amino acid, alanine, at position 14 instead of serine, and at position 42, valine was substituted with isoleucine. The differences for strain R1122 were obtained at position 13 and 15; asparagine was substituted with aspartic acid, and alanine was replaced with valine (**Fig. 3.4 E**). The sequence for plSp from R1122 did not show homology with plSp of any other reference strain, but was identical to a region displaying an integral membrane protein from *Lb. plantarum* WCFS1.

3.3.3.2 Amplification of putative bacteriocin genes in *O. oeni*

The genome of *O. oeni* PSU-1 was sequenced as part of a recent comparative-genomic project (Makarova et al., 2006). In the present study, the web server BAGEL (De Jong et al., 2006) was used to identify putative bacteriocin ORFs in the genome of *O. oeni* PSU-1, thus employing knowledge-based databases and motif databases. For the four most probable bacteriocin-encoding genes (OEOE0110, OEOE0116, OEOE0306, OEOE1548), primers were designed (see **Table 3.2**), to amplify the putative bacteriocin genes in the eight commercial *O. oeni* strains (see **Table 3.1**). PCR analysis rendered positive results with all four genes in all eight strains. The results obtained after gel electrophoresis are displayed in **Fig. 3.5**.

The PCR products were sequenced and showed 100% identity with the OEOE gene sequences of *O. oeni* PSU-1 deposited in GeneBank (accession number CP000411) (**Fig. 3.6**).

Table 3.6
PCR amplification of plantaricin genes from *Lb. plantarum* wine isolates

Lb. plantarum strain	Plantaricin gene				
	plnA	*plnEF*	*plnJ*	*plnK*	*plS* [b]
67.5[a]	+	+	+	+	
116.4					
85.2[a]		+			+
R1122[a]		+	+	+	+
65.1				+	
81.2		+			
130.1					
71.10		+			
74.9					
45.3[a]	+	+	+	+	
131.1		+			
81.9					
107.4					
131		+			
19.11	+	+	+	+	
120.1					
3.5[a]	+	+	+	+	
113.1		+	+	+	
21.11		+	+	+	
65.6			+	+	

(+) gene present, blank no presence of gene.
[a] Used for further studies.
[b] *plS* PCR analysis rendered correct amplification size, but sequenced product was incorrect.

3.3.4 PLASMID CONSTRUCTION AND HETEROLOGOUS EXPRESSION STUDY

3.3.4.1 Amplification of *nisA* and heterologous expression in *Lb. plantarum*

To achieve higher levels of bacteriocin production in *Lb. plantarum* a pBR322 vector was constructed containing the *nisA* gene and its native promoter from *Lact. lactis*. This plasmid, designated pBRNisA, was transformed via electroporation into two strains of *Lb. plantarum* (116.4, R1122) and heterologous expression studies were carried out with the 'colony-overlay' method. *Lb. hilgardii* and *P. pentosaceus* were used as sensitive indicator organisms. The transformed *Lb. plantarum* strains showed higher antibacterial activity than their untransformed parental strains (results not shown). However, when

freeze cultures of the transformed strains were inoculated again for further studies, their antibacterial activity was lost. This was observed despite the fact that the Lb. plantarum strains still harboured the plasmid, pBRNisA.

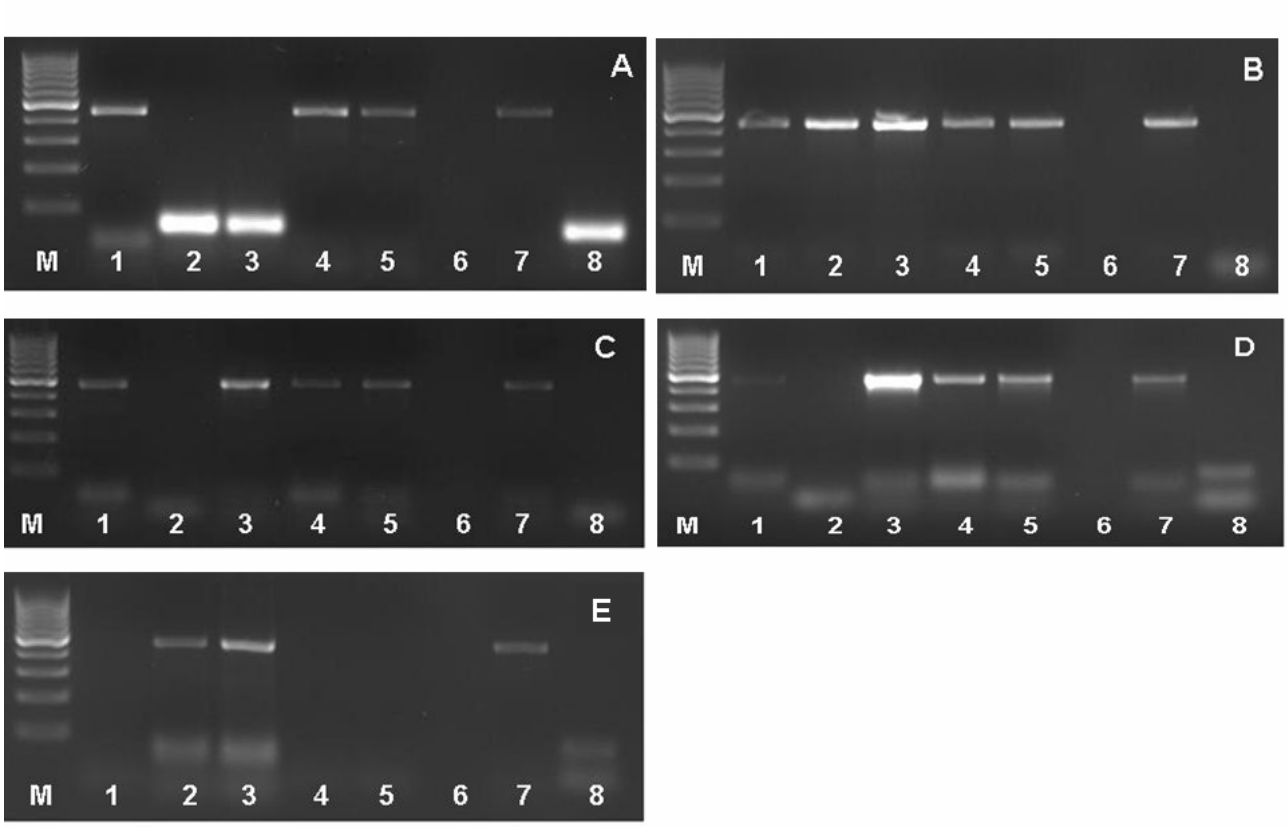

Fig. 3.3. PCR amplification fragments of purified DNA from *Lb. plantarum* strains 67.5, 85.2, R1122, 45.3, 3.5, using the primer pairs specific for *plnA* (A), *plnEF* (B), *plnJ* (C), *plnK* (D) and *plS* (E): lane M, molecular mass marker XIV; lane 1, strain 67.5; lane 2, strain 85.2; lane 3, strain R1122; lane 4, strain 45.3; lane 5, strain 3.5; lane 6, empty space; lane 7, positive control; lane 8, negative control.

3.3.4.2 *Heterologous expression study in E. coli*

In order to determine whether the genes OEOE0110, OEO0116, OEOE1548 and OEOE0306 are in fact encoding a bacteriocin gene, heterologous expression studies of the latter genes were performed in *E. coli*. For this purpose, the OEOE genes were fused to the T7 promoter in pET-14b, which was then transformed into *E. coli* strains DH5α, JM109, BL21-DE3, BL21-DE3 (pLysS) and Rosetta-gami (DE3)pLysS. None of the positive transformants showed antimicrobial activity when tested for bacteriocin production

against the indicator strains *Listeria monocytogenes* 127, *P. pentosaceus* NCDO 813 and *Lb. hilgardii* 51.

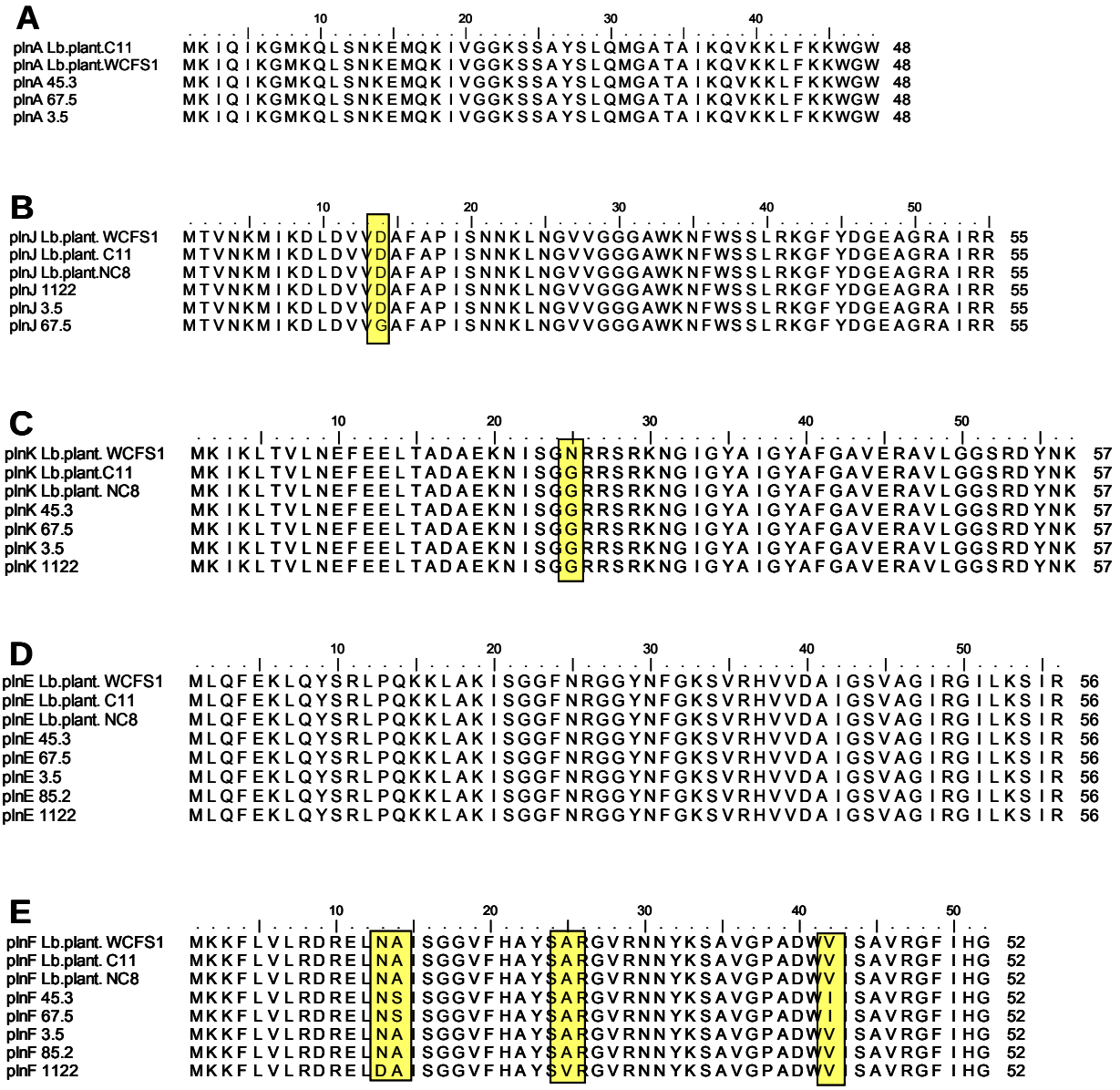

Fig. 3.4. Alignment of the amino acid sequences of the plantaricin encoding genes *plnA* (A), *plnJ* (B), *plnK* (C), *plnE* (D), *plnF* (E). The differences are highlighted in boxes.

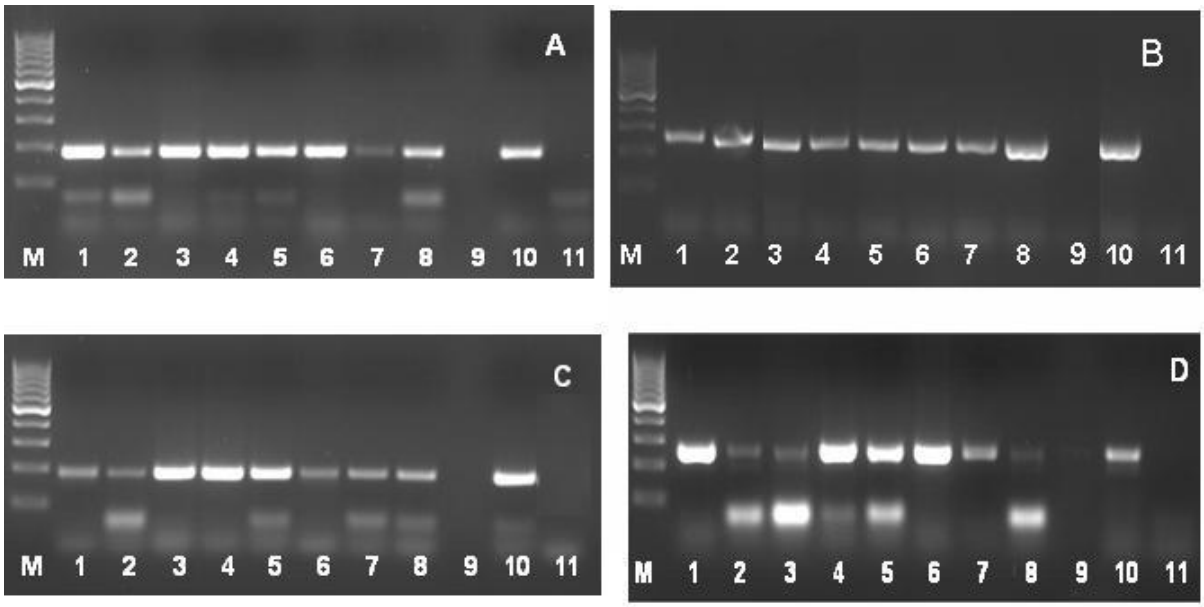

Fig. 3.5. PCR amplification fragments from *O. oeni* strains R 1098, R 1101, R1105, R1106, R 1108, R 1118, R 1123, R 1124, using the primer pairs specific for OEOE 0110 (A), OEOE 1548 (B), OEOE 0116 (C), OEOE 0306 (D): lane M, molecular mass marker XIV; lane 1, strain R 1123; lane 2, strain R 1124; lane 3, strain R 1101; lane 4, strain R 1105; lane 5, strain R 1106; lane 6, R 1108; lane 7, strain R 1118; lane 8, strain R 1098; lane 9, empty space; lane 10, positive control; lane 11, negative control.

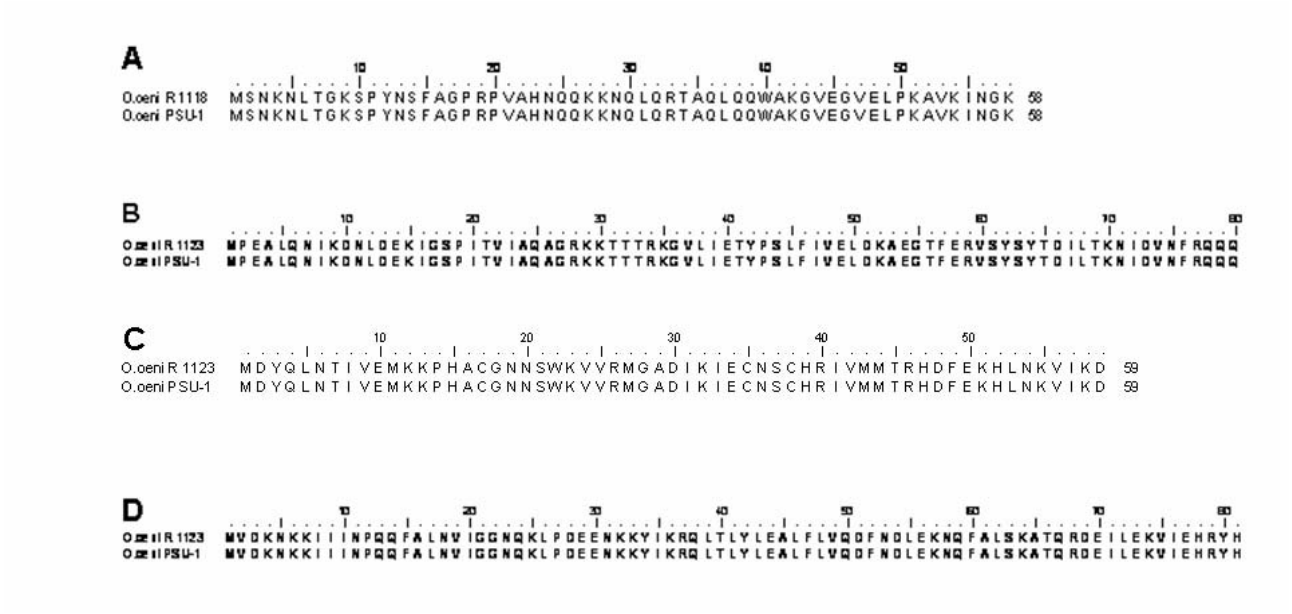

Fig. 3.6. Alignment of the amino acid sequences of the potential bacteriocin encoding genes OEOE 0110 (A), OEOE 1548 (B), OEOE 0116 (C), OEOE 0306 (D).

3.3.5 Protein extraction and evaluation

In order to determine whether the Rosetta-gami (DE3)pLysS transformants were producing an antimicrobial peptide, but not capable of secreting it, their cell walls were disrupted and the obtained supernatants evaluated for antimicrobial activity against the indicator organism *P. pentosaceus* NCDO 813 in a 'colony-overlay' test. None of the supernatants showed antimicrobial activity.

3.4. DISCUSSION

In this study the screening for antimicrobial activity of a wide variety of wine isolated LAB was performed in order to find novel bacteriocins or new LAB candidates which can be used as bacteriocinogenic cultures during the vinification process.

Eight percent (26 out of 330) of the screened LAB displayed antimicrobial activity. Among the 26 strains, *Lb. plantarum* comprised the largest lactobacilli species with antagonistic activity, followed by *Lb. hilgardii* and *Lb. paracasei*. Of all tested commercial starter cultures, including *O. oeni* and *Lb. plantarum*, all strains showed activity towards various, mostly wine-related, indicator strains. All strains showed similar inhibitory spectra against the full range of indicator organisms. Several *Lb. plantarum* and *Lb. paracasei* strains are known to produce bacteriocins (Diep, Havarstein and Nes, 1996; Maldonado et al., 2003; Pangsomboon, Kaewnopparat, Pitakpornpreecha and Srichana, 2006; Topisirovic, Kojic, Fira, Golic, Strahinic and Lozo, 2006), but no strains belonging to *Lb. hilgardii* and *O. oeni* have been reported to be bacteriocinogenic. Although numerous of the known bacteriocin-producing strains were isolated from wines, there is no information about bacteriocin production in wine available.

The use of bacteriocins as biopreservatives can be achieved either by adding the purified/semi-purified bacteriocin itself as a food additive, or by using a bacteriocin-producing culture as protective culture, or as starter culture (Deegan et al., 2006; Grande et al., 2007). The effectiveness of bacteriocins in food systems is highly dependent on various chemical and physical factors. Therefore, it is necessary to test the potential use of bacteriocins during the vinification process. It has been demonstrated that the addition of nisin to wine inhibits LAB (Daeschel et al., 1991; Radler, 1990a, 1990b). Moreover, a synergistic effect in combination with SO_2 has been reported (Rojo-Bezares et al., 2007b).

This study focused on the evaluation of bacteriocin production firstly in laboratory medium, secondly in synthetic wine medium and thirdly in wine or must. Hence, growth and production of plantaricin by *Lb. plantarum* R1122 was assayed in supplemented MRS broth and it was shown that the indicator bacterium, *Ent. faecalis*, was indeed inhibited within 72 hours. Nevertheless, the production of a certain bacteriocin in laboratory media does not ensure its effectiveness in a wine environment. When evaluating a bacteriocin-producing bacteria strain for wine fermentation, it is important to consider that wine is a complex and harsh medium with numerous factors influencing microbial growth and metabolite production. Cocolin, Foschino, Comi and Grazia Fortina (2007) observed that two strains of enterococci isolated from goats milk, produced a larger amount of bacteriocin in skim milk than in MRS medium during co-culture experiments. Additional to evaluating the production in different liquid media, it is necessary to test bacteriocin activity against potential realistic target bacteria from wine. However, the differentiation between closely related bacteria in mixed cultures displayed a major technical problem, because no selective media could discriminate between the different wine LAB genera on plates. Therefore, in this study *Ent. faecalis* was chosen as indicator organism, although it is not adapted for growth in a wine environment. Consequently, the production of plantaricin could not be tested in a wine model system. Other methods such as Real time PCR (Grattepanche, Lacroix, Audet and Lapointe, 2005; Neeley, Phister and Mills, 2005), DGGE (denaturing gradient gel electrophoresis) (Renouf, Claisse and Lonvaud-Funel, 2006), and TTGE (temporal temperature gradient gel electrophoresis) (Ogier, Son, Gruss, Tailliez and Delacroix-Buchet, 2002) could be considered to follow the survival of target bacteria in the presence of bacteriocin-producing bacteria in wine, while simultaneously determining the growth of the bacteriocinogenic strain at the same time. Fluorescence-based technology, such as fluorescence *in-situ* hybridisation (FISH), confocal laser microscopy and fluorescence ratio imaging microscopy (FRIM) could also be used to follow the microbial populations in wine (Fernandez de Palencia et al., 2004; Hornbaek, Brockhoff, Siegumfeldt and Budde, 2006).

In this work, 20 representative *Lb. plantarum* strains were evaluated for the presence of known genes encoding plantaricins. PCR amplification has shown that 14 out of 20 tested strains held either all or some of the genes *plnA*, *plnEF*, *plnJ* and *plnK*. No strain carrying plantaricin S, plantaricin W or *plNC8* were detected. *PlnA* encodes a peptide pheromone and is part of the regulatory operon (*plnABCD*). *PlnEF* and *plnJK* have been reported to encode two-peptide bacteriocins and to display complementary peptides

which are more active in combination than when present individually (Anderssen et al., 1998). Moreover, the production of several class II bacteriocins is regulated via the so-called Quorum-sensing, which is mediated by inducer peptides and has previously been described in *Lb. plantarum* C11 (Diep et al., 1996) and *Lb. plantarum* NC8 (Maldonado et al., 2003). This mechanism is regulated by three different proteins: the peptide pheromone, histidine protein kinase (HPK) and a response regulator (RR). Maldonado et al. (2003) recognized differences in the regulatory operon of *Lb. plantarum* NC8 compared to the one in *Lb. plantarum* C11 (**Fig. 3.7**). The regulatory operon of C11 consists of *plnABCD,* while the one of NC8 displays the genes *plnNC8IF*, *plnNC8HK* and *plnD*. Results obtained for strains R1122 and 85.2 did not show the presence of the *plnA* gene.

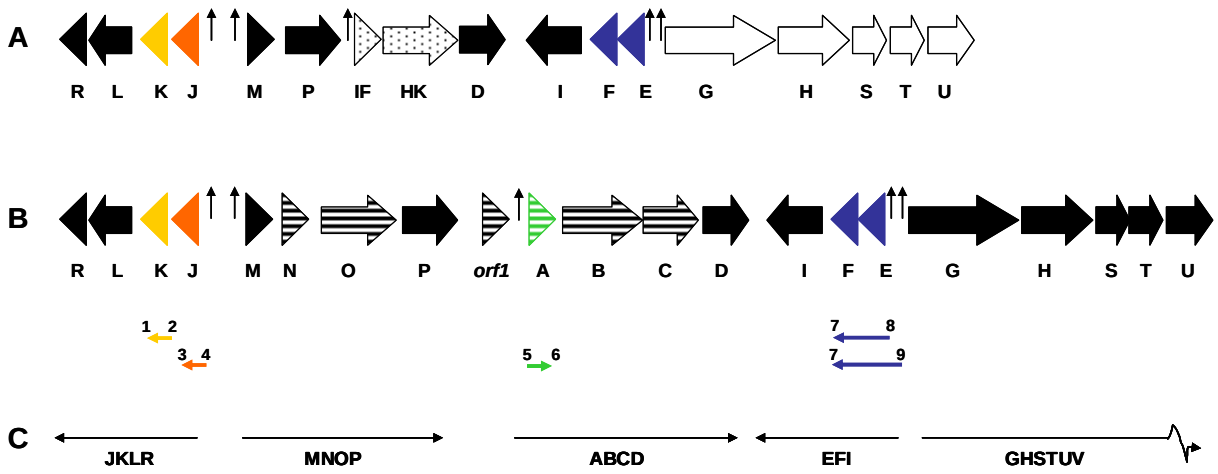

Fig. 3.7. Genetic map of the plantaricin gene cluster. Genetic map of the plantaricin gene cluster in *Lb. plantarum* NC8, showing the genes and ORFs that have been identified by Maldonado et al. (2003). Solid arrows, NC8 genes that share a high degree of homology with, or are virtually identical to genes found in *Lb. plantarum* C11. Dotted arrows, NC8 genes that have not been described elsewhere. Open arrows, genes that have been identified in NC8 by Maldonado et al.(2003) with specific PCR primers. Arrows pointing up, indicate the positions of putitative promoter sequences (A). Genetic map of plantaricin gene cluster in *Lb. plantarum* C11, showing the genes that have been described previously (Anderssen et al., 1998; Diep et al., 1996; 1994). Striped arrows, genes that have not been found in NC8. Numbered lines represent primers used in this study to amplify subsequently sequenced DNA fragments, as follows: 1, plnK-F; 2, plnK-R; 3, plnJ-F; 4, plnJ-R; 5, plnA-F; 6, plnA-R; 7, plnE-F; 8, plnE-R; 9, plnE-2R (B). 5 operons of the 20 ORFs in C11 (C).

To gain further insight on the regulatory region, additional molecular analysis is necessary. In this study, the identity of the PCR products obtained was confirmed by sequencing. Moreover, these sequences were translated *in silico* and aligned with published sequences of *Lb. plantarum* C11, *Lb. plantarum* NC8 and *Lb. plantarum* WCFS1. High homology of the genes was found in the five studied strains when compared

to the homologous genes in *Lb. plantarum* C11 and NC8. Maldonado et al. (2003) sequenced 13 ORFs belonging to the bacteriocin cluster of *Lb. plantarum* NC8 and compared them to the homologous ORFs in C11 (Anderssen et al., 1998; Diep et al., 1996; 1994). They showed that *plnA* function is "accomplished" by *plnNC8IF* and *plnB* by *plnNC8HK*. Furthermore, *plnC* was not found in NC8. However, a high percentage of homology was found between all the other homologous ORFs. This latter result was confirmed in the present study with the five *Lb. plantarum* strains. The only differences found for *plnJ* and *plnF* were a few amino acids. Since these peptides are short, it can be hypothesized that any difference may be relevant with regard to the corresponding activity. For *plnJ*, the only difference noticed concerned strain 67.5 where the Asp^{14} has been replaced by Gly^{14}, thereby changing a charged amino acid into a polar amino acid. For *plnF* more changes in the amino acid sequence have occurred. In strains 45.3 and 67.5 Ala^{14} has been replaced by Ser^{14}, thereby changing the amino acid from hydrophobic to polar. Moreover, Val^{42} was replaced by Ile^{42}, which are both hydrophobic amino acids. Two changes have also occurred for strain R 1122. Asn^{13} has been substituted by Asp^{13}, thereby changing a polar amino acid to a charged amino acid. Ala^{25} has been replaced by Val^{25}, which is another hydrophobic amino acid. For strain R 1122, a slightly weaker activity has been observed in the 'colony-overlay' assay (data not shown), which might confirm the influence of the above mentioned changes in the amino acid sequence. However, no significant differences in the activity of strains 45.3 and 67.5 have been noticed, despite the variation in their amino acid sequence. Nonetheless, a change of an amino acid might not influence the activity, but other properties of a bacteriocin. A change of one single amino acid in nisin Z resulted in a five-fold increase in the solubility of the peptide at pH 8, as well as an extension of its antimicrobial spectrum (Yuan, Zhang, Chen, Yang and Huan, 2004).

Nisin is a lantibiotic produced by *Lact. lactis* and belongs to the class I bacteriocins. In this study, heterologous expression of the structural *nisA* gene and its promoter was employed to enhance the antimicrobial activity of *Lb. plantarum* and to construct a multi-bacteriocinogenic strain. The constructed plasmid pBRNisA was successfully electroporated into two strains of *Lb. plantarum*. Although the transformants initially showed higher antimicrobial activity in the 'colony-overlay' test, the expression system was compromised after freeze cultures were made. First attempts in the construction of LAB strains with the capacity to co-produce two or more bacteriocins belonging to different classes have been reported previously. *Lact. lactis* strains with the ability to co-produce

nisin A and pediocin PA-1 were constructed by introducing the plasmid pFI2160 or a combination of the plasmids pFI2126 and pFI2148 into the nisin producing strain, *Lact. lactis* FI5876 (Dodd, Horn and Gasson, 1990; Horn et al., 1999). However, the choice of the expression and vector system is critical for the successful expression of the gene of interest. Moreover, for further applications of a possibly multi-bacteriocinogenic LAB strain in wine, a food-grade expression or integrative transformation system must be considered.

O. oeni is an important bacterium in the vinification process. It conducts MLF and contributes to the flavour profile of the wine. The eight commercial *O. oeni* starter cultures showed antimicrobial activity against all indicator strains tested, suggesting the secretion of a bacteriocin by these *O. oeni* strains. The web server BAGEL was used for *in silico* analyses of the *O. oeni* PSU-1 genome. Four putative bacteriocin-encoding genes were selected for heterologous expression studies in several *E. coli* strains, but no activity could be detected. The constructed vectors containing either one of the four putative bacteriocin-encoding genes might lack essential genes, such as the gene encoding for an accessory protein or an inducer peptide. These two genes have a relatively conserved genetic organization: the accessory gene is generally co-transcribed with and located immediately downstream of the ABC-transporter, gene while the inducer gene which is required for the activation of the two-component regulatory system, precedes the histidine protein kinase (HPK) (Diep et al., 1996). The *in silico* analyses of the genomic context of the four hypothetical proteins detected only an ABC-transporter eight ORF's downstream of OEOE1548 while the other putative bacteriocin genes had no ABC-transporter in their genomic context. A histidine kinase was found four ORF's downstream of the hypothetical protein OEOE0110 and four ORF's upstream of OEOE0116. Furthermore, the genes might contain mutations which could cause problems in the heterologous expression studies.

Previously it was shown that the secretion rate of the ABC-transporters depend to some extend on the amino acid sequence of the pre-sequence (Aucher, Lacombe, Héquet, Frère and Berjeaud, 2005). By site-directed mutagenesis, it was demonstrated that some point mutations in the pre-sequence could block the secretion completely. On the other hand, the chosen hosts and vector in this study might not be able to produce the potential bacteriocin. Several *E. coli* strains have been used for heterologous bacteriocin expression (Biet, Berjeaud, Worobo, Cenatempo and Fremaux, 1998; Bukhtiyarova, Yang and Ray, 1994; Coderre and Somkuti, 1999; Marugg et al., 1992). However, *E. coli* may

lack secretion mechanisms or heterologous proteins may be subject to degradation by cell proteases (Makrides, 1996). In this context, using other bacteria, such as LAB with either an ABC-dedicated transport system or a general secretory pathway, may help to overcome the problems. A recent data mining study reported that the genome of *P. pentosaceus* ATCC 25745 contains a gene cluster that was similar to a regulated bacteriocin system (Diep, Godager, Brede and Nes, 2006). The latter authors demonstrated that the putative bacteriocin operon can be heterologously expressed in a *Lb. sakei* host which contained the complete apparatus for gene activation, maturation and secretion. It was also shown that the bacteriocin operon was incomplete and *P. pentosaceus* ATCC 25745 was therefore a poor bacteriocin producer. However, this is the first report on the identification of the putative bacteriocin-encoding genes in different *O. oeni* wine isolates.

3.5. CONCLUSION

Different LAB species of oenological origins have the ability to produce bacteriocins. Yet, little is known about their impact on the bacterial ecology and interactions in the complex must and wine environment. Since the efficacy of bacteriocins in foods is dictated by environmental factors, it is necessary to determine more precisely the most effective conditions for the application of bacteriocins in wine or must.

However, given the effectiveness of bacteriocins against wine spoilage bacteria, they may represent an excellent option for use in combination with other preservatives and may lead to a reduction of total SO_2 in wine. Whether added or produced *in situ* by LAB, bacteriocins can play a beneficial role in controlling microbial flora during the vinification process.

Recent developments in molecular microbial ecology can help to better understand the overall effects of bacteriocins in a wine ecosystem. The progressing study of bacterial genomes may also lead to the discovery of new bacteriocins.

3.6 ACKNOWLEDGEMENTS

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Chapter 4

RESEARCH RESULTS

Influence of phenolic compounds on the activity of nisin and pediocin PA-1

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4. RESEARCH RESULTS

Influence of phenolic compounds on the activity of nisin and pediocin PA-1

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Abstract

Bacteriocins have previously been evaluated as biopreservatives in wine. However, in red wine, the phenolic compounds might have negative effects on the activity and stability of bacteriocins, and consequently limit the application of bacteriocins in red wine. In this study, the influence of phenolic compounds and polyphenols on the activity of nisin and pediocin PA-1 was investigated. No influence on stability and activity was detected. Furthermore, synergistic effects on bacterial growth inhibition were observed. Results showed that the combination of bacteriocin with the phenolic compounds or polyphenols decreased the bacterial cell numbers between 3 and 6 log units.

4.1 INTRODUCTION

Bacteriocins are bacterially produced antimicrobial peptides with narrow or broad host ranges (Klaenhammer 1988). Many bacteriocins are produced by food-grade lactic acid bacteria (LAB) which presents an opportunity to inhibit or prevent the development of specific bacterial species in beverages and food. They can be divided in three major classes, as detailed by Diep and Nes (2002). Nisin belongs to the lantibiotic group and is produced by some strains of *Lactococcus lactis* (De Vuyst 1994, Delves-Broughton 2005). It is one of the most industrially relevant bacteriocins and has been used for decades in many countries as a safe and effective food preservative (Delves-Broughton 2005). Pediocin PA-1 is a small heat-stable bacteriocin which is produced by *Pediococcus acidilactici* PAC1.0 (Marugg *et al.* 1992). Both nisin and pediocin inhibit food–borne pathogens and spoilage bacteria including LAB found in wine, such as *Lactobacillus plantarum, Lactobacillus casei*, *Lactobacillus brevis*, *Pediococcus damnosus* and *Oenococcus oeni* (Bauer *et al.* 2003, Nel *et al.* 2002, Radler 1990, Rojo-Bezares *et al.* 2007a, Strasser de Saad and Manca de Nadra 1993). Moreover, it was demonstrated that these peptides are stable under wine making conditions, and have no negative effect on either yeast growth or the sensorial wine profile.

The possibility of controlling bacterial growth during vinification and preservation by bacteriocins is a promising alternative to meet the consumer demands and preferences for minimally processed products that contain less chemical preservatives such as sulphur dioxide (SO_2). Nevertheless, one must keep in mind that although the total SO_2 amount could be lowered with the use of bacteriocins, a certain free SO_2 content in wine is still required for antioxidant and antioxidasic purposes. Bacteriocins could be used to promote quality by inhibiting the indigenous LAB microflora, thereby preventing the production of undesired compounds such as off-flavors and biogenic amines. Consequently, malolactic fermentation (MLF) could be conducted with a selected starter culture. Furthermore, suitable combinations of nisin and SO_2 could control the growth of spoilage bacteria in wine which thus allows a decrease in the amounts of SO_2 (Rojo-Bezares *et al.* 2007b).

However, Daeschel, *et al.* (1991) observed a decrease in nisin activity in Pinot Noir over a 4-month storage period to less than 90%, while little decrease was observed in Chardonnay. These authors suggested that nisin may be interacting with polyphenolic compounds which are present in red wine, but absent in white wines. Later on, Daeschel and Bower (1991-1992) verified that tannins caused an immediate decrease of nisin levels

when tested in a wine model system. Grapes and wine contain a large variety of phenolic compounds which originate either from initial grape material or from wood used during maturation (barrels, chips). Red wines contain numerous phenolic compounds (De Beer *et al.* 2002) such as phenol carboxylic acids (240 to 500 mg/L), *e.g.* gallic acid, p-coumaric acid; anthocyanins (40 to 470 mg/L); flavonols (65 to 240 mg/L) and flavan-3-ols (25 to 560 mg/L), *e.g*. catechin. Tannins are formed due to polymerization of elementary molecules with phenolic functions and are divided into two groups: hydrolyzable tannins and condensed tannins. Hydrolyzable tannins include gallotannins and ellagitannins and are not naturally found in grapes, but in wood. Condensed tannins in grapes are basically complex polymers of flavan-3-ols or catechins. Polyphenols, tannins in particular, are capable of forming stable combinations with proteins. Various chemical and physical factors, such as the amino acid composition of the protein, pH and temperature, may affect the formation of tannin-protein complexes (Ribéreau-Gayon *et al.* 2000). Bacteriocins could potentially be bound by polyphenols, since they are peptides.

For the possible use of bacteriocins to control MLFs, a loss or decrease in bacteriocin activity in red wines due to interaction between polyphenols and bacteriocins, would therefore be disadvantageous.

The purpose of this study was to investigate the short term influence of phenolic compounds on nisin and pediocin to better understand the factors which contribute to the decrease of bacteriocin activity.

4.2 MATERIALS AND METHODS

4.2.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

The bacterial strain used as sensitive test organism was *Pediococcus pentosaceus* NCDO 813. *Pediococcus acidilactici* PAC1.0 was used for the isolation and purification of pediocin PA-1. Both bacteria strains were grown at 30°C in MRS broth (Biolab, Merck, South Africa).

A modified synthetic wine medium (SWM) (Ugliano *et al.* 2003), 14% v/v and pH 3.5 was used as model wine system for the experiments. All compounds were added as described by the latter authors, except for glycoside extract.

4.2.2 PREPARATION OF THE SYNTHETIC WINE MEDIUM

Two phenolic acids, two flavan-3-ols, grape tannins and oak tannins were added individually or in combinations to aliquots of SWM according to **Table 4.1**.

The concentration of each component used in the synthetic wine medium was similar to the average concentration found in a red wine. Nisin and pediocin PA-1 were added to each sample at a concentration of 12,800 Activity Units/mL (AU/mL). Pediocin PA-1 was only tested in combination with p-coumaric acid, catechin and the oak and grape tannins.

The model wine system was then inoculated with an overnight culture (± 10^8 CFU/mL) of *P. pentosaceus* NCDO 813 and incubated at 30°C.

Nisin, p-coumaric acid, gallic acid, (+)-catechin and (-)-epi-catechin were obtained from Sigma-Aldrich (Johannesburg, South Africa). The grape tannins (VR Tannin Supra) were purchased from Laffort (Stellenbosch, South Africa), and the oak tannins (Oenotan Selection) from Columbit (South Africa).

Table 4.1 Concentration of compounds tested with Nisin or Pediocin PA-1 in SWM

Phenolic compound	Concentration (mg/L)
p-coumaric acid	200
gallic acid	200
catechin	200
epi-catechin	200
grape tannins	100
oak tannins	100
p-coumaric acid + gallic acid	100 + 100
p-coumaric + catechin	100 + 100
p-coumaric + epi-catechin	100 + 100
gallic acid + epi-catechin	100 + 100
gallic acid + catechin	100 + 100
catechin + epi-catechin	100 + 100
gallic acid + p-coumaric acid + catechin + epi-catechin	50 + 50 + 50 + 50

4.2.3 DETECTION OF ANTIMICROBIAL ACTIVITY

The bacteriocin activity was determined by measuring the cell density of the sensitive organism *P. pentosaceus* NCDO 813. Samples were taken at specific time intervals (immediately after inoculation and after 3h, 6h, 9h, 24h) and plated out in serial dilutions on MRS agar plates in duplicate. The plates were incubated at 30°C for 24 to 48 hours.

4.2.4 PURIFICATION OF PEDIOCIN PA-1

P. acidilactici PAC1.0 was cultured in 5 mL MRS broth at 30°C overnight and then re-inoculated into 1L MRS broth at 30°C overnight. Pediocin PA-1 was isolated from the culture by harvesting the cells (8000 x g, 10 min, 4°C) and precipitating the proteins from the cell-free supernatant with ammonium sulfate (56.8 g in total). The precipitate was pelleted by centrifugation (10000 x g, 10 min, 4°C), dissolved in deionized water and dialyzed against deionized water. The dialyzed sample was freeze dried and the AU/mL of the powder was determined.

4.3 RESULTS

Six phenolic compounds of grapes and wine were tested individually and in combination in a model wine medium.

4.3.1 BACTERIOCIN ACTIVITY IN PRESENCE OF ONE PHENOLIC COMPOUND

Both nisin and pediocin in combination with one single phenolic compound increased the inhibitory effect on the sensitive organism (**Figures 4.1**, **4.2**). However, the combinations with pediocin showed the strongest negative influence as the cell growth decreased with ± 10^6 CFU/mL, compared to the combination with nisin that reduced the cell numbers with ± 10^5 CFU/mL. The close parental relationship of the pediocin-producing strain *P. acidilactici* PAC 1.0 and sensitive organism *P. pentosaceus* 813, might be an explanation for this phenomenon. A specific bacterium exhibits high sensitivity to the bacteriocin produced by a closely related species and genera (Klaenhammer 1988).

Similar results were obtained by combining nisin with gallic acid or catechin (data not shown).

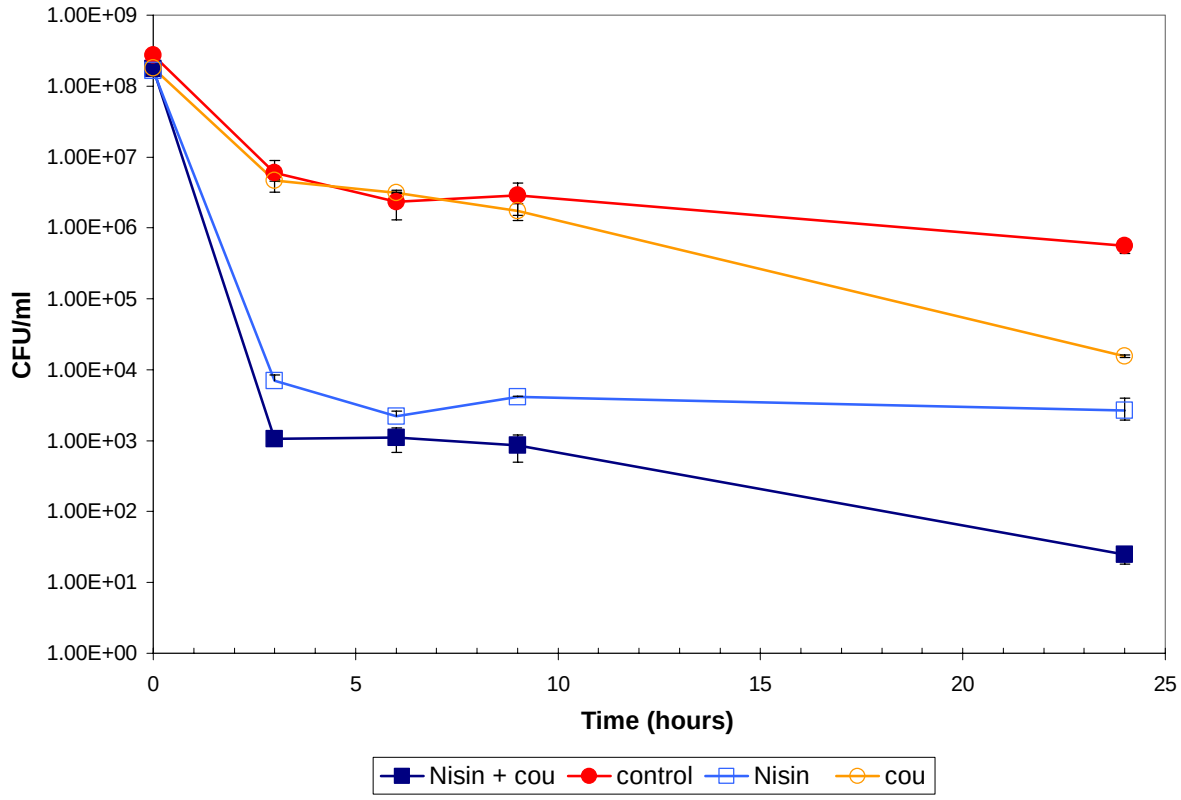

Figure 4.1. Effect of *p*-coumaric acid and nisin on the growth of *P. pentosaceus* NCDO 813. Error bars indicate standard deviation.

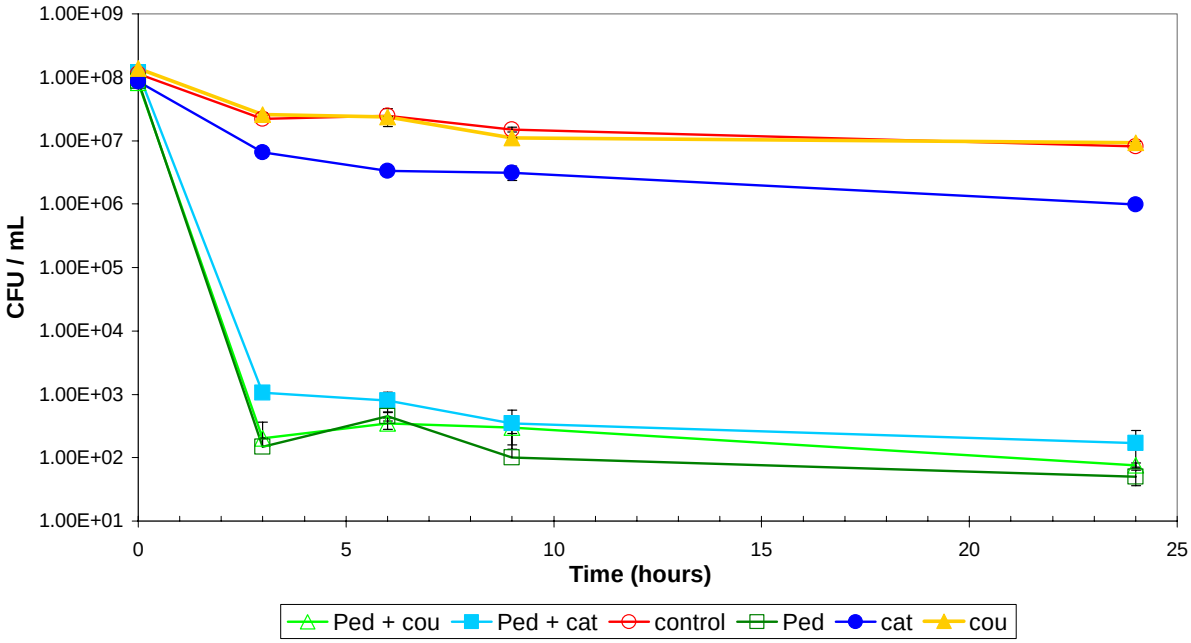

Figure 4.2. Effect of *p*–coumaric acid, catechin and pediocin on the growth of *P. pentosaceus* NCDO 813. Error bars indicate standard deviation.

4.3.2 NISIN AND PEDIOCIN COMBINED WITH OAK AND GRAPE TANNINS

The oak tannins in combination with nisin or pediocin showed the highest influence on the sensitive organism and almost completely inhibited growth (**Figure 4.3**). Similar results were obtained by using grape tannins (data not shown). Results showed that the tannins alone decreased the cell numbers with between 10^1 to 10^2 CFU/mL compared to the bacteriocin alone and the combinations that reduced the cell numbers with between 10^3 to 10^6 CFU/mL. It was also evident that nisin alone was less effective than the nisin-combinations.

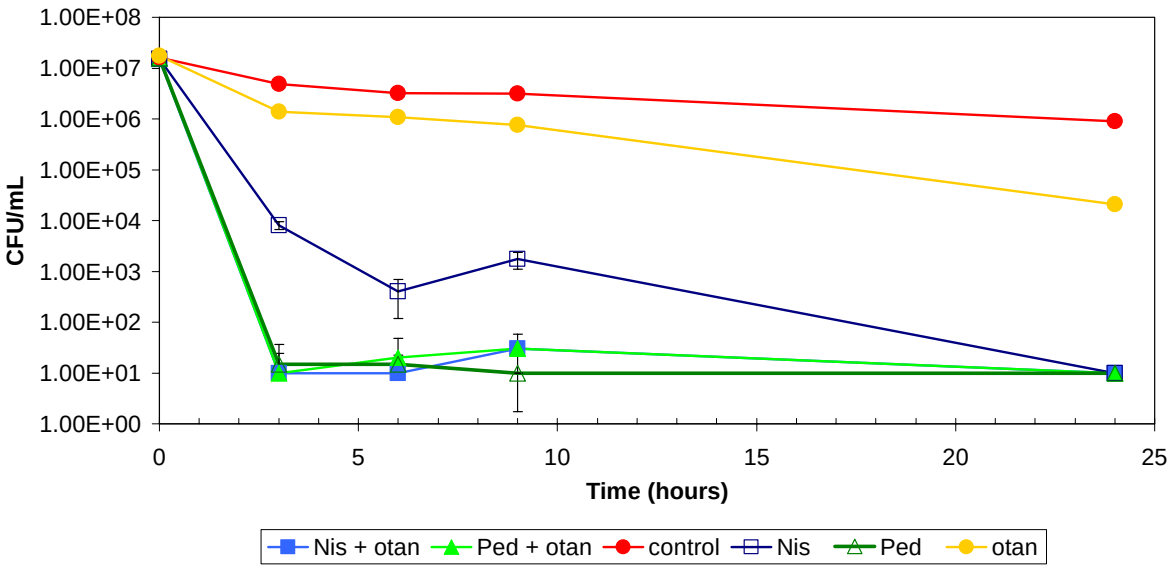

Figure 4.3. Effect of oak tannins, nisin and pediocin on the growth of *P. pentosaceus* NCDO 813. Error bars indicate standard deviation.

4.3.3 NISIN COMBINED WITH TWO AND FOUR OF THE PHENOLIC COMPOUNDS

Nisin activity was not influenced by the addition of p-coumaric acid and gallic acid, or *p*-coumaric acid and catechin. The combined phenolic compounds without nisin decreased the growth of the sensitive organism with ± 10^3 CFU/mL and the addition of nisin to the mixtures increased the inhibitory effect on the growth to ± 10^5 CFU/mL (**Figure 4.4**).

The combination gallic acid and epi-catechin did not affect the growth of the sensitive organism, whereas the combination of *p*-coumaric acid and epi-catechin as well as the

mixture of phenolic compounds with nisin showed a strong inhibitory effect on the growth of *P. pentosaceus* NCDO 813. In both latter cases the cell numbers decreased with ± 10^6 CFU/mL (**Figure 4.5**). The four compounds *p*-coumaric acid, gallic acid, catechin and epi-catechin all together, as well as the combinations of gallic acid and catechin, and catechin and epi-catechin had little affect on the sensitive organism. Nisin with the four compounds in combination had the strongest inhibitory effect on the growth of the sensitive organism and reduced the cell numbers with ± 10^5 CFU/mL. Any combination of two compounds with nisin decreased the cell numbers by ± 10^4 CFU/mL (data not shown).

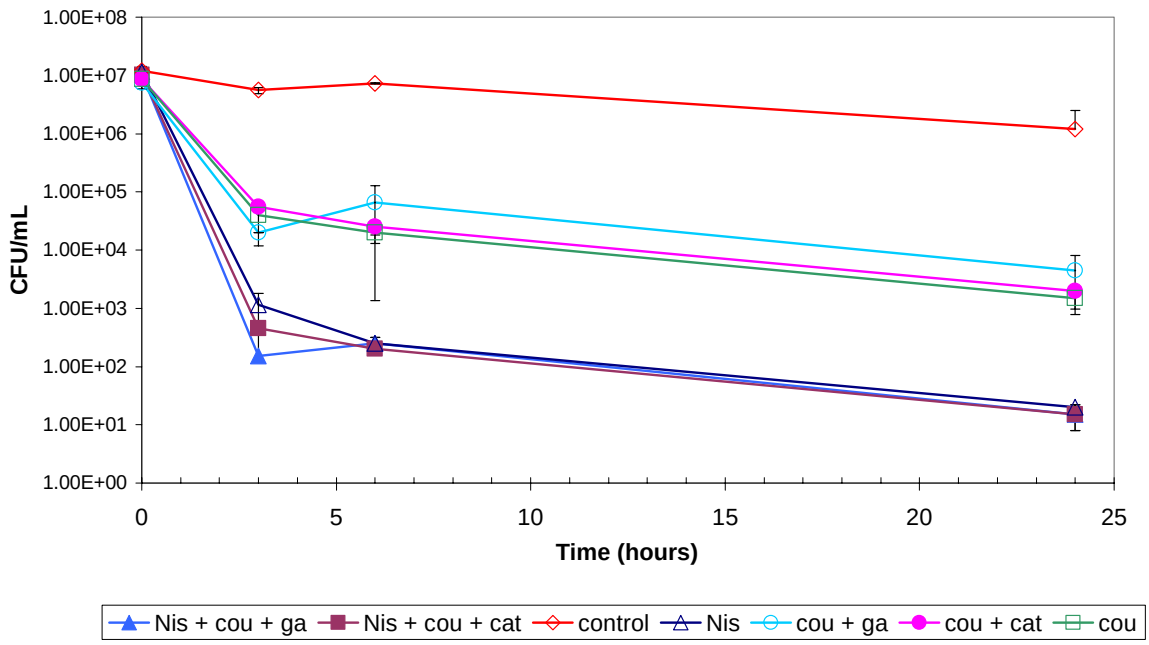

Figure 4.4. Effect of *p*-coumaric acid, gallic acid and nisin, and *p*-coumaric acid, catechin and nisin on the growth of *P. pentosaceus* NCDO 813. Error bars indicate standard deviation.

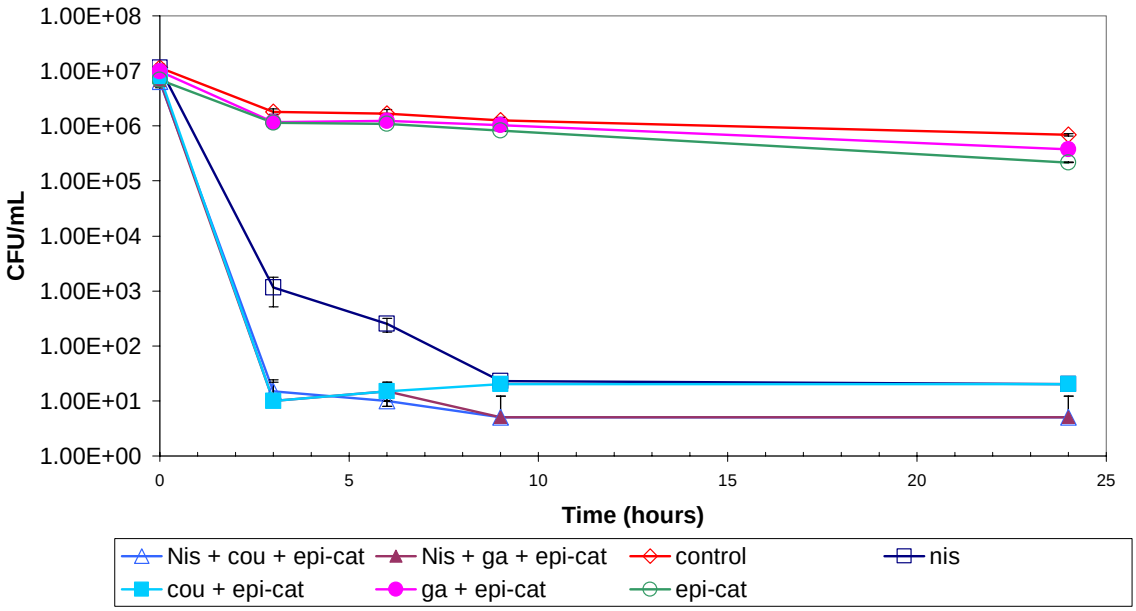

Figure 4.5. Effect of *p*-coumaric acid, epi-catechin and nisin, and gallic acid, epi-catechin and nisin on the growth of *P. pentosaceus* NCDO 813. Error bars indicate standard deviation.

4.4 DISCUSSION

Phenolic compounds are known to affect the growth and metabolism of bacteria either in an activating or inhibiting way (Lonvaud-Funel 2001, Reguant *et al.* 2000, Vaquero *et al.* 2007). Little is known about the interactions of phenolic compounds and bacteriocins.

In this study, most of the phenolic compounds tested had a negative influence on the bacteria and the combination of phenolic compounds and bacteriocin increased the inhibitory effect in the first three hours following the bacteriocin addition.

In previous studies, nisin activity has been observed to remain stable in white wines but to decrease in red wines over a four month period. Moreover, Daeschel and Bower (1991-1992) observed a decrease in nisin activity with grape tannins, but not with catechin or gallic acid over a six-week period. The latter study also indicated that the activity of nisin decreased more rapidly in mature wines than in younger wines. In our study, the activity of neither nisin nor pediocin PA-1 was inhibited by the phenolic compounds tested over a 24-h period. Moreover, synergistic effects were observed and after 24-h only five percent of the bacteria survived.

The purpose of this study was to investigate the short-term influence of phenolic compounds on the activity of nisin and pediocin in SWM. Thus, only one LAB species was used in our model system to test our hypothesis. The duration of the experiments might have been not long enough to have a negative effect on the activity and stability of the bacteriocins. Previous studies observed that not only the pH of wine but also the storage temperature and age had an effect on the activity of nisin (Daeschel and Bower 1991-1992). Time seems to be an important factor for the inhibition of bacteriocins in wine. Therefore, the use of bacteriocins might be an alternative tool to control fermentations. However, it might not be applicable as a long term preservative in red wine. Wine is a very complex medium. In the synthetic wine medium used in this study the interactions which naturally occur in red wine (e.g. polymerization reactions) might not happen in the model wine medium and further optimizations of the used medium are necessary.

4.5 CONCLUSIONS

In this study, no negative effect of phenolic compounds and polyphenols on nisin and pediocin was observed. Considering the advantages of natural inhibitors versus classical chemical control methods, bacteriocins present a beneficial and more ecologically friendly alternative or might possibly replace part of the total SO_2, once the interactions with wine are unraveled.

4.6 ACKNOWLEDGEMENTS

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Chapter 5

GENERAL DISCUSSION AND CONCLUSIONS

5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 GENERAL DISCUSSION AND CONCLUDING REMARKS

Numerous strains of LAB are used for food fermentations and for the preservation of fermented foods. In wine especially species belonging to the genera *Oenococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* are involved in the vinification process and have an impact on the organoleptic profile and quality of wine, especially those undergoing MLF. Although species of *Lactobacillus* and to a lesser extent *Pediococcus* species are capable of conducting MLF, it is more likely that these two genera produce undesirable by-products than positive sensory attributes in wine.

From a winemaker's perspective it is necessary to control the MLF to either enhance positive characteristics or to reduce possible negative impacts (Mills, Rawsthorne, Parker, Tamir and Makarova, 2005). The application of bacteriocins during the vinification process might help to prevent the production of undesired compounds by inhibiting the indigenous bacterial microflora and allowing MLF to be conducted by a selected bacterial population. Furthermore, the use of bacteriocins might allow the reduction of the total amount of SO_2 in wine.

In this study a broad screening of isolated LAB was performed in order to find novel bacteriocins or new candidates which can be used as bacteriocinogenic cultures to control the bacterial microflora during the vinification process. Moreover, to investigate concerns regarding limited bacteriocin activity in wine, the short term impact of other wine components on their activity was studied.

Out of 330 wine LAB strains screened in this study, 27 belonging to the species *Lb. plantarum*, *Lb. paracasei*, *Lb. hilgardii* and *O. oeni* showed antimicrobial activity towards numerous wine-related and non wine-related indicator strains. When the nisin A-encoding gene was successfully transformed into two strains of *Lb. plantarum*, an enhanced antimicrobial activity was observed; thereby suggesting gene functioning activity. However, the transformed *nisA* gene was not stable. Furthermore, a PCR-based screening revealed the presence of plantaricin structural genes in selected wine *Lb. plantarum* strains. The identity of the PCR products obtained was confirmed by sequencing. Moreover, these sequences were translated *in silico* and aligned with published sequences of *Lb. plantarum* strains. High homology of the genes was found when compared to the homologous genes of the published sequences for the five strains studied.

This study confirmed in many regards that different LAB species isolated from wine have the ability to produce bacteriocins. Natural isolates of wine LAB that produce well characterized bacteriocins could be used for winemaking. Likewise, natural isolates which are genetically well characterized could be a potential source of genes for the construction of new starter cultures or the improvement of existing ones through genetic engineering. Moreover, advances in identification of new bacteriocins and a better understanding of their natural diversity, capabilities and interactions will allow determining the regulation of their production. Although consumers are generally sceptic about the use of genetically modified organisms (GMO's) in the food industry, the creation of such GMO's has been beneficial with respect to gaining insights into the understanding of these peptides (Deegan, Cotter, Hill and Ross, 2006). Such techniques could provide an option to improve the stability and production of bacteriocins, so they might be more applicable in the winemaking process. However, bacteriocins used as food preservatives have relatively narrow spectra and are generally not active against yeast. Rojo-Bezares, Saenz, Zarazaga, Torres and Ruiz-Larrea (2007) reported a synergistic effect on LAB growth inhibition by nisin and metabisulphite. Nisin and lysozyme acted synergistically against Gram-positive bacteria (Chung and Hancock, 2000). The antimicrobial activity of enterocin AS-48 against *Staphylococcus aureus* in vegetable sauces was increased significantly in combination with phenolic compounds such as caffeic acid, *p*-coumaric acid and hydrocinnamic acid (Grande et al., 2007). Therefore, the simultaneous application of one or more bacteriocins and other antimicrobial compounds such as SO_2, lysozyme, organic acids and phenolic compounds could provide a beneficial opportunity for the wine industry (Chung et al., 2000; De Beer, Joubert, Geldenblom and Manley, 2002; Rojo-Bezares et al., 2007).

The use of bacteriocins during the vinification process might facilitate the inhibition or reduction of spoilage LAB which can potentially produce off-flavours and other undesired compounds (e.g. biogenic amines). Indeed, a more precise application and a better control of MLF could be achieved. Moreover, bacteriocins might provide an opportunity to meet the demand for cost-effective, sustainable and environmental friendly production of healthy minimally processed products, containing little or no chemical preservatives such as SO_2.

Little is known about bacteriocin production in wine and the influence of other wine compounds such as phenolic compounds on the activity and stability of bacteriocins. When nisin was used to control the microflora in wine, Daeschel , Jung and Watson (1991) observed a decrease in nisin activity in Pinot Noir over a four month storage period to less

than 90%, while little decrease was observed in Chardonnay. These authors suggested that nisin may be interacting with polyphenolic compounds which are present in red wine, but absent in white wines. Later on, Daeschel and Bower (1991-1992) verified that tannins caused an immediate decrease of nisin levels when tested in a wine model system. This study focused on the evaluation of bacteriocin production firstly in laboratory medium, secondly in synthetic wine medium and thirdly in wine or must. Furthermore, the short term influence of different phenolic compounds on the activity of nisin and pediocin was investigated. No negative effect of phenolic compounds and polyphenols on nisin and pediocin was observed. Most of the phenolic compounds tested had a negative influence on the bacteria and the combination of phenolic compounds and bacteriocin increased the inhibitory effect. These results indicate that bacteriocins can be used to control the bacterial microflora during fermentation processes.

In situ bacteriocin production offers several advantages such as costs and legal aspects compared to the addition of purified or semi-purified bacteriocins. In this study, the bacteriocin production by a wine *Lb. plantarum* strain in liquid media was evaluated. Indeed, production of plantaricin and inhibition of an indicator bacterium was observed in laboratory medium. However, technical problems regarding the availability of selective and differential media for distinguishing between different LAB, made a repetition in a wine medium impractical. For further investigations, there is a need for development of selective agar media to be able to discriminate between different genera of LAB. On the other hand, other methods such Real Time-PCR (Grattepanche, Lacroix, Audet and Lapointe, 2005; Neeley, Phister and Mills, 2005), DGGE (denaturing gradient gel electrophoresis) (Renouf, Claisse and Lonvaud-Funel, 2006) or TTGE (temporal temperature gradient gel electrophoresis) (Ogier, Son, Gruss, Tailliez and Delacroix-Buchet, 2002) could be considered to follow the survival of target bacteria in the presence of bacteriocin-producing bacteria in wine, while determining the growth of the bacteriocinogenic strain at the same time.

Bacteriocins are a miscellaneous group of antimicrobial peptides which work differently on different targets under different ecological conditions (Grande et al., 2007). Since the efficacy of bacteriocins in foods is dependent on environmental factors, it is necessary to determine the precise and most efficient conditions for their application in wine. However, it is generally observed that bacteriocin production supplies a competitive advantage to the bacteriocinogenic organism (Dykes, 1995; Riley and Wertz, 2002). Bacteriocins might be exploited as anti-competitors permitting invasion of a strain into an established microbial

environment. They might also be used as a defence mechanism to prohibit establishment of other strains or species into an occupied niche (Riley et al., 2002). Yet, it needs to be explored whether LAB produce bacteriocins in most ecological niches under most growth conditions or whether they only produce it under particular conditions, e.g. in nutrient poor conditions (Eijsink, Axelsson, Diep Dzung, Håvarstein, Holo and Nes, 2002). Wine is a harsh environment with decreasing oxygen and nutrient levels and increasing pH and alcohol levels during the course of the vinification process. To date it is not known, whether LAB also rely on the production of bacteriocins to gain a competitive advantage in the wine environment or whether well-adapted species such as *O. oeni* solely exploit their high ethanol and low pH tolerance, for instance, to out-compete other potential MLF bacteria during the later stages of vinification.

However, this is the first report on *O. oeni* strains with antimicrobial activity and on the identification of putative bacteriocin-encoding genes. Indeed, this might be an additional way of *O. oeni* to gain dominance in the wine environment and to inhibit the establishment of other strains.

The under-utilization of bacteriocins in food may be caused by a combined lack of awareness of what bacteriocins can accomplish in food systems and a lack of enthusiasm to retreat from existing food preservation methods (Cotter, Colin and Ross, 2005). However, this study confirmed that whether added or produced *in situ* by LAB, bacteriocins could provide a beneficial prospect for the inhibition of spoilage LAB and the control of MLF in wine. It may represent an excellent option for use in combination with other preservatives. Cotter et al. (2005) described the capability of bacteriocins to regulate the microflora as a type of programmable innate immunity for food.

Despite the tremendous amount of research being done on bacteriocins, further research regarding their application and impacts in wine is necessary. Further investigations are required regarding their long term activity and stability in wine, especially in red wines. The bacterial ecology, their interactions and the production of bacteriocins in the complex must and wine environment needs to be further examined in large scale experiments. Moreover, if a bacteriocinogenic starter culture is used, conditions for an optimized production of bacteriocins need to be investigated.

As for other fermented food, LAB are necessary to obtain quality, but their control is an important part of modern commercial winemaking. In order to prevent MLF, physical (filtration, low temperature) or chemical stabilization is required. For a long period of time in winemaking, SO_2 was the only authorized and efficient agent for the microbial

stabilization of wine. Previously, the use of lysozyme, another antimicrobial protein, was approved by the OIV (resolution OENO 15/2001). Currently, the addition of bacteriocins to wine is not authorized. However, the application of bacteriocins during the vinification process might provide a valuable additional tool to prevent the production of undesired compounds by inhibiting or reducing the indigenous LAB microflora and allowing the MLF to be conducted by a selected bacterial strain. Moreover, the use of bacteriocins could lead to a reduction of the total sulphur dioxide amount in wine, which will help to meet the increasing consumer demand for wines containing less chemical preservatives.

Much knowledge is still needed on *O. oeni*. Apart from its originality in the LAB group with respect to its adaptation to the wine environment, the identification is the most interesting aspect yet to be studied. It has scientific and technological impacts (Lonvaud-Funel, 1999). With the identification of its antimicrobial activity against various wine related LAB and the identification of putative bacteriocin-encoding genes, this study contributed further beneficial insight into *O. oeni*.

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Chapter 6

ADDENDUMS

6. ADDENDUM A

A.1 HETEROLOGOUS EXPRESSION OF BACTERIOCIN GENES IN LAB

The third objective of this study was the transformation and expression of heterologous bacteriocin genes in LAB strains in order to achieve an enhanced bacteriocin activity and a broader inhibition spectrum. Due to restricted time, not all aims in this objective could be achieved. Therefore this part of the study is presented in this addendum.

Lb. plantarum and *O. oeni* were chosen for the heterologous expression study. *O. oeni*, most often responsible for MLF in wine and tolerant to various wine related stresses (low pH, high ethanol), was especially attractive for the heterologous bacteriocin expression, because it was not known to produce bacteriocins. As mentioned in previous chapters of this thesis, an antimicrobial activity was nevertheless detected. *O. oeni* strains are often used as starter cultures to conduct MLF in wine, because among the wine-related LAB it is the least associated with off-flavours and other undesired metabolites (Mills, Rawsthorne, Parker, Tamir and Makarova, 2005). Thus, a MLF starter culture with the ability to produce a bacteriocin to inhibit spoilage bacteria will offer a great benefit during the vinification process.

However, *O. oeni* still presents a challenge when applying molecular tools for genetic manipulation and only few successful transformations have been reported (Beltramo, Oraby, Bourel, Garmyn and Guzzo, 2004; Dicks, 1994; Zúñiga, Pardo and Ferrer, 1996a, 1996b; 2003).

With few exceptions, all of the successful commercial starter cultures are strains of *O. oeni*. One exception is the starter culture of a *Lb. plantarum* strain. Because of an increasing interest in new starter cultures and its ability to conduct MLF, two *Lb. plantarum* strains were chosen for this study.

A.2 MATERIALS, METHODS AND STRATEGIES

A.2.1 STRAINS, MEDIA AND GROWTH CONDITIONS

Lb. plantarum strains R1122 and 116.4 were grown in MRS medium (Biolab Diagnostics (PTY) Ltd, Wadeville, Gauteng, SA); *O. oeni* strains R1124 and 1101 were grown in MRS supplemented with 5 g/L fructose and 4 g/L malic acid and the pH was adjusted to 4.5.

Ent. faecalis and *O. oeni* ATCC BAA 1163 were grown in FT80 medium. *Escherichia coli* DH5α was grown in Luria-Bertani broth (Biolab Diagnostics) at 37°C on a rotary wheel at 50 rpm.

Selective antibiotic concentrations were as follows: for 100 µg/mL of ampicillin (Sigma Aldrich, Johannesburg, South Africa); 10 µg/mL of vanomycin (Sigma Aldrich); 5µg/mL of lynomycin (Sigma Aldrich); 5 µg/mL of chloramphenicol (Roche, Mannheim, Germany) and 5 µg/mL of erythromycin (Sigma Aldrich) for *O. oeni* ATCC BAA 1163, 15 µg/mL of erythromycin for *Ent. faecalis* and 200 µg/mL of erythromycin for *E. coli* carrying the pGID052 plasmid; when appropriate, 30 µg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma Aldrich) were added.

A.2.2 CLONING STRATEGY

The bacteriocins, nisin A from *Lact. lactis* subsp. *lactis* and pediocin PA-1 from *P. acidilactici* PAC 1.0, were chosen for the heterologous expression study. Plasmid pBR322 was used for transformation into *Lb. plantarum.* Plasmid pGID052 (Beltramo et al., 2004) previously constructed for *O. oeni* was selected, because it was reported to be segregationally stable and seemed to be promising for transformation purposes.

Moreover, different promoters were decided on, because it was uncertain whether the native bacteriocin promoter could be recognized by *Lb. plantarum* and *O. oeni.* Therefore, different promoters were included: (i) the native bacteriocin promoter, (ii) a specific synthetic promoter p11 as designed by Rud, Jensen, Naterstad and Axelsson (2006) for *Lb. plantarum* and (iii) the *mle* promoter of *O. oeni* for the latter strain which resulted in the construction of 6 different plasmids (**Fig. A.1**).

A.2.3 PCR AMPLIFICATION

A.2.3.1 Pediocin PA-1 and Nisin A genes

Purified plasmid DNA of pSRQ220 (Marugg et al., 1992) containing the 5.6-kbp *Sal*I-*Eco*RI – pediocin PA-1gene fragment served as template to amplify the pediocin a-d (*ped a-d*) genes and purified genomic DNA of *Lact. lactis* DSM 20729 served as template to amplify Nisin A (*nisA*).

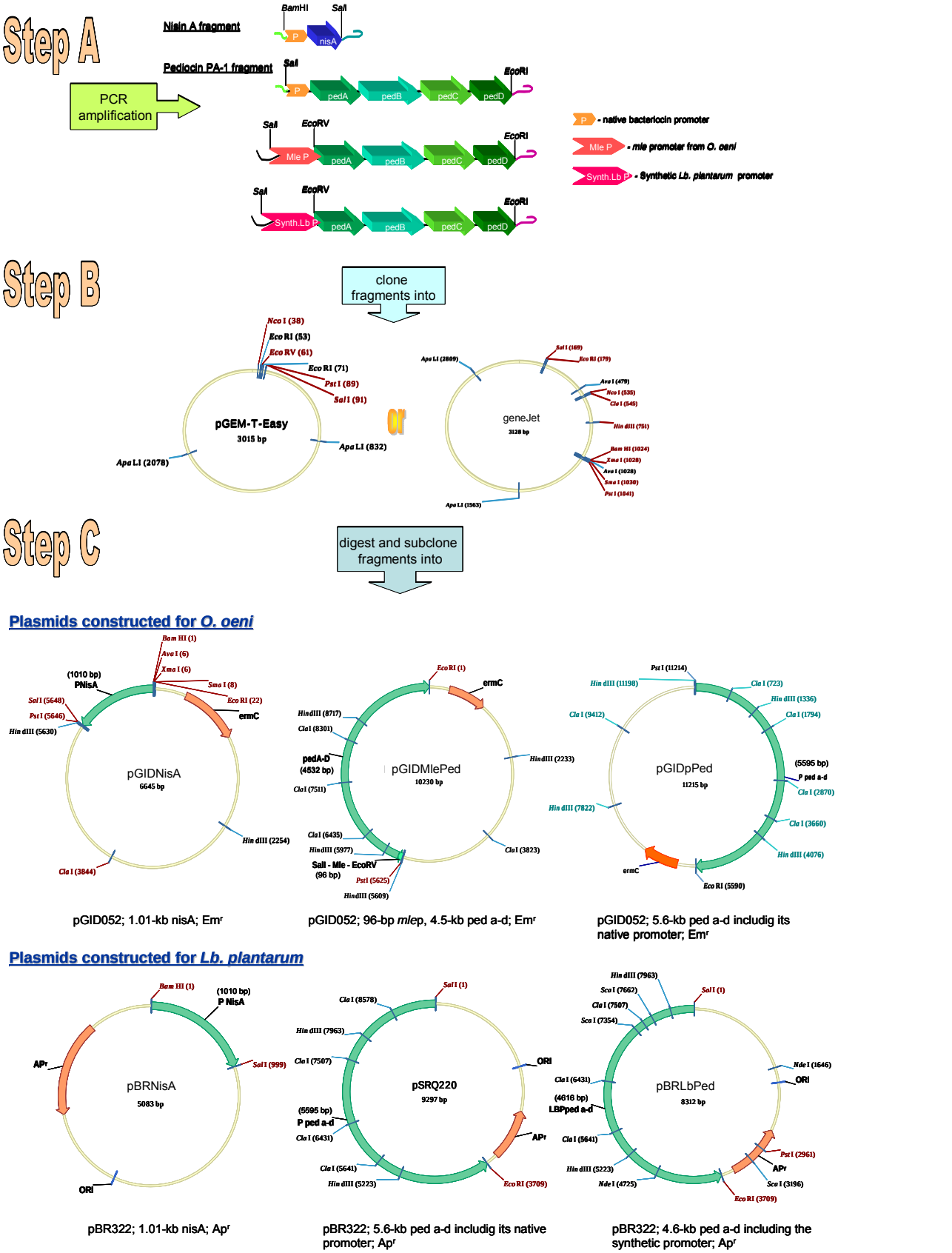

Fig. A. 1. Cloning strategy. Step A, PCR amplification of the fragments *nisA*, ped, Pped, *mlep* and LbPed; Step B, cloning of fragments into the pGEM-T easy or GeneJET vector; Step C subcloning fragments into the pBR322 vector for subsequent transformation into *Lb. plantarum* and into the pGID052 vector for subsequent transformation into *O. oeni*. The relevant genotypes are indicated underneath each plasmid.

Primers designed to amplify *nisA* included the native promoter. The primers designed for pediocin amplified a region spanning a *Lb. plantarum* specific synthetic promoter p11 and the *ped a-d* genes (Lbped).

To allow subsequent cloning, *Sal*I and *Eco*RI sites were introduced at the 5' and 3' ends of the pediocin primers and *Bam*HI and *Sal*I for nisin primers. The primers are described in **Table A.1**.

Amplifications consisted of denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing temperature see in **Table A.1** for 30 s, and polymerization at 72°C for 1 min for *nisA* and 3 min 40 s for *ped a-d*. An extra final elongation step was performed at 72°C for 5 min.

A.2.3.2 *mle* promoter

Purified genomic DNA of *O. oeni* R1124 served as template to amplify the *mle* promoter (*mle*p). Amplification comprised denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and polymerization at 72°C for 1 min. An extra final elongation step was performed at 72°C for 5 min.

Table A.1
Primers used in this study

Species/ plasmid	Designation	Sequence (5' - 3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
Lact. lactis	NisA-Bam - F	GATGGATCCTAGGCACGTTCGGCAGTAAC (*Bam*HI)	47	1010	This study
	NisA-SalI - R	GCCGTCGACTTCAGTAAAACTCCGTTTATCG (*Sal*I)			
pSRQ220	LbPed - F	GCCGTCGACAGCGCTATAGTTGTTGACAGAATGGACAT ACTATGATATATTGTTGCGTATTAAGGATAATTTAAG (*Sal*I)	56	4616	This study
	Ped-EcoRI - R	GCCGAATTCCTAGCCAAATTATTAAAC (*Eco*RI)			This study
O. oeni	Mle-Sal-F	GCCGTCGACGATAATTTTATCTCTTTTATAAG (*Sal*I)	52	96	This study
	Mle-ECORV-R	GGCGATATCATTTTCTCCTCAAGAACCAC (*Eco*RV)			

Underlined fragments indicate introduced restriction sites and the corresponding enzyme is mentioned in brackets after the sequences.

A.2.4 DNA MANIPULATIONS AND PLASMID CONSTRUCTION

All cloning steps were conducted according to standard procedures as described in Sambrook and Russell (2001). T4 DNA ligase was purchased from Roche (Mannheim, Germany) and all restriction enzymes were obtained from Promega (Madison, WI, USA).

A.2.4.1 *Lb. plantarum*

Plasmids pSRQ220, pBRNisA and pBRLbPed

The amplified PCR product for Nisin A was cloned into the pGEM-T easy vector (Plasmid pGEM-T Easy System I, Promega, Cape Town, South Africa), while the amplified fragment for pediocin was cloned into the GeneJET vector (GeneJET^{TM} PCR Cloning Kit, Inqaba Biotechnical Industries (PTY) LTD, Pretoria, South Africa). Two different vector system were used, because after several attempts with different ligation ratios and times, the ~5.6 kb pediocin fragment could not be successfully cloned into the pGEM-T easy vector. The GeneJET vector is a slightly bigger blunt cloning vector which allowed cloning of the pediocin fragment with the synthetic *Lactobacillus* promoter.

The fragments were then digested with the appropriate enzymes and subcloned into the pBR322 plasmid (Bolivar, Rodriguez, Greene, Betlach, Heyneker and Boyer, 1977), which was digested with *Sal*I and *Eco*RI or *Bam*HI and *Sal*I, respectively. The resulting plasmids, designated pBRNisA and pBRLbPed, as well as plasmid pSRQ220 were used to transform *E. coli* DH5α cells. Plasmid DNA was then purified with the QIAprep® Spin Miniprep Kit, according to the protocol supplied by the manufacturer and used for electroporation (see section 3.2.4) into *Lb. plantarum* strains R1122 and 116.4.

A.2.4.2 *O. oeni*

Plasmid pGIDNisA

The amplified and cloned nisin A fragment was digested with the appropriate enzymes and subcloned into the pGID052 plasmid which was digested with *Bam*HI and *Sal*I respectively.

Plasmid pGIDMlePed

The amplified PCR product for the *mle* promoter was cloned into the pGEM-T easy vector (Promega) (designated pGMleP). To obtain the pediocin fragment (ped) without its native promoter, plasmid pSRQ220 was digested with *Eco*RV and *Eco*RI. The pediocin fragment was then cloned into the GeneJET blunt cloning vector. In order to insert the *mle* promoter in front of the pediocin gene, the *mle*p gene was digested with the appropriate enzymes and subcloned into the GeneJET vector containing the pediocin gene fragment.

Plasmid pGIDpPed

To obtain the pediocin fragment with its native promoter (pPed), plasmid pSRQ220 was digested with *Sal*I and *Eco*RI and cloned into the pGID052 plasmid, which was digested respectively with *Sal*I and *Eco*RI.

The resulting plasmids were used to transform *E. coli* DH5α cells. Plasmid DNA was then purified with the QIAprep® Spin Miniprep Kit, using the protocol supplied by the manufacturer and used for transformation into *O. oeni.*

A.2.5 TRANSFORMATION STRATEGY FOR *O. oeni*

As transformation strategy for *O. oeni*, the conjugal method described by Beltramo et al. (2004) was chosen. The latter authors successfully performed conjugal experiments from *Lact. lactis* to *O. oeni* with a new constructed plasmid, pGID052, via mobilization. This transformation system was chosen, because pGID052 was shown to be transferred and could replicate in *O. oeni*. A conjugal transformation seemed to be promising, because only one successful electroporation has been reported by Dicks (1994), but has not been repeated since.

A.3 RESULTS AND DISCUSSION

All subsequent fragments (*nisA*, ped, Pped, *mle*p, LbPed) were successfully amplified by PCR and cloned into the pGEM-T Easy or GeneJet vector. In the last cloning step, only the plasmid pBRNisA was successfully constructed and transformed as described in Chapter 3 section 3.3.5.1.

The remaining fragments could not be successfully subcloned into the plasmids pBR322 and pGID052 due to restricted time. The large size of these fragments could be one of the reasons for the difficulties in the cloning procedure. Although various concentration ratios of the vector to insert and different ligation times have been tested, further optimization of the cloning process is necessary.

A.4 FUTURE PERSPECTIVE

The construction of new malolactic starter cultures or the improvement of existing ones by genetic engineering has beneficial aspects regarding the improvement of wine microbial stability and sensorial characteristics and are of great interest for the wine industry.

As soon as the cloning methods have been optimized and the plasmids successfully constructed, the plasmids can be transformed into the bacteria strains. Because of the challenging transformation of *O. oeni* strains, the transformation strategy will possibly have to be optimized. *O. oeni* is the most desired bacterial species for MLF during the vinification process and has been the focus of much recent research. A successful and repeatable transformation for *O. oeni* method will be beneficial and would provide new possibilities and opportunities for both science and industry.

Once the *Lb. plantarum* and *O. oeni* strains are successfully transformed, they must be tested for antimicrobial activity on plate assays, as well as in liquid media. Moreover, it can be determined which of the used promoters is the most efficient.

For further application in the wine industry, food grade plasmids without antibiotic selection markers will also have to be considered.

A.5 ACKNOWLEDGEMENTS

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6. ADDENDUM B

B.1 INDUCIBLE PLANTARICIN PRODUCTION BY *Lb. plantarum*

In this study all bacteriocin producing bacteria strains showed antimicrobial activity on solid media with the 'spot on the lawn' method. However, no antimicrobial activity was detected in any liquid cultures at any time of incubation when aliquots of cell free supernatant samples were tested.

Previously, *Lb. plantarum* NC8 was shown to produce plantaricin NC8, an inducible class IIb bacteriocin, only when it was co-cultured with other Gram-positive bacteria (Maldonado, Jimenez-Diaz and Ruiz-Barba, 2004a; 2004b). Induction was promoted by both living and heat-killed cells and no relationship between the sensitivity of the strains and their ability to induce bacteriocin production could be drawn.

To investigate the possible need of induction for plantaricin production by *Lb. plantarum*, one *Lb. plantarum* strain was chosen to gain more insight into the plantaricin production.

B.2 MATERIALS AND METHODS

B.2.1 STRAINS, MEDIA AND GROWTH CONDITIONS

All strains used are listed in **Table B.1** and were grown in MRS medium (Biolab Diagnostics (PTY) Ltd, Wadeville, Gauteng, SA) at 30°C.

B.2.2 DETECTION OF ANTIMICROBIAL ACTIVITY

The "spot on the lawn" method (Schillinger and Lücke, 1989) was used to detect antimicrobial activity as described previously in section 3.2.2.

Table B.1
Bacteria strains used in this study

Purpose	Species	Strain	Source
Bacteriocin producing bacteria			
	Lb. plantarum	R1122	Lallemand, France
Indicator bacteria			
	P. pentosaceus	813	NCDO
Bacteria tested for plantaricin induction			
	Lb. sake	2714	NCDO
	Lb. fermentum	20092	DSM
		9328	ATCC
		23271	ATCC
		13554	LMG
	P. pentosaceus	1356	LMG
		848	NCDO
		1859	NCDO
		813	NCDO
	Lact. lactis subsp. *lactis*	20729	DSM
	Lb. brevis	27305	ATCC
		14869	ATCC
	Lb. hilgardii	8290	ATCC
		20176	DSM
	Lc. mesenteroides	529	NCDO
		Ta33a hastings	NCFB
	E. faecalis	13566	LMG
	Lact. lactis subsp. *cremoris*	CNRZ 105	INRA
		CNRZ 106	INRA
		CNRZ 113	INRA
		CNRZ 114	INRA
		CNRZ 158	INRA
		CNRZ 163	INRA
		CNRZ 1352	INRA
		CNRZ 489	INRA

ATCC American Type Culture Collection (Rockville, Md., USA)
BfE Bundesanstalt für Ernährung (Karlsruhe, Germany)
INRA Institut National de la Recherche Agronomique (Jouy-en-Josas, France)
NCDO National Collection of Dairy Organisms (Reading, UK)
NCFB National Collection of Food Bacteria (c/o NCIMB Ltd.) (Aberdeen, Scotland, UK)
LMG Culture Collection of the Laboratory of Microbiology (University of Gent, Belgium)

A.2.3 INDUCTION OF PLANTARICIN PRODUCTION

The bacteriocin induction assay was adapted from the method described by Maldonado et al. (2004b). Production of plantaricin by *Lb. plantarum* strain R1122 in co-culture with Gram-positive bacteria listed in **Table B.1** was determined as follows:

50 mL of MRS broth were inoculated with 2% of an overnight culture of *Lb. plantarum* and 0.5% or 1% of an overnight culture of each strain to be tested for induction. Both living and heat-killed (55°C, 1h) cells were used. The controls as well as the combined cultures were incubated at 30°C for 8 h, centrifuged (10,000 rpm, for 10 min at 4°C), and the supernatants adjusted to pH 7.0 with 5 M NaOH and filter-sterilized (0.22-µm-pore size). 10 µL of the supernatant were then spotted onto a lawn containing *P. pentosaceus* NCDO 813 as indicator. The plates were incubated at 30 °C for 24 h.

B.3 RESULTS

Various Gram-positive bacteria belonging to the genera *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Pediococcus* and *Leuconostoc* were tested both as living and heat-killed inducer cells. Maldanado et al. (2004a; 2004b) reported bacteriocin activity of the supernatant after 3 h and a maximum of activity after 8 h, with a minimum of inoculum of 10^4 CFU/mL inducer cells and a maximum of 10^6 CFU/mL. In this study, different amounts of inducer cells, as well as different incubation times (8, 12, 24, 48h) of the co-culture were tested. Moreover, various preparation methods of cell free supernatant were investigated. However, no antimicrobial activity could be detected in the cell free supernatant of the liquid co-cultures. Then again, in section 3.2.3 the production of plantaricin in liquid media was investigated and an inhibition of the sensitive indicator organism was observed.

B.4 DISCUSSION AND FUTURE PERSPECTIVE

A possible cause could be the preparation of the supernatant. Different preparation methods of the supernatant were tested, such as using different temperatures to centrifuge the culture, different centrifugation speed and heat inactivation instead of filter sterilization. However, no difference could be observed. Furthermore, the amount of plantaricin in the liquid culture could be insufficient to be detected in the supernatant. A possible concentration and purification of the supernatant as described in section 4.2.4 could help to detect antimicrobial activity. For future investigations the preparation methods of the supernatant need to be optimized. Moreover, other detection methods such as RT-PCR for analysis of the expression of the bacteriocin (Maldonado et al., 2004a) should be considered to gain more insight into the induction phenomenon. Different environmental factors and signals have shown to play an important role in the regulation of bacteriocin

production and need to be further investigated (Brurberg, Nes and Eijsink, 1997; Cotter, Colin and Ross, 2005; Delgado, Lopez, Brito, Peres, Fevereiro and Garrido-Fernandez, 2007).

In the case of *Lb. plantarum* NC8, it has been shown that the presence of other bacteria in the same environment triggers bacteriocin production (Maldonado et al., 2004b). This type of regulatory system could be part of a defense strategy that includes sensing of other bacteria and subsequent production of bacteriocin (Gobbetti, De Angelis, Di Cagno, Minervini and Limitone, 2007; Riley and Wertz, 2002). Competing LAB in the wine environment might also make use of this type of regulatory mechanism to gain a competitive advantage. However, to date, it is not known whether LAB also make use of bacteriocin production to defend their ecological niche or if the harsh wine environment with decreasing oxygen and nutrient levels and increasing pH and alcohol levels is efficient enough for well-adapted species to out-compete other bacteria strains. Better understanding of the wine microbial ecology, population dynamics and interactions between the microbes will assist in the control of wine microbes. It is therefore important to gain further insight into the bacterial ecology and their defense mechanisms.

B.5 ACKNOWLEDGEMENTS

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