CHARACTERIZATION AND IDENTIFICATION OF BACTERIOCINS FROM TWO *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* STRAINS

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

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In this study, bacteriocins from two *L. lactis* subsp. *lactis* isolates of Turkey origin designated OC1 and OC2, respectively, were characterized and identified. The activity spectra of the bacteriocins were determined by using different indicator bacteria including *Listeria, Bacillus* and *Staphylococcus* spp. Bacteriocins were tested for their sensitivity to different enzymes, heat treatments and pH values. Loss of bacteriocin activities after α-amylase treatment suggested that they form aggregates with carbohydrates. Molecular masses of partially purified bacteriocins

were determined by SDS-polyacrylamide gel electrophoresis. PCR amplification was carried out with different primers for the detection of structural genes of lactococcal bacteriocins. As a result of these studies, the two bacteriocins were characterized as nisin and lacticin 481, respectively. Association of the bacteriocin production with plasmid DNA was examined by using acriflavine as a plasmid curing agent. Plasmid profiles of the wild type and its non-bacteriocin producing mutants were determined by using the alkali lysis method followed by agarose gel electrophoresis. The genetic nature of industrially important characteristics of *Lactococcus lactis* strains were investigated through gene transfer studies via conjugation. According to the results of plasmid curing and conjugal transfer trials, it was concluded that in *Lactococcus lactis* subsp. *lactis* OC1 strain a 39,7 kb plasmid is responsible for nisin production, lactose fermentation and proteolytic activity. In *Lactococcus lactis* subsp. *lactis* OC2 strain, on the other hand, a 16 kb plasmid appeared to be responsible for lacticin 481 production and lactose fermentation.

Key Words: *Lactococcus*, bacteriocins, nisin, lacticin 481, plasmid, conjugation, plasmid curing

ÖZ

İKİ AYRI *LACTOCOCCUS LACTIS* ALTTÜR *LACTIS* SUŞUNDAN BAKTERİYOSİN KARAKTERİZASYONU VE TANIMLANMASI

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Bu çalışmada, Türkiye'den izole edilen iki ayrı *L. lactis* alttür *lactis* suşu tarafından üretilen bakteriyosinler karakterize edilmiş ve tanımlanmıştır. Bakteriyosinlerin aktivite spektrumları *Listeria, Bacillus* ve *Staphylococcus* suşlarını içeren farklı indikatör bakteriler kullanılarak belilenmiştir. Bakteriyosinler, farklı enzimlere, sıcaklık muamelelerine ve pH değerlerine hassasiyetleri bakımından test edilmişlerdir. Bakteriyosinlerin α-amilaz muamelesiyle etkinliğini kaybetmesi, tanımlanan bakteriyosinlerin karbonhidratlarla agregat oluşturduğunu

düşündürmüştür. Kısmi olarak saflaştırılmış bakteriyosinlerin moleküler ağırlıkları SDS-PAGE yöntemiyle belirlenmiştir. Laktokokal bakteriyosinlerinin yapısal genlerinin saptanması için spesifik primerlerle PCR amplifikasyonu yapılmıştır. Bakteriyosin üretiminin plazmid DNA ile olan ilgisi, plazmid kaybettirici ajanlardan birisi olan akriflavin kullanılarak incelenmiştir. Suşlar tarafından üretilen iki bakteriyosin, sırasıyla nisin ve lactisin 481 olarak tanımlanmıştır. Doğal tip ve onun bakteriyosin üretmeyen mutantlarının plazmid profilleri alkali liziz ve ardından jel elektroforezi yöntemleri kullanılarak belirlenmiştir. *Lactococcus lactis* suşlarının endüstriyel olarak önem taşıyan bakteriyosin üretme özelliklerinin genetic doğası konjugasyon çalışmalarıyla incelenmiştir. Plazmid giderme ve konjugal transfer denemeleri sonucunda *Lactococcus lactis* subsp. *lactis* OC1 suşunda 39.7 kb büyüklükteki plazmidin nisin üretimi, laktoz fermentasyonu ve proteolitik activiteden; *Lactococcus lactis* subsp. *lactis* OC2 suşunda ise 16.0 kb büyüklükteki plazmidin lactisin 481 ve laktoz fermantasyonundan sorumlu olduğu saptandı.

Anahtar Kelimeler: *Lactococcus*, bakteriyosinler, nisin, lacticin 481, plazmid, konjugasyon, plasmid kaybettirme

To My Parents

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LIST OF SYMBOLS

AF	Acriflavine
AU	Arbitrary Activity Unit
CFU	Colony Forming Units
cm	Centimeter
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylene diamine tetraacetic acid
g	Gram
GRAS	Generally Regarded as Safe
kb	Kilobase
kDa	KiloDalton
L	Liter
LAB	Lactic Acid Bacteria
mA	Miliamper
mg	Miligram
min	Minute
mL	Mililiter
mM	Milimolar
MRS	Man-Ragose-Sharp
MW	Molecular Weight
RNA	Ribo Nucleic Acid
rpm	Revolution per minute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
µL	Microliter
µm	Micrometer

CHAPTER I

INTRODUCTION

Since food safety has become an increasingly important international concern, the application of antimicrobial peptides from lactic acid bacteria (LAB) that target food pathogens without toxic or other adverse effects has received great attention. The consumption of more food that has been formulated with chemical preservatives has also increased consumer concern and created a demand for more "natural" and "minimally processed" food. As a result, there has been a great interest in naturally produced antimicrobial agents.

LAB have been used for centuries in the fermentation of food, not only for flavour and texture, but also due to their ability of starter-derived inhibitors to prevent growth of pathogenic microorganisms [1,2]. Over the last two decades, bacteriocins produced by LAB have been the subject of considerable research and industrial interest due to their potential as food biopreservatives. Some bacteriocins produced by LAB, such as nisin, inhibit not only closely related species but are also effective against food-borne pathogens such as *Listeria monocytogenes* and many other gram-positive spoilage microorganisms.

Bacteriocins generally exert their antimicrobial action by interfering with the cell wall or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing pore formation, subsequently resulting in death. The incorporation of bacteriocins as a biopreservative ingredient into model food systems has been studied

extensively and has been shown to be effective in the control of pathogenic and spoilage microorganisms [3].

1.1. Lactic Acid Bacteria

LAB, which include the genera *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Carnobacterium* and *Propionibacterium* [4], play an essential role in food fermentations given that a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat and vegetable products. The most important contribution of these microorganisms to the product is to preserve the nutritive qualities of the raw material through an extended shelf life and the inhibition of spoilage and pathogenic bacteria. This is due to competition for nutrients and the presence of inhibitors produced by the starter, including organic acids, hydrogen peroxide and bacteriocins [5]. There are many reported examples of the inhibition of spoilage and pathogenic bacteria by LAB including, for example, that of Daly *et al*. [6] who demonstrated the inhibition of the food-borne pathogens *Staphylococcus aureus* and *Clostridium perfringens* by inoculation with the starter *Streptococcus diacetylactis.* This study demonstrated that *S. aureus* numbers were reduced by more than 99% in foods such as ham sandwich spread, chicken gravy and ground beef, and that the inhibition was most likely as a result of acid production by the starter *L. lactis* subsp. *lactis* biovar. *diacetylactis* strain. Extensive investigations, over the last number of decades, into the antagonistic behaviour of such strains have led to the identification and characterization of numerous bacteriocins produced by LAB [4,7,8]. Such investigations have led to the discovery of a range of different bacteriocin-producing strains, many of which have potential in food applications (Table 1.1). Given the ease with which bacteriocin-producing strains can be isolated from food sources, it is clear that many of these bacteriocins have been safely consumed for decades and thus it could be argued that reintroduction of such cultures should have negligible associated safety or toxicological problems when consumed [9]. Even though there has been a dramatic increase in the number of novel bacteriocins discovered in the last decade, the potential applications are limited by individual properties such as spectrum of inhibition, heat stability, solubility, etc.

Table 1.1. Examples of bacteriocins produced by members of the LAB with potential as biopreservatives in foods [3].

Bacteriocins of LAB	**Inhibition**
Lactococcus sp.	
Nisin	Broad spectrum
Lacticin 3147	Broad spectrum
Lacticin 481	Medium spectrum
Lactococcin A, B and M	Narrow spectrum
Lactobacillus sp.	
Lactocin 27	Narrow spectrum
Sakacin A	Narrow spectrum
Sakacin B	Narrow spectrum
Plantaricin C	Broad spectrum
Pediococcus sp.	
Pediocin A	Broad spectrum
Pediocin AcH (PA-1)	Broad spectrum
Leuconostoc sp.	
Leucocin A-UAL187	Broad spectrum
Enterococcus sp.	
Enterocin A	Narrow spectrum
Cornobacterium sp.	
Carnocin U149	Broad spectrum
Piscicolin	Broad spectrum
Diversin V41	Broad spectrum

In general, the followings should be considered when selecting bacteriocin-producing strains for food applications: (1) the producing strain should preferably have a GRAS status, and the bacteriocin should (2) have a broad spectrum of inhibition which includes pathogens such as *Listeria monocytogenes* and *Clostridium botulinum*, or else specificity against a particular pathogen; (3) be heat stable; (4) pose no associated health risks, (5) lead to beneficial effects in product such as improved safety, quality and flavour, and (6) have a high specific activity [10].

1.2. Characteristics of Genus *Lactococcus*

Members of the *Lactococcus* genus are Gram-positive cocci that can depending on growth conditions, appear ovoid and are typically 0.5–1.5 μm in size. They do not form spores and they are not motile. *Lactococcus* species grow in pairs or in short chains and unlike many members of the *Streptococcus* genus, these organisms do not grow in long chains [11]. They have a fermentative metabolism and as expected for lactic acid bacteria, they produce copious amounts of lactic acid. They have complicated nutritional requirements and are auxotrophic for a number of amino acids and vitamins. Their optimum growth temperature is 30°C and they can grow at temperatures as low as 10°C, but not at 45°C. They also cannot grow in 0.5% NaCl. Both their maximum growth temperature and their failure to tolerate salt are somewhat diagnostic of this genus as compared to closely related members of the *Streptococcus* genus, most notably *S. thermophilus*. The lactococci are usually members of the Lancefield serological Group N [12,13]. Although serotyping was historically used for taxonomic purposes, it is now less important except to identify potentially pathogenic streptococci. Lactic acid bacteria have as a common feature of forming lactic acid as a major end product of their fermentation of hexoses. Beyond is lactic acid bacteria diverge into a wide array of microorganisms that have few other common features. Initial classification schemes as proposed by Orla-Jensen [14] have proven relatively accurate even in the face of challenges raised by the advent of molecular classification methods.

The genus *Lactococcus* is relatively new and most members of this genus previously belonged to the genera *Streptococcus* and *Lactobacillus*. In 1985, Schleifer and colleagues [11] proposed the genus *Lactococcus* and included the species formerly known as *Streptococcus lactis*, *Streptococcus raffinolactis*, *Lactobacillus hordniae* and *Lactobacillus xylosus*. The fact that the latter two species were formerly classified within the genus *Lactobacillus* is curious and suggests that the pleomorphic nature of these organisms can confound classification based on cell shape. The former *Lactobacillus xylosus* has in fact been reclassified to *Lactococcus lactis* subsp. *lactis*, with the single distinguishing characteristic being the ability of the former *Lactobacillus xylosus* to metabolize xylose, whereas most strains of

Lactococcus lactis subsp. *lactis* fail to metabolize that sugar. Reclassification was on the basis of cell wall structure, fatty acid composition and menaquinone composition. In addition to the lactococci, other 'new' genera including *Vagococcus* and *Enterococcus* were established [15].

The characteristics that distinguish the lactococci from other lactic acid bacteria including former members of the *Streptococcus* genus include pH, salt and temperature tolerances for growth [11,16]. Whereas some lactic acid bacteria produce D-lactic acid, L-lactic acid and/or a combination of D and L, the lactococci produce only L-lactic acid. The other characteristics are presented in Table 1.2.

There are five major species within the genus *Lactococcus*. They can be distinguished by their ability to grow above 40°C and at > 4% sodium chloride. In addition, they differ in their ability to produce acid from sugars including lactose, mannitol and raffinose. The ability to ferment lactose is an important characteristic especially for those species used to produce dairy products [17]. Specific tests to distinguish among the various *Lactococcus* species are shown in Table 1.3.

Table 1.2. The characteristics that distinguish lactococci from other lactic acid bacteria [18].

Character	*Carno-bacterium*	*Lactobacillus*	*Aerococcus*	*Entero-coccus*	*Lactococcus/ Vagnococcus*	*Leuconostoc/ Oenococcus*	*Pedio-coccus*	*Strepto-coccus*	*Tetragen-o-coccus*	*Weissella*
Tetrad formation	–	–	+	–	–	–	+	–	+	–
CO_2 from glucose	–	±	–	–	–	+	–	–	–	+
Growth at 10°C	+	±	+	+	+	+	±	–	+	+
Growth at 45°C	–	±	–	+	–	–	±	±	–	–
Growth at 6.5% NaCl	ND	±	+	+	–	±	±	–	+	±
Growth at 18% NaCl	–	–	–	–	–	–	–	–	+	–
Growth at pH 4.4	ND	±	–	+	±	±	+	–	–	±
Growth at pH 9.6	–	–	+	+	–	–	–	–	+	–
Lactic acid	L	D, L, DL	L	L	L	D	L, DL	L	L	D, DL

Table 1.3. Specific tests to distinguish among various *Lactococcus* species [18].

Test	*L. garvieae*	*L. lactis*			*L. piscium*	*L. plantarum*	*L. raffinolactis*
		subsp. *cremoris*	subsp. *hordniae*	subsp. *lactis*			
Growth at 40°C	+	–	–	(+)	–	–	–
Growth with 4% NaCl	+	–	–	+	ND	+	–
Arginine hydrolysis	+	–	+	+	–	–	(–)
Acid from lactose	+	+	–	+	+	–	+
Acid from mannitol	(+)	–	–	(–)	+	+	ND
Acid from raffinose	–	–	–	–	+	–	+
Pyrrolidonyarylamidase	+	–	–	(–)	ND	–	–

ND: not determined

Lactococci are GRAS (Generally Regarded as Safe) microorganisms. Their isolation sources are milk, dairy products and plant materials [11,19]. The physiological properties of lactococci that makes them as an acceptable starter culture can be summarized as below [20]:

-*Acid production:* The major fermentable sugar of milk, lactose, is fermented to an end product of L (+) lactic acid leading to a decrease in pH. By decreasing pH, clotting phenomenon of milk and reduction in the number of the microflora other than the starter were obtained.

-*Proteolytic activity:* Lactococci possess a proteolytic system that is responsible for the conversion of casein into peptides and amino acids together with other protein hydrolyzing enzymes like chymosin [21].

-*Aroma formation:* Lactococci produce flavor compounds such as organic acids, lactic and acetic acid in fermented milk products and in matured cheeses. On the other hand, the biovariety *diacetylactis* produce diacetyl from the citrate of the milk.

-*Exopolysaccharide (EPS) formation:* EPS can be as a capsule attached to the bacterial cell. This property provides, for example, an increase in the rheological quality of yoghurt.

-*Production of inhibitory compounds:* Bacteriocin producing lactococcal strains have been used successfully in starter cultures for cheese-making in order to improve safety and quality properties of cheese. One of the bacteriocin produced by lactococci is nisin [22].

1.3. Bacteriocins of Lactococci

Tagg et al. [7] defined bacteriocins as 'proteinaceous compounds which kill closely related bacteria', with a bactericidal mode of action. Even though many characterised bacteriocins concur with this definition, it has become apparent that some have a broad host-range, inhibiting many different species.

Bacteria are sources of antimicrobial peptides, which have been examined for applications in microbial food safety. The bacteriocins were first characterized in Gram-negative bacteria. The colicins of *E. coli* are the most studied ones [23]. The colicins constitute a diverse group of antibacterial proteins, which kill closely related bacteria by various mechanisms such as inhibiting cell wall synthesis, permeabilizing the target cell membrane, or by inhibiting RNase or DNase activity. Among the Gram-positive bacteria, the lactic acid bacteria have been comprehensively exploited as a reservoir for antimicrobial peptides with food applications [24].

Numerous bacteriocins have been isolated from LAB over the last three decades, and vary in size from small (~3 kDa), heavily post-translationally modified peptides to large heat labile proteins. The continual emergence of new bacteriocins has necessitated a continual updating on the classification of bacteriocins.

Bacteriocins produced by LAB are commonly divided into three main groups [8,25] (Table 1.4). Nisin was discovered in 1928 [26], and subtilin, a nisin analogue differing by 12 amino acid residues, was discovered in 1948 [27]. Both belong to Class I, termed lantibiotics. The classification of bacteriocins is currently being revised to reflect similarities and differences observed in the discovery of new molecules. Class I is being further subdivided into Class Ia and Class Ib. In general, Class I peptides typically have from 19 to more than 50 amino acids. Class I bacteriocins are characterized by their unusual amino acids, such as lanthionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine. Class Ia bacteriocins, which include nisin, consist of cationic and hydrophobic peptides that form pores in target membranes and have a flexible structure compared to the more rigid Class Ib. Class Ib bacteriocins, which are globular peptides, have no net charge or a net negative charge [28].

Class II contains small heat-stable, non-modified peptides, and can be further subdivided. According to conventional classification, Class IIa includes Pediocin-like *Listeria* active peptides with a conserved N-terminal sequence Tyr–Gly–Asn–Gly–Val and two cysteines forming a S–S bridge in the N-terminal half of the peptide. Bacteriocins composed of two different peptides comprise Class IIb. The

two peptide bacteriocins need both peptides to be fully active. The primary amino acid sequences of the peptides are different. Though each is encoded by its own adjacent genes, only one immunity gene is needed. Class IIc was originally proposed to contain the bacteriocins that are secreted by the general sec-system [25]. Since this proposal, it has been shown that Class IIa bacteriocins can use this secretory system and consequently the sub-class. IIc should be eradicated [29].

The large (>30 kDa) and heat labile bacteriocins e.g. helveticin J [30] make up the Class III bacteriocins for which there is much less information available.

Table 1.4. Classification of bacteriocins [24]

Group		**Features**	**Bacteriocins (Group Representatives)**
I	Ia	Lantibiotics, small (< 5kDa) peptides containing lanthionine and β-methyl lanthionine	Nisin
	Ib	Globular peptides with no net charge or net negative charge	Mersacidin
II	IIa	Small heat-stable peptides, synthesized in a form of precursor which is processed after two glycine residues, active against *Listeria*, have a consensus sequence of YGNGV-C in the N-terminal	Pediocin PA-1, sakacins A and P, leucocin A, carnobacteriocins, etc.
	IIb	Two component systems: two different peptides required to form an active poration complex	Lactococcins G and F, lactacin F, plantaricin EF and JK
III		Large molecules sensitive to heat	Helveticins J and V, Acidophilucin, lactacins A and B

1.3.1. Bacteriocins versus antibiotics

Bacteriocins are often confused in the literature with antibiotics [26,27]. This would limit their use in food applications from a legal standpoint. In some countries, it is critical to make the distinction between bacteriocins and antibiotics. The main differences between bacteriocins and antibiotics are summarized in Table 1.5. Bacteriocins, which are clearly distinguishable from clinical antibiotics, should be safely and effectively used to control the growth of target pathogens in foods. In this respect, Hurst (1981), in his review, proposed the term biological food preservatives since bacteriocins, unlike antibiotics, are not used for medicinal purposes.

Table 1.5. Bacteriocins versus antibiotics [24].

Characteristic	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Host cell immunity	Yes	No
Mechanism of target cell resistance or tolerabce	Usually adaptation affecting cell membrane composition	Ususally a genetically transferrible determinant affecting different sites depending the mode of action
Interaction requirements	Sometimes docking molecules	Specific target
Mode of action	Mostly pore formation, but in a few cases possibiby cell wall biosynthesis	Cell membrane or intracellular targets
Toxicity / side effects	None known	Yes

1.3.2. Mechanism of action of bacteriocins

In general, the action of bacteriocins produced by Gram-positive bacteria is directed primarily against other Gram-positive species. The range of organisms inhibited by each bacteriocin varies greatly; while nisin is active against a broad variety of bacteria including strains of *Lactococcus, Streptococcus, Staphylococcus, Listeria* and *Mycobacterium,* as well as the vegetative cells and outgrowing spores of *Bacillus* and *Clostridium* species [31-33], the Class II bacteriocin, lactococcin A, spesifically kills lactococci [33,34]. Under normal circumstances, bacteriocins produced by Gram-positive bacteria do not have a bactericidal effect on Gram-negative species. However, in some cases, activity against Gram-negatives can be observed on disruption of the outer membrane, as reported for nisin [35]. It has been established that the primary target for many of these small, cationic peptides is the cytoplasmic membrane of sensitive cells [36-41], where they act to dissipate the proton motive force (PMF) through the formation of discrete pores in the cytoplasmic membrane, and thus deprive cells of an essential energy source [42]. The PMF, which is composed of a chemical component (the pH gradient; ΔpH) and an electrical component (the membrane potential; $\Delta\psi$), drives ATP synthesis and the accumulation of ions and other metabolites through PMF-driven transport systems in the membrane [43]. Collapse of the PMF, induced by bacteriocin action, leads to cell death through cessation of energy-requiring reactions. Such a mode of action has

been demonstrated for the type-A lantibiotics and the Class II bacteriocins. The type-A lantibiotics act in a voltage-dependent manner without the requirement for a specific protein receptor [44-48]; however, recent work has shown that the activity of nisin is dependent on the concentration of lipid II (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)- GlcNAc) in the membrane of sensitive cells [49,50]. The Class II bacteriocins are thought to interact with membrane receptor proteins prior to insertion into the cytoplasmic membrane in a voltage-independent fashion [36-38]. In contrast, the type-B lantibiotics do not form membrane pores; instead, these peptides act by interfering with essential enzyme activities [51].

1.3.3. Bacteriocin immunity

The immunity of the cell synthesizing the bacteriocin to its product is a phenomenon that distinguishes bacteriocins from antibiotics. Genes coding for "immunity proteins" are in close genetic proximity to other bacteriocin structural and processing genes [52]. It is common for the structural bacteriocin gene and the immunity gene to be located on the same operon and often next to each other [25,53]. The immunity of lantibiotics was initially thought to be due to an immunity gene, such as *nis*I for nisin and *spa*I for subtilin, which code for NisI and SpaI immunity proteins, respectively. It appears, however, that immunity to these bacteriocins is the result of the influence of several proteins, since the deletions of other genes result in altered host immunity [53] . For example, non-nisin producing nisin-resistant strains of *L. lactis* do not have the genetic elements coding for NisI protein, but do have sequences similar to *nis*F, *nis*E and *nis*G [54]. These are thought to render the strains resistant to nisin. Deletion of *nis*G makes the cells less resistant to nisin. The complexity of host immunity with respect to nisin is reviewed by Saris et al. (1996) [55] but as of yet, there is not a clear uderstanding of how immunity proteins serve to protect the producing organism from its bacteriocin. The phenomenon of immunity is simpler in the nonlantibiotics, Class II bacteriocins. One gene encodes for the immunity protein. Usually, it is a basic protein between 50 and 150 amino acid residues long that is loosely associated with the membrane. The lactococcin A immunity protein LcnI is by far the most studied one, yet the basic mechanism behind the immunity is still not understood [56-58].

1.4. Lantibiotics

The production of ribosomally synthesized linear antimicrobial peptides is well conserved in nature and almost all groups of organisms have been shown to produce such peptides [59,60]. The lantibiotics (from '*lan*thionine-containing an*tibiotic*') are unique in that they are produced on the ribosome as a prepeptide which undergoes extensive post-translational modification to form the biologically active peptide [61-63]. The term is used to encompass peptides containing unusual amino acids normally not found in nature, e.g. the thioether aminoacids Lan and/or MeLan, in addition to a number of modified residues, such as 2,3 didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) [64]. The presence and influence of these residues on the structure and activity of lantibiotics, and the novel enzymes found in their biosynthetic pathways responsible for the specific amino acid modifications has attracted significant research interest.

1.4.1. Structural aspects

Examples of lantibiotics described to date are listed in Table 1.6. According to a proposal by Jung in 1991 [65], lantibiotics are grouped into type-A and type-B peptides based on their structural and functional features. In general, type-A lantibiotics are elongated, cationic peptides up to 34 residues in length that show similarities in the arrangement of their Lan bridges. These peptides primarily act by

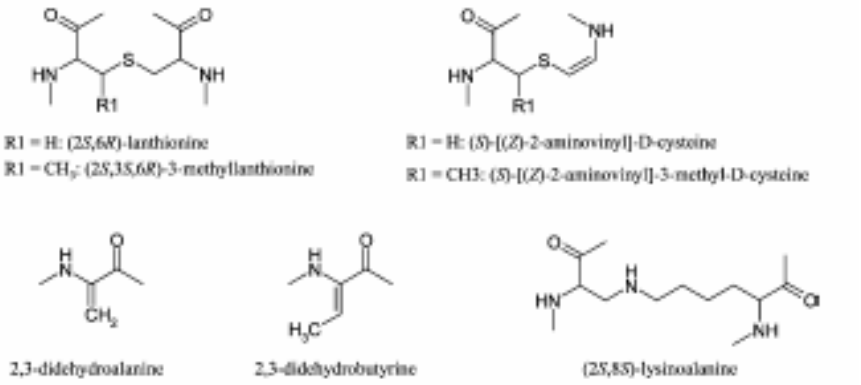

Figure 1.1. Structures of some of the modified amino acids found in lantibiotics [67]

Table 1.6. Examples of lantibiotics characterized so far [67].

Lantibiotic	**Producing strain(s)**
Type-A Lantibiotics	
Type-A(I)	
Nisin A	*L. lactis* NIZOR5, 6F3, CFB894, ATCC11454
Nisin Z	*L. lactis* N8, NIZO22186
Subtilin	*B. subtilis* ATCC6633
Epidermin	*Staphylococcus epidermidis* Tu3298
Gallidermin	*Staphylococcus gallinarum* Tu3928
Mutacin B-Ny266	*S. mutans*
Mutacin 1140	*S. mutans* JH1000
Pep5	*S. epidermidis* 5
Epicidin 280	*S. epidermidis* BN280
Epilancin K7	*S. epidermidis* K7
Type-A(II)	
Lacticin 481	*L. lactis* CNRZ481, ADRIA85LO30
Cytolysin	*E. faecalis* DS16
Lacticin 3147	*L. lactis* DPC3147
Staphylococcin C55	*S. aureus* C55
Salvaricin A	*Streptococcus salvarius* 20P3
Lactocin S	*L. sake* L45
Streptococcin A-FF2	*Streptococcus pyogenes* FF22
Sublancin 168	*B. subtilis* 168
Carnocin U149	*C. pisicola*
Variacin 8	*Micrococcus varians* MCV8
Cypemycin	*Streptomyces* ssp.
Type-B lantibiotics	
Cinnamycin	*Streptomyces cinnamoneus*
Duramycin B	*Streptoverticillium* ssp.
Duramycin C	*Streptomyces griseoluteus*
Ancovenin	*Streptomyces* ssp.
Mersacidin	*B. subtilis* HIL Y-85, 54728
Actagardine	*Actinoplanes*

disrupting the membrane integrity of target organisms, and include nisin, subtilin, and epidermin. Type-B peptides are globular, up to 19 residues in length, and act through disruption of enzyme function, e.g. inhibition of cell wall biosynthesis. Examples are the duramycins produced by Streptomyces species, mersacidin and actagardine [68]. A number of lantibiotics, however, do not fall into either category suggesting that as more are discovered; classification will undoubtedly become more complex.

1.4.2. Organisation of lantibiotic gene clusters

The events which lead to the production of a lantibiotic include formation of the prelantibiotic, dehydration and cross-linkage reactions, cleavage of the leader, and secretion. In addition, the cell must be immune to the lantibiotic that it produces [68]. The genetic determinants flanking the structural gene(s) for several linear (type-A) and few type-B lantibiotics have been characterised to date [61,69], and the organisation of a representative number is summarised graphically in Fig. 1.2. Comparison of the gene clusters indicate the presence of a number of conserved genes proposed to encode similar functions. Following the generic nomenclature used for all lantibiotics, as reported by de Vos et al. [61], these include the precursor peptide LanA and enzymes responsible for the specific modification reactions LanB,C/LanM, accessory proteins including processing proteases responsible for removal of the leader peptide (LanP), ABC-superfamily transport proteins involved in peptide translocation (LanT), regulatory proteins (LanR, K) and dedicated self-protection (immunity) mechanisms (LanI, FEG), in addition to genes with no homologues in the database [62,69,70].

Two classes of genetic organisation have been identified; nisin, epidermin, subtilin and Pep5 are grouped on the basis that they are modified by separate LanB and LanC enzymes, whereas this function is performed by a single LanM enzyme in the subclass containing lacticin 481, lactocin S, cytolysin and mersacidin. Interestingly, the gene cluster of the two-component lacticin 3147 contains two lanM genes. In addition, transporters with an associated protease activity have not been found in the gene clusters of the nisin-like lantibiotics [67].

Lantibiotic genetic determinants may be chromosomally encoded, as in the case of subtilin [71] and SA-FF22 [72], although in most cases, lantibiotic gene clusters are found on large plasmids, e.g. epidermin [73], Pep5 [74], cytolysin [75], and lacticin 3147 [76]. Dufour et al. [77] have recently shown the lacticin 481 gene cluster to be present on a composite transposon, Tn5271, on a 70-kb plasmid. The nisin genes are encoded on a number of conjugative transposons, Tn5301 from *L. lactis* NCFB894 [78-80] and Tn5276 from *L. lactis* NIZO R5 [81,82], on a large (~70 kb) plasmid, which also harbour the genes for sucrose utilisation and integrate into the recipient chromosome following conjugal transfer [8]. There is evidence that at least parts of the gene clusters of some lantibiotics are organised as operons. Many consist of several transcriptional units [83-91] and a weak terminator structure is often found in the intergenic region between the structural gene(s) and downstream genes. Interestingly, an intragenic triple stem-loop structure has been identified within a crucial gene in the lacticin 3147 biosynthetic operon; this structure acts to control the level of the downstream biosynthetic genes [91]. This transcriptional organisation allows moderate readthrough from the lanA promoter, thus ensuring a high level of transcription of the prepeptide mRNA in comparison to the mRNA encoding the biosynthetic enzymes.

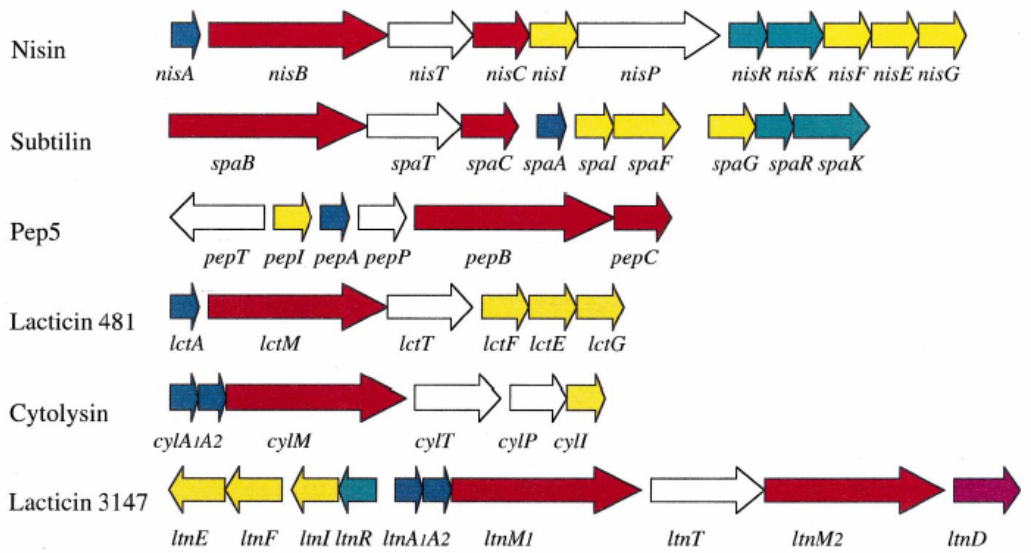

Figure 1.2. Organisation of biosynthetic gene clusters of well characterised lantibiotics. Structural genes (not drawn to scale) are highlighted in blue; genes with similar proposed functions are highlighted in the same colour (yellow for immunity, white for transport/processing, green for regulatory red for modification, and blue for unknown function) [62].

1.4.3. Nisin

Of all the antimicrobial peptides known, only a very few of them are actually allowed to be used either as a preservative in the food industry or as an antibiotic in health care. Nisin is undoubtedly the most well known and characterized bacteriocin and the only one to have realized widespread commercial use. This FDA-approved bacteriocin produced by the organism *Lactococcus lactis* has consequently been the subject of a wide variety of fundamental studies as to its structure and genetics [18,92,93].

Nisin being a member of the type A Class is composed of 34 amino acids and has a pentacyclic structure [94,95] with one lanthionine residue (ring A) and four β-methyllanthionine residues (rings B, C, D and E). The peptide shares similar characteristics with other antimicrobial peptides. It is overall positively charged (+4) and its structure possesses amphipathic properties. However, some structural properties make nisin rather special. Nisin is post-translationally modified such that serine and threonine residues are dehydrated to become dehydroalanine and dehydrobutyrine. Subsequently, five of the dehydrated residues are coupled to upstream cysteines, thus forming the thioether bonds that produce the characteristic lanthionine rings. Two naturally occurring nisin variants that have similar activities, nisin A and nisin Z, have been found [50,96,97] (Figure 1.3, Figure 1.4). Nisin A differs from nisin Z in a single amino acid residue at position 27, being a histidine in nisin A and an asparagine in nisin Z [96,97]. The thioether bonds give nisin two rigid ring systems, one N-terminally and one C-terminally located. A hinge region (residues 20-22), that often is found in antimicrobial peptides, separates the ring systems. Due to the ring structures, the nisin molecule is maintained in a screw-like conformation that possesses amphipathic characteristics in two ways: the N-terminal half of nisin is more hydrophobic than the C-terminal half; and the hydrophobic residues are located at the opposite side of the hydrophilic residues throughout the screw-like structure of the nisin molecule [18].

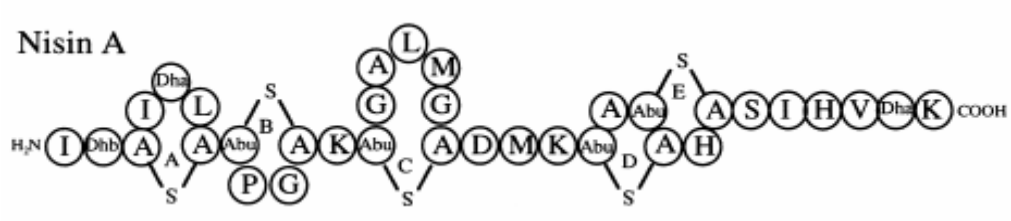

Figure 1.3. Nisin A [67]

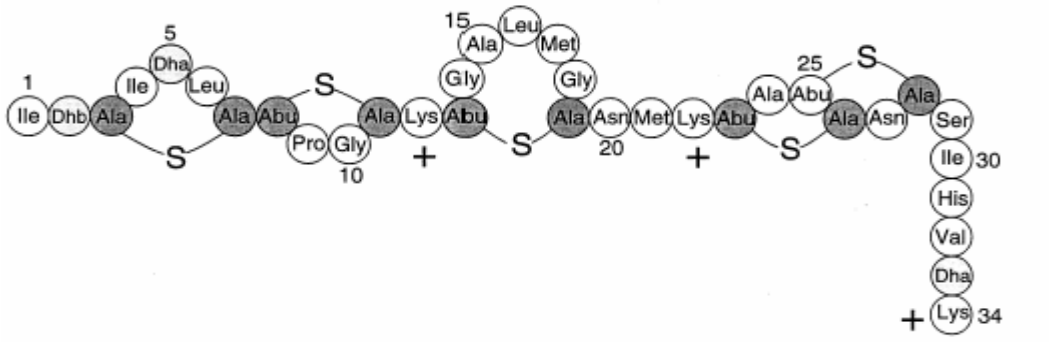

Figure 1.4. The primary structure of nisin Z [92].

An interesting feature of nisin is its unusually high specific activity. Nisin can be effective at nanomolar concentrations depending on the target strain under investigation. Nisin kills its target by pore formation in the target membrane. Over the past 10 years, much insight into the mechanism of pore formation has been achieved [7,98,99]. Earlier studies with nisin demonstrated that it inhibited peptidoglycan biosynthesis [100] and that it interacted with either lipid I or lipid II [101]. It was later found that nisin caused pore formation in the membranes of sensitive bacteria [44,102,103]. More recently, it was shown that nisin interacts with a docking molecule, lipid II, which is a membrane-bound precursor for cell wall biosynthesis (Figure 1.5). Indeed, in the absence of this precursor, significantly higher concentrations of nisin are required to form pores [49,104]. Significantly, mutations in the N-terminal rings of nisin indicated that these are involved in lipid II binding, whereas mutations in the flexible hinge region severely affected the ability of the bacteriocin to form pores. Such experiments have revealed the dual functionality of the nisin molecule involving initial binding to lipid II followed by pore formation resulting in rapid killing of the target cell [105].

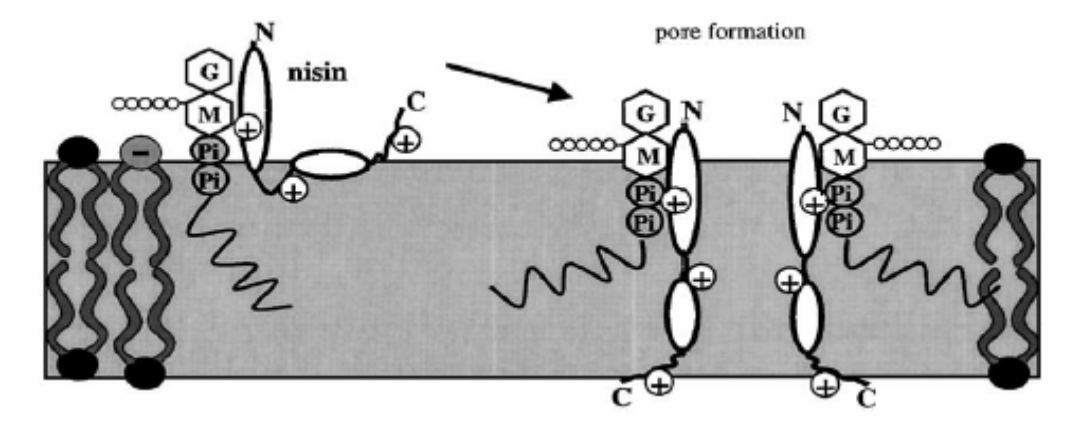

Figure 1.5. A schematic representation of the mode of action of nisin [104].

A number of genes are involved in the production and export of nisin as well as immunity [80]. These genes are tightly linked together in the nisin cluster made up of a total of 11 genes of which *nis*A encodes the nisin precursor itself. Interestingly, the genes responsible for nisin A production and immunity are carried on a 70-kb conjugative transposon called Tn5301 from *L. lactis* NCFB894 [106,107] or Tn5276 from *L. lactis* NIZO R5 [85], while the genetic determinants for nisin Z are on the transposon Tn5278 [108]. These transposons also encode sucrose utilization genes *sac*A, *sac*B and *sac*R. Nisin synthesis is regulated by a two-component regulatory system made up of the membrane-bound histidine kinase sensor protein NisK and the regulator NisR. This regulatory system responds to extracellular nisin, which leads to the expression of genes involved in immunity and synthesis/posttranslational modification [109]. Indeed, this regulatory system is the basis for the nisin-induced controlled expression system (NICE) which is a very useful overexpression system for heterologous expression of proteins in many Gram-positive bacteria [110].

1.4.4. Lacticin 481

Lacticin 481 is a lactococcal lantibiotic [111] representative of type AII lantibiotics [81] or of the so-called lacticin 481 subgroup [112,113], depending on the classification used (Figure 1.6). Lacticin 481 is significantly different, both biophysically and biochemically from nisin, it has a narrower spectrum of activity, a higher stability at neutral pH, a different structure and another system of immunity.

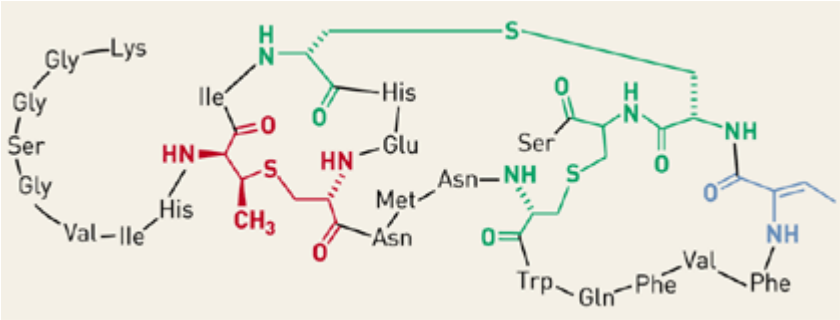

Figure 1.6. Structure of lacticin 481

The lacticin 481 gene cluster consists of the single six gene operon lctAMTFEG [77,114-116]. Whereas the first three genes are responsible for lacticin 481 biosynthesis (*lct*A encodes the lacticin 481 precursor peptide, which modified and secreted by the lctMT products) [114,115,115], *lct*FEG encode an immunity system protecting the producer strain from its own bacteriocin [115]. Counterparts of these genes are found in many other lantibiotic gene clusters [67], but the lacticin 481 operon is unusual since it does not include any regulator gene [116,117]. It is thus the simplest characterized lantibiotic gene cluster, since seven 13 genes have been found in other cases [67,72,91,118]. As the transcriptional organization of the lacticin 481 operon was investigated, it was shown that lacticin 481 production is stimulated by low pH and that this regulation occurs via the two main promoters of the operon [114,115].

1.5. Industrial Importance of Bacteriocins

There is a significant dilemma at present in modern food processing in that there is an expectation for long shelf-life and safety of foods and beverages, at a time when consumer preferences are veering towards foods which are minimally processed and free from chemical preservatives (due to safety concerns). Consequently, there has been renewed interest in so-called ‘‘green technologies’’ including novel approaches for minimal processing and the exploitation of microbial metabolites such as bacteriocins for biopreservation. Undoubtedly, the most well-known and studied bacteriocin is nisin, the lantibiotic which has found application as a shelf-life

extender in a broad range of dairy and nondairy products worldwide, ranging from processed and cottage cheese to dairy desserts and liquid egg [18]. This bacteriocin was also found to be effective in such applications as inhibiting spoilage bacteria during beer and wine fermentations, while the exploitation of nisin-producing strains has been shown to improve certain vegetable fermentations. The success of nisin has prompted many research groups to search for novel bacteriocin-producing strains over the last 20 years. This has resulted in a growing arsenal of potential biopreservatives which may be used either singularly or in combination to protect food from spoilage and safety problems. These biopreservatives can be used in a number of ways in food systems, ranging from the use of the bacteriocin-producing strains directly in food as starter or protection cultures to the use of concentrated bacteriocin preparations as food additives [105].

1.5.1. Nisin as a biopreservative

Since nisin is produced by a lactococcal culture, one of the principal applications of nisin-producing strains is in the manufacture of cheese, where it has been investigated for the inhibition of both spoilage and pathogenic microorganisms. From a food safety point of view, the pathogen of primary concern in a number of cheeses is *L. monocytogenes*, which is capable of growing at refrigeration temperatures [119] and has the ability to survive the acidic conditions of cheese manufacture [120]. Moreover, the pathogen can also resume growth in cheeses exhibiting a pH rise during ripening, such as on the surface of mould-ripened cheese. Furthermore, *Listeria* has been shown to survive the manufacturing process of cottage cheese, Camembert and Cheddar [121]. Indeed, listeriosis outbreaks linked to the consumption of contaminated dairy products are well documented [122]. A number of studies have been performed nisin-producing cultures demonstrating the effectiveness of nisin for the inhibition of *L. monocytogenes* in cheese [123-125]. Nisin has also been demonstrated to be effective in a range of food products which include processed cheese and cheese spreads, milk products, canned foods, fish and meat products, brewing, wine manufacture, liquid egg and confectionery.

1.5.2. Future prospects

Fuelled mainly by the success of nisin, recent and enormous research efforts have led to a wealth of information on both modified and unmodified bacteriocins. Despite this, an understanding of the relevant molecular mechanisms is far from complete. This is especially true for the functions of dehydrated amino acids, the enzymatic reactions responsible for their formation, and mechanism of action which remain unanswered. The understanding of such molecular mechanisms will be particularly important in the area of biotechnology. Already, the potential of genetic engineering for rational drug design has been demonstrated for subtilin [126], resulting in enhanced stability and activity. With the continuing development of expression systems, the production of mutant peptides will accelerate our knowledge of the biological activities of lantibiotics. Moreover, it may one day be possible to introduce modification systems *in vitro* to lantibiotic producers with the aim of obtaining novel, modified peptides displaying unique properties. Although the commercial exploitation of bacteriocins to date is mainly restricted to the food applications of nisin, potential novel applications for lantibiotics and unmodified bacteriocins continue to be developed [127]. In particular, the problems such as low production levels and instability of bacteriocins in certain foods/environments need to be addressed. In addition, the cytolytic abilities of these peptides must be assessed, especially in the wake of the identification and characterisation of the haemolysin/bacteriocin, namely cytolysin, produced by *E. faecalis*. Possibly one of the main obstacles to the use of other bacteriocins in food is a regulatory one. To this end, use of bacteriocin-producing cultures in food might be of considerable advantage over using purified bacteriocin preparations as food additives. In these respects, bacteriocins, both modified and unmodified, deserve further, scientific attentions.

1.6. Objectives of the Present Study

Bacteriocin production has been well documented for most of the LAB and has been reviewed by various researchers [18,24,63]. Many bacteriocins produced by LAB are active not only against other LAB, but also against foodborne pathogens. This makes them attractive for use as natural food preservatives. Nisin has thus far been the only bacteriocin to find wide spread application in the food industry. Potential use of bacteriocins as biopreservatives has been studied in meat, dairy products or vegetable products [92]. Besides being used as natural preservatives, bacteriocin producing LAB were used as starter cultures in dairy fermentations to control the fermentation and achive high quality, as well.

The objective of this study is to characterize and identify bacteriocins from two different local isolates of *Lactococcus lactis* subsp. *lactis* strains. Our aim is to determine the activity spectra of these bacteriocins, their biochemical characteristics, molecular weights and their genetic nature. Bacteriocin-producing lactic acid bacteria studied in the present work will extend the number of available cultures, and probably the number of available bacteriocins, offering a useful biopreservation of the traditional fermented milk products in Turkey.

CHAPTER II

MATERIALS AND METHODS

2.1. Bacterial Cultures and Media

In this study, two bacteriocin producing *L. lactis* subsp. *lactis* isolates from Ankara University Faculty of Agriculture, Food Engineering Department were used. Indicator strains used for the characterizaion bacteriocin were obtained from Laboratory of Microbial Gene Technology, NHL, As, Norway.

L. lactis subsp. *lactis* strains OC1 and OC2, isolated from raw milk, were grown in M17 [128] medium supplemented with 0.5% glucose (GM17) at 30^{o}C. *Listeria innocula*, *Pediococcus pentosaceus*, *Enterococcus* and *Lactococcus* strains were grown in GM17 Broth at 30^{o}C. *Lactobacillus* strains were grown in Man-Ragosa-Sharp (MRS) medium (Oxoid Ltd., UK) at 37^{o}C. *Bacillus cereus*, *Staphylococcus cornosus*, *Pseudomonas fluorescens*, *Salmonella enterica typhimurium*, *Escherichia coli* were grown in Tryptic Soy medium (Merck, Germany) at 37^{o}C. Bacterial stocks were stored at -80^{o}C in their respective broths supplemented with 20% glycerol.

2.2. Detection of Antimicrobial Activity

Bacteriocin producing *L. lactis* subsp. *lactis* strains were grown at 30°C for overnight in M17 broth medium. By using sterile toothpicks, *L. lactis* subsp. *lactis* strains were transferred to glucose-M17 plates. After growth overnight, an indicator lawn of 3 mL of soft glucose-M17 agar (0.5% agar), containing 100 μL of an overnight indicator strain was poured on the surface. Colonies were examined for inhibition zones at the end of overnight incubation [129].

2.3. Effect of Heat, Enzymes, and pH on Bacteriocin Activity

To determine the effect of pH on bacteriocin activity, cell free culture supernatant (CFNS) was adjusted to pH levels between pH 2.0 and 11.0 by using 6 N NaOH or HCl. Samples were assayed for activity after 24 h at 4°C, using the critical dilution method (Figure 2.1). *Lactococcus lactis* subsp. *lactis* SIK83 was used as the indicator strain for OC1 and *Lactococcus lactis* JC17 was used as the indicator strain for OC2. As the control for pH inhibition, the samples treated with proteinase K before adjusting pH were tested against the same indicator.

To evaluate the effect of heat on bacteriocin activity, CFNS was heated at 100°C for 5, 10, 15 and 20 min and 121°C for 15 minutes. CFNS was also treated with the following enzymes at a final concentration of 1mg/mL: trypsin (pH 7.0, Merck, Germany), α-chymotrypsin (pH 7.0, typeII, Sigma, Chem. Co. USA), proteinase K (pH 7.0, Sigma), pepsin (pH 3.0, Sigma), catalase (pH 7.0, Sigma), lipase (pH 7.0, Sigma), α-amylase (pH 7.0, Sigma) and lyzozyme (pH 7.0, Sigma). Following incubation at 37°C for 2 h, enzyme activity was terminated by heating at 100°C for 5 min. Untreated samples were used as the controls [130]. After heat or enzyme treatment, the remaining bacteriocin activity was assayed by the critical dilution method. One arbitrary activity unit (AU) was defined as the reciprocal of the highest dilution yielding a clear zone of inhibition on the indicator lawn, and was multipled by a factor of 100 to obtain the AU/mL of the sample [130].

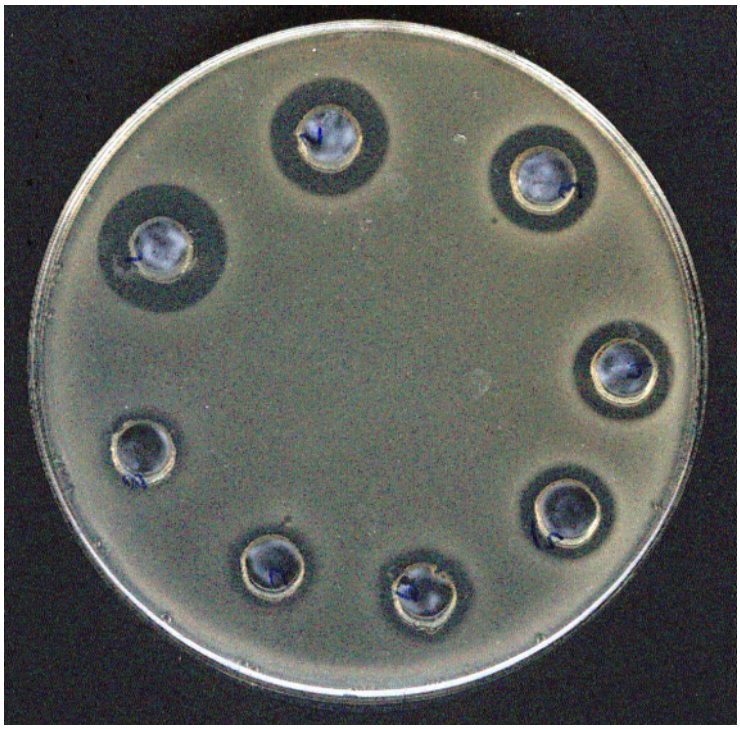

Figure 2.1. A typical result from the critical dilution method.

2.4. Purification of Bacteriocins

All the purification steps were performed at room temperature, and all the chromatographic equipment was obtained from Amersham Pharmacia Biotech. Uppsala, Sweden. Bacteriocin purification was done via four-step procedure by using 1 L cell-free supernatant of 18 h culture in GM17 medium. Bacteriocin was precipitated from the supernatant by adding 400 g ammonium sulphate and the precipitate was collected by centrifugation at 10.000 x *g* for 30 min and resuspended in 10 mmol.L^{-1} sodium phosphate buffer (pH 5.0); loaded on a column of SP-Sepharose (Amersham Pharmacia Biotech. Uppsala, Sweden) equilibrated with 10 mmol.L^{-1} sodium phosphate buffer (pH 5.0). Bacteriocin was eluted with 1 mL of 1 mol.L^{-1} NaCl [131].

2.5. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis of bacteriocins was performed as described by Laemmli [132]. Before electrophoresis, glass plaques for gel preparation were cleaned with 70% ethanol and dried. 15 mm thick spacers were placed between the glass plaques and 1% agarose was pipetted and solidified around the spacers to avoid the possible leakage of gel solutions. The glass plaques were than placed into the electrophoresis tank and separation gel solution was pipetted between the glass plaques up to gel-comb level. Distilled water was added immediately after separation gel solution pipetted. After the polimerization of separation gel, water was discarded by a filter paper. Stacking gel solution was pipetted and the gel-comb was placed. After polimerization electrode buffer was poured into the electrophoresis tank and the gel-comb was removed. 25 µL of the samples containing purified bacteriocins were loaded to the stacking gel. Electrophoresis was performed at 25 mA / 100-120 V for 30 min (the time for protein bands to reach the separation gel) and 35 mA/300 V for 2.5 h. The gel was removed from the glass plaques and stained overnight with Comassie blue dye solution. Gel was destained at room temperature for 1-1.5 h.

2.6. Total DNA Isolation

Total DNA was extracted according to protocol of Head *et al*. (1993) and Utaker *et al.* (1998). 1-5 mL cell culture was grown up overnight (volume depends on cell density). The cells were harvested by centrifugation and washed once with TE buffer (10 mM-Tris/HCl, 1 mM EDTA, pH 8.0). The pellet was resuspended in ~100 µl lysis buffer (containing 1%, v/v, Tween 80), volume depending on the amount of cells. Resuspended pellet transferred to another microfuge tube. The tubes were locked and placed in an open plastic rack. The whole rack was then placed on liquid nitrogen. The rack was left until all the samples are frozen. After that, they were thawn on a boiling water bath. The last two steps were repeated five times, after which 1-5 µL of the sample was used as template in PCR amplification [133,134].

2.7. Identification of the Respective Genes of the Bacteriocins by PCR Amplification

PCR amplification was carried out to detect the structural genes of nisin, lacticin 481 using the primers listed in Table 2.1. Reaction parameters were 94^{o}C for 45 s, 56^{o}C for 45 s and 72^{o}C for 1 min, for a total of 30 cycles. Amplified fragments were visualized on 1% agarose gels by staining with ethidium bromide using 100 bp ladder (Gibco, BRL) as molecular weight Standard [135].

Table 2.1. Sequences of oligonucleotide primers used for the identification of bacteriocin encoding genes.

Primer	Specifity	5'-sequence-3'	Size	Reference
1	Nisin	AAGAATCTCTCATGAGT	898	[135]
2	Nisin	CCATGTCTGAACTAACA		
3	Lactisin481	TGATTTCGAAGGTAAG	585	[136]
4	Lactisin481	AAAGCTTTACCTGTACT		

2.8. Plasmid DNA Isolation

2.8.1. Plasmid Extraction

The strains from which plasmid DNA was to be extracted were grown overnight (18h) in the appropriate M17 broth at 30°C. The resulting culture provided a 10% inoculum for a M17 broth growth medium. Strains were propagated for 3-3.5 h at 30°C and were harvested by centrifugation (10,000 rpm, 10 min). Dried pelleted cells were resuspended in 379 µL sucrose buffer (6.7% sucrose-50 mM Tris-1 mM EDTA, pH 8.0) and warmed to 37^{o}C. 96.5 µL lysozyme solution was added (10 mg/mL in 25 mM Tris, pH 8.0) and incubated in 37^{o}C waterbath for 5 min. After application of 48.2 µl Tris-EDTA-1 (0.25 M EDTA-50 mM Tris, pH 8.0), 27.6 µL SDS solution (20% [wt/vol] in 50 mM Tris-20 mM EDTA, pH 8.0) was added to the tubes. In this stage, an increase in viscosity of the medium shows that the lysis has

begun. Tubes were incubated for 10 min at 37°C for complete lysis. At the end of this time, tubes were vortexed at highest setting for 30 s to break chromosomal DNA. 27.6 μL of freshly prepared 3.0 N NaOH was added and mixed gently by intermittent inversion or swirling for 10 min for alkali denaturation of chromosomal DNA. After denaturation, 49.6 μL 2.0 M Tris-Cl, pH 7.0 solution was added to centrifuge tubes and mixing was continued for 3 minutes. 71.7 μL 5 M NaCl kept at 4^{o}C and 700 μL phenol saturated with 3% NaCI were added into the tubes and the tubes were mixed thoroughly and centrifuged at 4^{o}C 15000 rpm for 20 minutes. The upper phase was removed and extracted with 700 μL isoamyl-alcohol (24:1) solution. Again the upper phase was removed and precipitated with equivalent volume of isoropanol. The extracts were kept at –20^{o}C for overnight. The plasmid DNA was precipitated by centrifugation at 15,000 rpm for 20 minutes. In the last stage, liquid phase was poured off and DNA precipitate was dried. Excess propanol was removed and precipitates were resuspended in 20 μL of 10 mM Tris-1 mM EDTA-2, pH 7.5. Before examining by agarose gel electrophoresis, precipitates were incubated at 37^{o}C for 45 min by adding 2 μL of RNase A stock solution [137].

2.8.2. Agarose gel electrophoresis

DNA samples were subjected to electrophoresis in 0.7% agarose gels. Agarose was dissolved in Tris-asetate electrophoresis buffer in boiling waterbath. The solution was cooled to 45^{o}C and poured into the electrophoresis tanks to introduce approximately 30-50 mL. A 2 μL dye solution consisting of bromophenol blue (0.07%), SDS (7%), and glycerol (33%) in water was added at 5 μL per sample to DNA samples prior to electrophoresis. Electrophoresis was carried out at 60 mA, 100 V, for 3-3.5 h until the dye reached to the bottom of the gel. The gel was then placed in a solution of ethidium bromide in water (0.2 μg/mL) and stained for 1 h.

2.9. Plasmid Curing

Plasmids were cured by using acriflavine (AF; Sigma Chem. Co., USA) [138]. Two loopsful of a 24 h-old culture in lactic broth of each organism were inoculated into 2.0 mL of lactic broth containing various concentrations of AF. After 24 h at 21, 32, or 37^{o}C, each culture was diluted and spread over the surface of the fast-slow differential agar for total bacterial count and for bacteriocin (-) mutants. For the determination of the direct effect of AF on lactose-fermenting cells of *L. lactis* strains, a culture was diluted to contain 50 to 100 colony-forming units (CFU) per 0.1 mL of the test solution. The test solution consisted of 0.2 mL of lactic broth containing 1.2 g of AF. The tubes were incubated at 32^{o}C for 3 hr, and the 0.2 mL samples were spread over the surface of the indicator agar. Control tubes, plated after 1.5 hr at 32^{o}C, lacked AF and initially contained 10 to 20 CFU per 0.1 mL. Cultures were incubated at 32^{o}C after receiving an inoculum to give ca.103 CFU per mL. The samples were taken at periodic intervals and diluted. The total count was determined by using pour plates of lactic agar. The plates were incubated at 32^{o}C for 48 hr.

2.10. Conjugation

Recipient and donor strains were grown in M17 broth medium at 30^{o}C for 18 h. In conjugation trials, dilutions of 10^{-1} level were used from these active cultures. For the recipient strain *L. Lactis* subsp. *cremoris,* streptomycin (500 μg/mL) and rifamycin (50 μg/mL) were added to this medium. From these dilutions, 2 mL the of donor and 3 mL of the recipient culture were taken and mixed, after that of the cells were collected on sterile membrane filters (0.45 μm, Sartorius, Germany). The filters containing the recipient and donor cells were put on the M17 agar plates, and kept at 30^{o}C for 18 h. At the end of this time filters were taken from M17 agar plates and dissolved in 1ml sterile Ringer's solution and serial dilutions were performed to 10^{-8} level. For each dilution, the fast-slow differential agar plates containing the antibiotics (to which recipient strains used are resistant) were streaked, and left for incubation at 30^{o}C for 48 h. The conjugation frequency was determined according to

the ratio of the number of transconjugants per mL to the number of donors per mL. The donor strains grown on fast-slow differential agar that did not contain any antibiotic constituted the controls. During the conjugation process, filters were treated with 2 mL DNase I (100 μg/mL, Sigma Chem. Co., USA) solution and kept at 37^{o}C for 15 minutes in order to prevent a possible transformation. In addition, the sterile physiologic waters that were used to dissolve the membrane filters were treated with DNase I (100 μg/mL) [139,140].

2.11. Determination of Lactose Fermentation and Proteolytic Activity

In the Lactococcus strains, lactose fermentation and proteolytic activity were determined on Fast-Slow Differential (FSD) agar [141] and Milk agar plates [142].

Lactococci strains were inoculated onto FSD agar plates under anaerobic conditions (Oxoid anaerobic system), and grown at 30^{o}C for 48 h. At the end of the incubation, lactose fermentation (Lac^{+}) and proteolytic activity (Prt^{+}) of colonies were defined according to the morphologic and cultural properties of the colonies. The Lac^{+} and Prt^{+} colonies were defined according to the criteria of a colony diameter of 2-3 mm, white concave morphologic structure and red zone around the colony; the Lac^{-} and Prt^{-} colonies were defined according to criteria of having the diameters of less than 1mm, flatness, half transparency and no red zone around the colonies [141,143].

Lactose fermentation and proteolytic activity were determined also in Milk agar medium to make a double check. The strains were inoculated onto the Milk agar and grown at 30^{o}C for 48 h. At the end of incubation the colonies that turn from night-blue to yellow in colour (due to acid production) were defined as Lac^{+}. Prt^{+} strains in this medium were determined by the presence of clear zones around the colonies.

CHAPTER III

RESULTS & DISCUSSION

3.1. Detection of Antimicrobial Activity

Both of the lactococcal isolates (*Lactococcus lactis* subsp. *lactis* OC1 and OC2) were found to show inhibitory activity against a broad range of bacteria including lactobacilli, lactococci and staphylococci (Table 3.1). In addition, they showed inhibitory activity against *Listeria innovia*, and *Enterococcus feacalis* and *Pediococcus pentosaceus*. No inhibitory activity could be detected against *Bacillus cereus* in the strains. While activity against *Staphylococcus aureus*, *Enterococcus faecalis* NCDO581 and lacticin 3147 producers was seen in OC1 strain, no activity was observed in the OC2 strain against those indicator organisms.

When compared with the respective control strains (*Lactococcus lactis* SIK83: nisin producer, *Lactococcus lactis* JC17: lacticin 481 producer), OC1 and SIK83 showed similar inhibitory activity against indicator organisms except for *Bacillus cereus* FM1. *Bacillus cereus* FM1 was resistant to the bacteriocin produced by OC1, but sensitive to SIK83 (nisin producer-control). Similarly, OC2 and JC17 showed similar inhibitory activity against indicator organisms except for *Lactococcus lactis* SIK83, *Enterococcus faecalis* and *Staphylococcus cornosus* MC1B. *Lactococcus lactis* SIK83, *Enterococcus faecalis* and *Staphylococcus cornosus* MC1B were resistant to the bacteriocin produced by OC2, but sensitive to that produced by JC17.

These differences in inhibitory spectra of bacteriocins of OC1 and OC2 may lead to a misinterpretation in that the bacteriocins produced by these strains are different than nisin and lacticin 481. In literature, such differences in inhibitory spectra were generally attributed to other products of LAB such as organic acids, hydrogen peroxide, diacetyl and inhibitory enzymes [144-146]. Thus, interpretation of spectra of inhibitory activity in terms of specific bacteriocin activities can sometimes be difficult if the producer strains release more than one bacteriocin-like agent [129, 145] or if inhibitory activities of other metabolic products such as acids and hydrogen peroxide are not eliminated [147].

Table 3.1. Inhibitory spectrum of bacteriocin producing *Lactococcus lactis* strains

	TESTED STRAINS		CONTROL STRAINS	
INDICATOR STRAINS	OC1	OC2	SIK83	JC17
Lactococcus lactis SIK-83 (nisin producer)	-	+	-	-
Lactobacillus sake NCDO2714 (nisin sensitive)	+	+	+	+
Lactococcus lactis IL1403 (nisin sensitive)	+	+	+	+
Enterococcus faecalis (nisin sensitive)	+	+	+	-
Listeria innovia (nisin sensitive)	+	+	+	+
Lactococcus lactis 105 (lactisin producer)	+	-	+	-
*Lactococcus lactis*1 (Bac-)	+	-	+	-
Lactococcus lactis T1 (Bac-)	+	+	+	+
Lactococcus lactis 731 (lactisin 3147 producer)	+	-	+	-
Lactococcus lactis 2 (lactisin 3147 producer)	+	-	+	-
Lactococcus lactis 1(lactisin 3147 producer)	+	-	+	-
E.coli CFAI (ETEC)	-	-	-	-
Salmonella enterica typhimurium SL1344	-	-	-	-
Pseudomonas fluorescence P1 (Matfask)	-	-	-	-
Lactobacillus plantarum	+	+	+	+
Lactococcus lactis JC17 (lactisin481 producer)	+	-	+	-
Bacillus cereus FM1	-	-	+	-
Staphylococcus cornosus MC1B	+	+	+	-
Pediococcus pentosaceus FBB61.1 (pediocin producer)	+	+	+	-
Staphylococcus pentosaceus FBB61.1 (pediocin producer)	+	-	+	-
Enterococcus faecalis NCDO581	+	-	+	-
S. aureus FRI100	+	-	+	-
L. cremoris (LcnA+B producer clone) 2132	+	+	+	+
Lactococcus lactis	-	+	-	+

+ : sensitive
- : resistant

Supernatants of both strains were cross reacted with their respective controls and other bacteriocin producing strains (Table 3.2). For the strain OC1, there was no cross reactivity with nisin and for OC2 there was no cross reactivity with lacticin 481. Thus, OC1 was immune to nisin and OC2 was immune to lacticin 481 at the concentration used in the study, and the strains were immune to their own culture supernatants (data not shown).

Table 3.2. Cross test of bacteriocins

	TESTED STRAINS			
INDICATOR STRAINS	SIK83	731	JC17	2132
OC1	-	+	+	+
OC2	+	+	-	+
SIK83	-	+	+	+
JC17	+	+	-	+

+ : sensitive

- : resistant

Bacteriocin production is invariably linked to the expression of specific immunity proteins required to protect the producing strain against the inhibitory action of its own product [1,148]. To date, the mechanisms by which these proteins confer immunity remain relatively unknown. Two distinct systems of lantibiotic immunity have been identified to date. Protection can be mediated by the first of these, the so-called ‘immunity’ proteins, LanI [53,83,87,149-151], while the second constitute specialised ABC-transport proteins, LanFEG [52,53,72,152], which can be encoded on two or three separate ORFs.

3.2. Effect of Heat, Enzymes, and pH on Bacteriocin Activity

The effect of heat and enzymes on bacteriocins is shown in Table 3.3. The antagonistic compounds produced by the lactococcal isolates were all considered heat stable, as activity remained even after heating at 121 ^{o}C for 15 min (Table 3.3). Inhibitory compounds of both lactoccocal strains had the same enzyme inactivation pattern. They were inactivated by α-chymotrpsin and proteinase K, but not by trypsin, lipase, catalase or lysozyme. This confirmed that the inhibitory compounds had a proteinaceous nature and must be classified as bacteriocins.

Table 3.3. Effect of enzymes and heat treatment on bacteriocin activity

	BACTERIOCIN ACTIVITY (AU/mL)			
TREATMENT	**OC1**	**OC2**	**SIK-83**	**JC17**
Control	12800	3200	12800	3200
α-Chymotrypsin (Sigma, no C-6423)	0	0	0	0
Trypsin (Merck, no T-8253)	6400	3200	6400	3200
Proteinase K (Sigma, no P-0390)	0	0	0	0
α-Amylase (Sigma, type VIIA)	0	0	12800	3200
Lipase (Sigma, no L-17714)	12800	3200	12800	3200
Catalase (Sigma, noC-10)	12800	3200	12800	3200
Lysozyme (Sigma no L-7651)	12800	3200	12800	3200
100'C 5 min	12800	3200	12800	3200
100'C 10 min	12800	3200	12800	3200
100'C 15 min	12800	3200	12800	3200
100'C 20 min	6400	3200	6400	3200
121'C 15 min	3200	1600	3200	1600

Tramer (1964) reported that nisin retained its activity after autoclaving at 115.6^{o}C at pH 2.0 [153]. Autoclaving nisin in solution at pH 6.8, however, decreased activity by 90% [153]. In our study, the activity of nisin-like bacteriocin produced by lactoccus lactis OC1 strain also decreased after autoclaving neutralised culture supernantants, but the decrease in its activity was not as high as 90%. It was previously noted that large molecules present in milk and broth had a heat protective effect, so that the degree of inactivation of nisin in these media may be less pronounced than in buffer [32]. OC2 was relatively more heat resistant. Its biological activity was unchanged after 20 min at 100^{o}C, but 50% of its activity was lost after 15 min at 121^{o}C. Similar

results were obtained for lacticin 481 by Piard *et al*. (1990). The activity did not change after 1h at 100^{o}C at pH 4.5 or 7.0, but the activity loss detected after 20 min at 115^{o}C was 50% [154].

The bacteriocin produced by the strain OC1 was resistant to inactivation by trypsin as was reported earlier for nisin [155]. However, bacteriocin activity disappeared upon treatment with α–chymotrypsin, as was also the case with nisin [32,156]. The chemical nature of lacticin 481 was first studied by testing the ability of various enzymes to inactivate it [155]. Partial or total inactivation of the bacteriocin produced by OC2 by non-specific proteinases, e.g proteinase K, confirmed its proteinaceous nature, as was also the case in lacticin 481. The ability of α–chymotrypsin but not trypsin to eliminate the activity of lacticin 481 suggested the presence of aromatic amino acids and the absence of basic amino acids in the primary sequence [156].

One drawback of bacteriocins produced by OC1 and OC2 was that they were inactivated by α-amylase. The activity of OC1 and OC2 bacteriocins was lost upon treatment with α–amylase which is not seen in the case of control strains. Although by definition all bacteriocins are made of proteins, some have been reported to consist of combinations of different proteins or are composites of proteins together with lipid or carbohydrate moieties. These bacteriocins form large complexes with other macromolecules [8]. The existence of this class was supported mainly by the observation that some bacteriocin activities obtained from cell-free supernatant, exemplified by the activity of *Lactobacillus plantarum* LPCO10, were abolished not only by protease treatments, but also by glycolytic and lipolytic enzymes [157]. However, presently, no such bacteriocins have been purified and there is good reason to believe that this type of bacteriocin can be an artifact due to cationic and hydrophobic properties of bacteriocins which result in complexing with other macromolecules caused by interaction between constituents from the cells or growth medium. This view is strongly supported by the experiments which showed that proper purification of such activities indeed leads to the isolation of regular peptide bacteriocins [157]. For example, plantaricin S was first claimed to be a large

complex molecule, but later the activity was purified as a small peptide, and the complex disintegrated while the activity was maintained [157].

Taken together, the loss of bacteriocin activities of OC1 and OC2 when treated with α-amylase shows that these bacteriocins are active only when they form aggregates with carbohydrate residues. As mentioned above, the main bacteriocin activity results from peptide units. When carbohydrate residues that bond these peptide units to each other are isolated, peptides cannot reach to sufficient concentration to show their inhibitory activity in liquid medium [25,157].

Table 3.4. Effect of pH on bacteriocin activity

pH	BACTERIOCIN ACTIVITY (AU/mL)			
	OC1	**OC2**	**SIK-83**	**JC17**
2	25600	3200	25600	3200
3	25600	3200	25600	3200
4	25600	3200	25600	3200
5	25600	6400	25600	6400
6	12800	6400	12800	6400
7	12800	6400	12800	6400
8	6400	6400	12800	6400
9	6400	3200	6400	3200
10	6400	3200	6400	3200
11	1600	1600	1600	1600

The effect of pH on bacteriocins produced by OC1 and OC2 is shown in Table 3.4. The OC1 was the most active at pH < 5.0. Activity decreased, but could still be detected at higher pH values. The nisin molecule is acidic in nature and exhibits greatest activity under acidic conditions [158,159]. It was reported that the nisin activity decreased drastically and lost at basic pH values between 8.0 and 11.0 [160]. The other bacteriocin produced by OC2 was the most active between pH values of 5.0 and 8.0. Activity decreased below pH 5.0 and above pH 8.0, but could be still detectable. The pH stability of the OC1 and OC2 bacteriocins was identical to that of the bacteriocins (nisin and lactisin 481) produced by *Lactococcus lactis* SIK83 and *Lactococcus lactis* JC17, used as experimental controls, respectively (Table 3.4).

3.3. Determination of the Molecular Weight of the Purified Bacteriocins by SDS-PAGE

The purified bacteriocins were run on SDS-PAGE and their molecular weights are determined using standard molecular weight markers. The molecular weights of the bacteriocins produced by the strains OC1 and OC2 was found to be 3.5 kDa and 3.4 kDa, repectively. The purified controls, nisin and lactisin 481 were also 3.5 and 3.4 kDa in their molecular weight, respectively as found previously by Gross & Morell [161,162] and Piard *et al*., Lui & Hansen [154, 160].

Total soluble protein patterns of the *Lactococcus lactis* SIK83 and JC17 which were the reference bacteriocin producing strains showed a high degree of similarity, confirming the identity of the bacteriocins of the local isolates to nisin and lactisin 481, respectively.

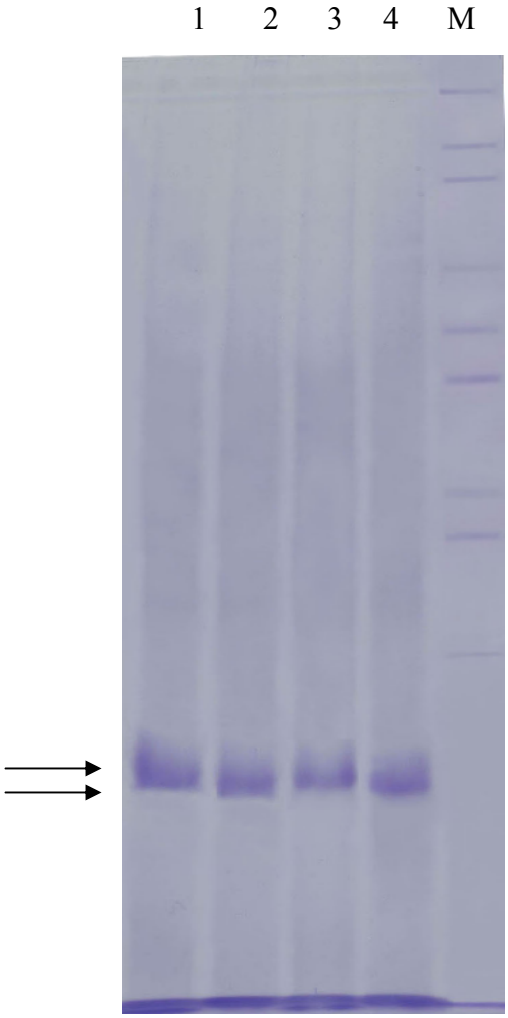

Figure 3.1. SDS-PAGE analysis of bacteriocins from OC1 (lane1), OC2 (lane2), SIK83 (lane3) and JC17 (lane4). Marker lane contains proteins of 180, 116, 97, 58.1, 39.8, 29.0, 20.1, 4.3, 6.5 kDa, respectively.

3.4. Amplification of Bacteriocin Genes by PCR

PCR has been a powerful tool to identify bacteriocins [163-166]. In our study, PCR using the primers specific to nisin and lactisin 481 led to a fast identification of bacteriocins produced by OC1 and OC2. The PCR amplification using OC1 and OC2 total DNA as the templates yielded 536 bp and 300 bp fragments, respectively. The fragments were of expected sizes, as amplified also from the total DNA of the standard strains SIK83 and JC17, the producers of nisin and lacticin 481,

respectively. Thus, the two bacteriocin-producing lactococci were identified as nisin and lacticin 481 producers according to the results of the PCR analysis, OC1 containing *nis*Z gene and OC2 harbouring the structural gene of lactisin 481.

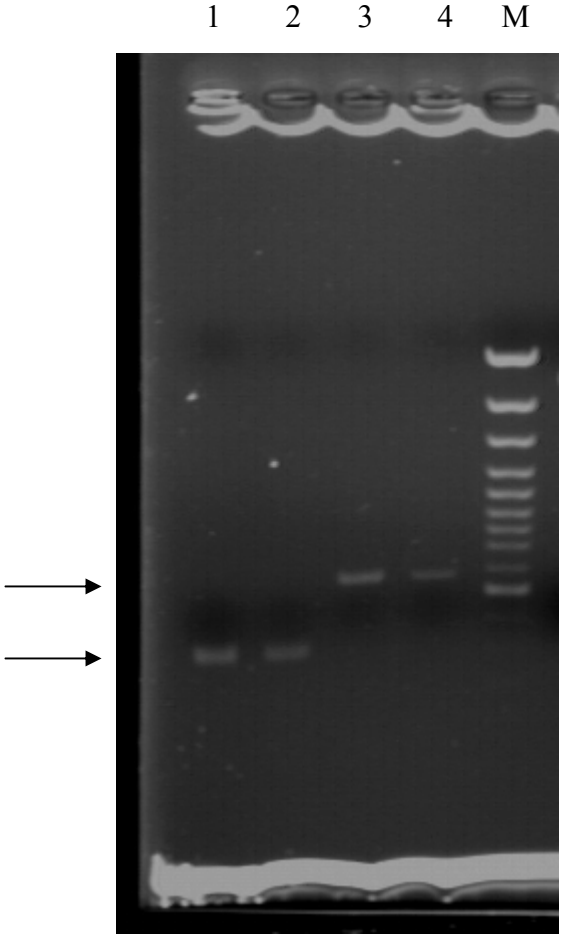

Figure 3.2. PCR amplification of bacteriocin gene fragments from total genomic DNAs of OC1 (lane3), SIK83 (lane4), OC2 (lane1), JC17 (lane2) strains. *Nis*Z gene specific primers were used for OC1 and SIK83 total DNA and lacticin 481 gene specific primers were used for OC2 and JC17 total DNA. The marker DNA fragments were of 3000, 2000, 1500, 1200, 1030, 900, 800, 700, 600 and 500 bp, respectively.

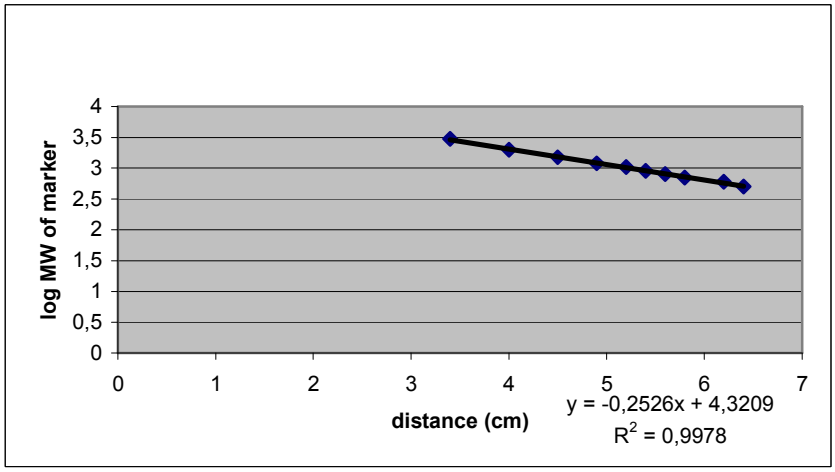

Figure 3.3. Log MW of the marker DNAs versus migration (distance) in cm calibration curve for the gel run for the detection of PCR products.

3.5. The Genetic Nature of Industrially Important Characteristics of *Lactococcus lactis* strains

In certain *Lactococcus lactis* strains some industrially important properties such as sucrose utilization, nisin production and proteolytic activity are encoded on the same plasmid [8,87]. Similarly, lactose fermentation, lacticin 481 and immunity genes are also encoded by a specific plasmid [167,168]. Genetic analysis of industrially important characteristics (lactose fermentation, nisin production and proteolytic activity) in nisin and lacticin 481 producer two *Lactococcus lactis* subsp. *lactis* strains were made by using plasmid curing method. Examination of plasmid contents of the strains OC1 and OC2 capable of producing nisin and lacticin 481, respectively, showed that *Lactococcus lactis* subsp. *lactis* OC1 strain has 9 distinct plasmids with MWs varying between 2.0 and 39.7 kb and *Lactococcus lactis* subsp. *lactis* OC2 strain has 9 plasmids with MWs varying between 2.0 and 23.3 kb. Our plasmid curing attempt with wild OC1 strain resulted in the loss of 39.7 kb plasmid as well as the formation of mutant phenotypes in that the cured derivatives were unable to ferment lactose and incapable of nisin and protease production. This indicated that these three characteristics were linked to the 39.7 kb plasmid. Likewise, the loss of

16.0 kb plasmid in the strain OC2 was associated with the the loss of the ability to ferment lactose and lacticin 481 production (Figure 3.4).

These results suggested that lactose fermentation, nisin production and proteolytic activity might be encoded by the 39,7 kb plasmid in *L. lactis* subsp. *lactis* OC1 whereas lacticin 481 production and lactose fermentation ability might be encoded by the 16.0 kb plasmid in *L. lactis* subsp. *lactis* OC2.

In order to confirm above-mentioned plasmid association of industrially important traits of Lactococci, conjugation trials were made. As a consequence of these trials, one plasmid from each of the wild strains OC1 and OC2 was found to be transferred to the recipient *Lactococcus lactis* subsp. *cremoris* MG1363 incapable of producing bacteriocin and possessing no plasmids. The transconjugants became lac^+, prt^+ and nis^+ via the transfer of 39.7 kb plasmid from OC1 strain to MG1363 strain. This proved that lactose fermentation ability, proteolytic activity and nisin production are encoded by the 39.7 kb plasmid in OC1 (Figure 3.6). Similary; the transconjugants of the cross between OC2 and MG1363 became $lct481^+$ and lac^+ via the transfer of the 16.0 kb plasmid from OC2 to MG1363 (Figure 3.7), proving that lactose fermentation ability and lacticin 481 production characters are encoded by the 16.0 kb plasmid in OC2.

Various reseachers have found that nisin genes are present on a number of conjugative plasmids [77,86,87,106,107] usually a ~70 kb plasmid which also harbour the genes for sucrose utilisation and which integrate into the recipient chromosome following conjugal transfer [8]. In one publication [77], lacticin 481 gene clusters has been showed to be present on a composite transposon, Tn5271, on a 70 kb plasmid. Lacticin 481 production was found to be linked to a conjugative plasmid in our study too, yet the plasmid involved is a much smaller one, only being 16.0 kb. In our study, for the first time in the literature, we showed that nisin production ability and lactose fermentation ability are coded by the same plasmid. Lactose fermentation is much more important than sucrose fermentation in industry since Lactococci perform the preferred rapid acid formation by using lactose sugar. Thus having nisin genes on a lactose plasmid is very important for the improvement

of industrial starter cultures. An investigation of the transposon involment, on the other hand, should be among the scopes of further studies with our isolates.

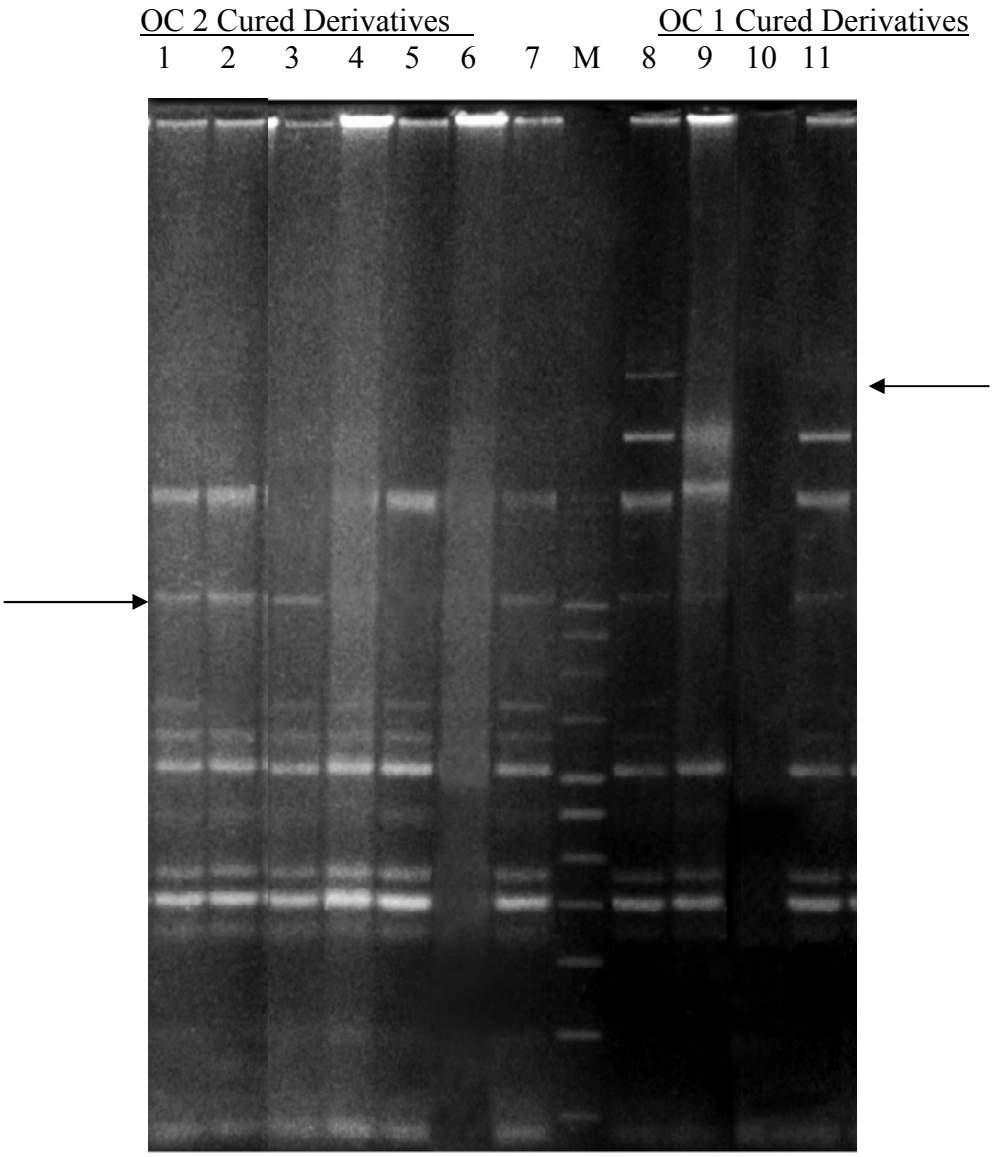

Figure 3.4. Plasmid contents of bacteriocin producing *L. lactis* subsp. *lactis* OC1 (lane 8) and OC2 (lane 7) strains and their cured derivatives which were incapable of producing bacteriocin for both OC1 and OC2.

Table 3.5. Plasmid complements of OC1 and OC2 and their cured plasmids

OC2 Cured Derivatives						OC2		OC1	OC1 Cured Derivatives		
1	**2**	**3**	**4**	**5**	**6**	**7**	**M**	**8**	**9**	**10**	**11**
Lct481^{+}, Lac^{+}, Prt^{+}	Lct481^{+}, Lac^{+}, Prt^{-}	Lct481^{+}, Lac^{+}, Prt^{+}	Lct481^{-}, Lac^{-}, Prt^{+}	Lct481^{-}, Lac^{-}, Prt^{+}	Lct481^{-}, Lac^{-}, Prt^{-}	Lct481^{+}, Lac^{+}, Prt^{+}		Nis^{+}, Lac^{+}, Prt^{+}	Nis^{-}, Lac^{-}, Prt^{-}	Nis^{-}, Lac^{-}, Prt^{-}	Nis^{-}, Lac^{-}, Prt^{-}
23.3	23.3	16.0	10,5	23.3		23.3	16.210	**39.7 ***	29.550		29.6
16.0	16.0	10,5	9.4	10,5		**16.0 ****	14.174	29.6	23.918		23.9
10.5	9.4	9.4	8.2	9.4		**10.5*****	12.138	23.9	16.0		16.0
9.4	8.2	8.2	5.5	8.2		9.4	10.102	16.0	8.4		10.7
8.2	5.5	5.5	4.7	5.5		8.2	8.066	10.7	5.5		8.4
5.5	4.7	4.7	4.5	4.7		5.5	7.045	8.4	4.9		5.5
4.7	4.5	4.5	2.0	4.5		4.7	6.030	5.5	2.1		4.9
4.5	2.0	2.0		2.0		4.5	5.012	4.9			2.1
2.0						2.0	3.990	2.1			
							2.972				
							2.067				

* responsible for nisin production, lactose fermentation and proteolytic activity in OC1

** responsible for lacticin 481 production and lactose fermentation in OC2

*** responsible for proteolytic activity in OC2

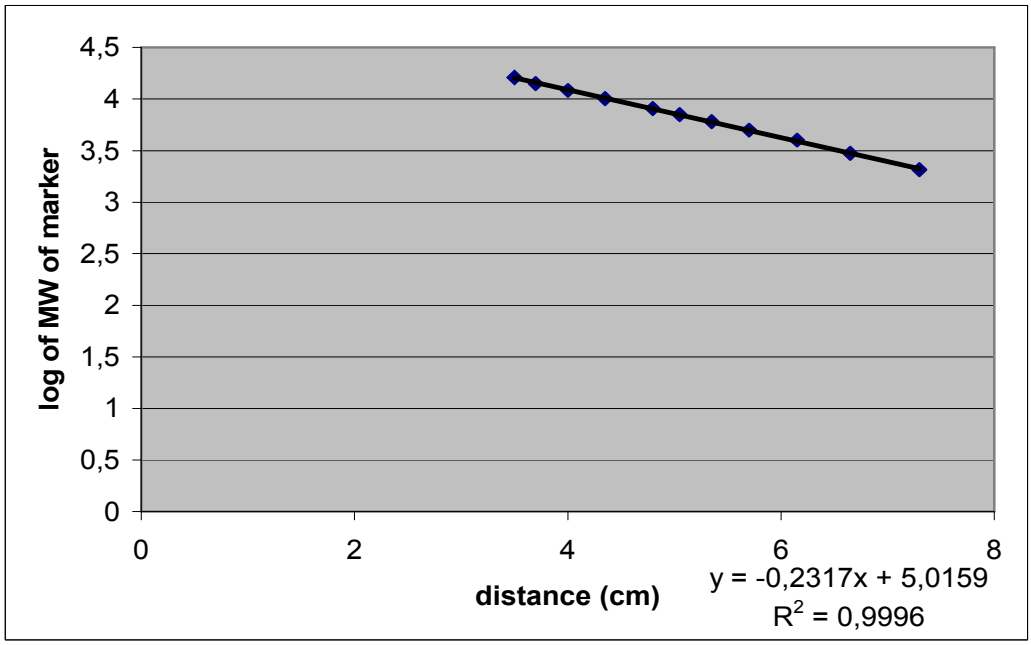

Figure 3.5. Log of MW of the marker DNAs versus migration (distance) in cm calibration curve for the gel run for plasmid curing experiment

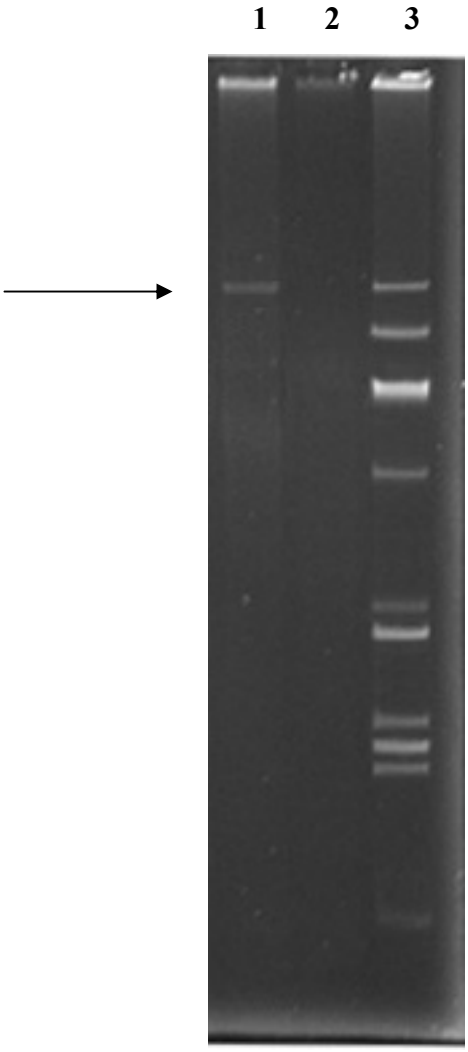

Figure 3.6. The result of conjugation (lane1) between *L. lactis* subsp. *lactis* OC1 (Lac^+, Prt^+, Str^s, Rif^s, Nis^+) as the donor strain (lane3) and *L. lactis* subsp. *cremoris* MG1363 (Lac^-, Prt^-, Str^r, Rif^r, Nis^-) as the recipient strain (lane2). The arrow points the 39.7 kb plasmid isolated from one of the transconjugants (Lac^+, Prt^+, Str^r, Rif^r, Nis^+, lane1)

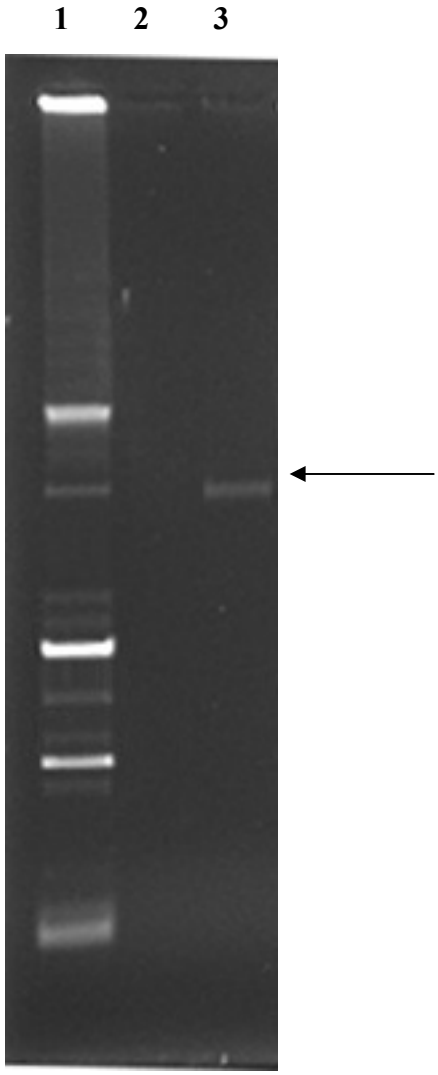

Figure 3.7. The result of conjugation between *L. lactis* subsp. *lactis* OC2 (Lac^+, Prt^+, Str^s, Rif^s, $Lct481^+$) as the donor strain (lane1) and *L. lactis* subsp. *cremoris* MG1363 (Lac^-, Prt^-, Str^r, Rif^r, $Lct481^-$) as the recipient strain (lane2). The arrow points the 16.0 kb plasmid isolated from one of the transconjugants (Lac^+, Prt^-, Str^r, Rif^r, $Lct481^+$, lane3)

CHAPTER V

CONCLUSION

The results of the present study revealed that the bacteriocins produced by the *Lactococcus lactis* subsp. *lactis* strains OC1 and OC2 were nisin and lactisin 481, respectively.

OC1 and OC2 showed a very similar inibitory spectrum with their reference strains towards indicator bacteria. The effects of heat and pH on bacteriocin activities were also the same when compared to those on the bacteriocins produced by the reference strains. The effects of various enzymes on bacteriocin activity were the same too for our isolates and the reference strains except for that of the enzyme α-amylase. The sensitivity to α-amylase of OC1 and OC2 bacteriocins can be explained by the aggregate formation with carbohydrates which is not the case with the respective bacteriocins of the reference strains.

The bacteriocins produced by OC1 and OC2 showed no cross reactivity with nisin and lactisin 481, respectively. This findings suggested that the bacteriocins produced by OC1 and OC2 should structurally be quite similar or identical to nisin and lactisin 481 as structuraly unrelated bacteriocins did not respond in cross reactivity test. Bacteriocin purification and subsequent SDS-PAGE further strengthened their identity to nisin and lacticin 481, respectively. In addition, PCR with nisin and lacticin 481 specific primers using OC1 and OC2 total DNA, respectively, resulted in

the amplification of single fragments of a size identical to those obtained by using the total DNA of their respective reference strains.

Examination of plasmid contents of the isolates and the results of plasmid curing and conjugation experiment showed that in *Lactococcus lactis* subsp. *lactis* OC1 strain the 39.7 kb plasmid is responsible for nisin production, lactose fermentation and proteolytic activity, whereas the 16.0 kb plasmid is responsible for lacticin 481 production and lactose fermentation in *Lactococcus lactis* subsp. *lactis* OC2 strain.

Since above mentioned characters of the strains are encoded by the same conjugative plasmid, the strains present promise for strain improvement studies. Conjugative nature of the plasmids facilitate related genetic manipulations, therefore transfer of these characters to other strains will provide developments in industrial starter cultures and bring about an economical gain in fermentation industry. Especially, the further investigation of the phage resistance in these two strains will be of great importance for the determination of the potential as starter cultures of the strains.

Bacteriocin-producing lactic acid bacteria characterized in the present work extend the number of available cultures, and probably the number of available bacteriocins, offering a useful protection against eventual contamination of foods with pathogenic or spoilage microorganisms. Their Turkey origin also offers reliability to their use in traditional fermented milk products in the country.

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APPENDIX A

CULTURE MEDIA COMPONENTS

Table A.1. Composition and preparation of M17 broth and agar.

M17 Broth and Agar

Polypeptone	5 g
Fitopeptone	5 g
Yeast extract	2.5 g
Meat extract	5 g
β-Na-glycerophosphate	19 g
Lactose (%10)	50 mL
$MgSO_4.7\ H_2O$ (1 M)	1 mL
Ascorbic acid	0.5 g
dH_2O	950 mL
pH (prior to sterilization)	6.9±0.2

Media contents were dissolved in 950 mL dH_2O and before agar addition pH was set to 6.9. After sterilization at 121^{o}C for 15 min. it was cooled down to 45^{o}C and sterile lactose solution (50 mL) was added [127].

M.R.S. Broth and Agar

Peptone	5 g
Yeast extract	4 g
Meat extract	8 g
Tween 80	1 mL
K_2HPO_4	2 g
D(+) Glucose	20 g
Sodium acetate.$3H_2O$	5 g
$MgSO_4$.7 H_2O	0.2 g
$MgSO_4$.4 H_2O	0.05 g
Triammonium citrate	2 g
dH_2O	1000 mL
pH (prior to sterilization)	6.2±0.2

Lactose Indicator Agar

Triptone	20 g
Gelatine	2.5 g
Dextrose	2.5 g
Lactose (%1)	100 mL
NaCl	4.0 mL
Sodium acetate	1.5 g
Ascorbic acid	0.5 g
$CaCl_2.6H_2O$	10 mL
Bromocresolpurple(%0.004)	10 mL
Agar	15 g
dH_2O	880 mL
pH (prior to sterilization)	6.8

Fast Slow Differential Agar

Fat-free milk powder	100 g
Sodiumglycerophosphate	19 g
Bacto litmus	1 g
Bromocresolpurple (%0.004)	10 mL
Agar	10 g
dH_2O	990 mL

The agar transferred to the erlenmayer containing 540 mL distilled water was dissolved in boiling waterbath and mixed with adding sodiumglycerophosphate and bacto litmus. In another erlenmeyer containing 450 mL distilled water, fat-free milk powder was dissolved and erlenmayers were sterilized separately at 121^{o}C for 17 minutes. The mediums were cooled to 55^{o}C, mixed and 10 mL of stock bromocresol purple solution was added and poured to Petri plates. Media were kept at 30^{o}C for 48 h.

Milk Agar

11% fat-free milk	50 mL
Sodium sitrate	0.5 g
Bromocresolpurple (%0.004)	5 mL
Agar	3.75 g
dH_2O	195 mL

In 50 mL fat-free sterile milk and 75 mL distilled water, 0.5 g of sodium sitrate was dissolved and pH is adjusted to 6.8 and terilized at 110^{o}C for 10 minutes. In another erlenmayer, agar prepared in 120 mL distilled water and bromocresol purple were sterilized at 121^{o}C for 15 minutes. Both of them were cooled to 50^{o}C and mixed at the last step and poured to Petri plates. Media were kept at 30^{o}C for 48 h.

APPENDIX B

CHEMICAL SOLUTIONS

Table B.2. Chemical solutions used in SDS-PAGE electrophoresis.

Separation Gel	
Acrylamide	12 mL
Tris (2M, pH 8.8)	5.62 mL
SDS (10%)	300 mL
dH_2O	12.1 mL

200 µL 10% amonium persulphate (APS) and 20 µL tetramethyl etylendiamine (TEMED) were added to the gel mixture.

Stacking Gel	
Acrylamide	1.8 mL
Tris (0.5M, pH6.8)	3.65 mL
SDS (10%)	9.4 mL

100 µL10% APS and 10 µL TEMED were added to the gel mixture.

Table B.2. (continued)

Tris-SDS Buffer	
Tris	5.9 g
SDS	0.4 g
dH_2O	100 mL
pH	6.7

Acrylamide Solution	
Acrylamide	30 g
Bis-acrylamide	0.8 g
dH_2O	100 mL

The solution was kept at +4^{o}C.

Electrode Buffer	
Glycine	8 g
Tris	12.64 g
SDS	2 g
dH_2O	2 L

Sample Buffer	
Tris-SDS buffer	1 mL
SDS (25%)	0.8 mL
β-merchaptoethanol	0.5 mL
Glycerol	1 mL
Brome-phenol-blue	0.01 mL

Commasie Brillant Blue Dye Solution	
Commasie brillant blue	1.25 g
Methanol	250 mL
Glacial acetic acid	50 mL
dH_2O	200 mL

Table B.2. (continued)

Gel Fixation Solution	
Glacial acetic acid	70 mL
Methanol	50 mL
dH_2O	880 mL

Table B.3. Chemical solutions used in DNA isolation

Tris -EDTA Buffer	
Tris	0.60 g
EDTA	09.31 g
dH_2O	100 mL
pH	8.0

Lysis Buffer
TE (pH 8.0) with 1% Tween 80

Table B.4. Chemical solutions used in Agarose Gel Electrophoresis

Tris - Acetate Buffer	
Tris	4.84 g
Sodium Acetate	4.08 g
EDTA	0.37 g
dH_2O	1000 mL
pH	8.0

Marker Dye	
Bromophenol blue	0.25 g
Saccharose	40 g
dH_2O	100 mL

Table B.5. Chemical solutions used in Plasmid Isolation

Saccharose Solution	
Tris	0.655 g
EDTA	0.0372 g
Saccharose	6.7g
dH_2O	100 mL
pH	8.0

Lysozyme Solution	
Tris	0.655 g
Lysozyme	0.1 g
dH_2O	10 mL
pH	8.0

Tris – EDTA -1	
Tris	0.60 g
EDTA	9.31 g
dH_2O	100 mL
pH	8.0

Tris – Cl	
Tris - Cl	31.52 g
dH_2O	100 mL
pH	7.0

SDS Solution	
Tris	0.60 g
EDTA	0.74 g
SDS	20 g
dH_2O	100 mL
pH	8.0

Table B.5. (continued)

Tris – EDTA -2	
Tris	0.121 g
EDTA	0.037 g
dH_2O	100 mL
pH	7.5

Preparation of RNase Solution : the pH of 0.05 M sodium acetate solution prepared in 5 mL distilled water was adjusted with acetic acid to 5.0 and 5 mg RNase A was added and then kept in boiling waterbath for 5 minutes and stored at -18^{o}C.

Preparation of Phenol Solution Saturated with 3% NaCl : 20 mL distilled water and 3 g NaCl were added onto 100 g phenol and dissolved in 45^{o}C waterbath. 0.1 g of hydroxiguinoline was added to the medium, mixed and kept at room temperature.

Table B.6. Chemicals used in PCR reaction

PCR Reaction	
10x buffer	5 µL
$MgCl_2$	3 µL
dNTP (10mM)	1,5µL
Primer I (100mM)	1 µL
Primer II (100mM)	1 µL
Template	1 µL
Taq Polymerase	0,5 µL
dH_2O	37 µL
Total :	50 µL