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Characterization of bacteriocins from two *Lactococcus lactis* subsp. *lactis* isolates

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In this study, bacteriocins from two *Lactococcus lactis* subsp. *lactis* isolates from raw milk samples in Turkey 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 the purified bacteriocins were determined by SDS-PAGE. PCR amplification was carried out with specific primers for the detection of their structural genes. As a result of these studies, the two bacteriocins were characterized as nisin and lacticin 481, respectively. Examination of plasmid contents of the isolates and the results of plasmid curing and conjugation experiments showed that in *L. 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 *L. lactis* subsp. *lactis* OC2 strain.

Keywords: Bacteriocins / Lacticin 481 / *Lactococcus* / Nisin / Plasmids

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1 Introduction

Because 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 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 [1]. Potential use of bacteriocins as biopreservatives has been studied in meat, dairy products or vegetable products [2].

LAB have been used for centuries in the fermentation of food, not only for flavor and texture, but also due to their ability of to prevent the growth of pathogenic microorganisms [3–5]. 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. Bacteriocin production has been well documented for most of the LAB and has been reviewed by various researchers [1, 6–8]. Many bacteriocins produced by LAB are active not only against other LAB, but also against food borne pathogens. This makes them attractive for use as natural food preservatives. Nisin, as approved by FDA, has thus far been the only bacteriocin that find widespread application in the food industry.

The objective of this study is to characterize bacteriocins from two different local isolates of *Lactococcus lactis* subsp. *lactis* strains. For this aim, their activity spectra, biochemical characteristics and molecular weights were determined. Their identity to nisin and lacticin 481 was confirmed with PCR amplification of the respective structural gene. The relations of bacteriocin biosynthesis, lactose fermentation and protease production to the specific plasmids that the isolates harbor were also investigated.

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Abbreviations: LAB, lactic acid bacteria; **Prt**, proteolytic activity; **TE,** Tris-EDTA

2 Materials and methods

2.1 Bacterial strains and culture media

Lactococcus lactis subsp. *lactis* strains were isolated by inoculating raw milk samples onto NRCLA medium [9].

Two positive colonies were identified as the strains of *L. lactis* subsp. *lactis* by performing 16S rDNA analyses [10] and designated OC1 and OC2, respectively. Their screening for bacteriocin production was done as described in Section 2.2. All indicator strains used for the characterization of bacteriocins were provided by the Laboratory of Microbial Gene Technology, NLH, As, Norway.

The strains OC1 and OC2 were grown in M17 medium [11] supplemented with 0.5% glucose (GM17) at 30°C. *Listeria innocua*, *Pediococcus pentosaceus*, *Enterococcus* and *L.* strains were grown in GM17 broth at 30°C. *Lactobacillus* strains were grown in De Man-Rogosa-Sharpe medium (Oxoid, UK) at 37°C. *Bacillus cereus*, *Staphylococcus carnosus*, *Pseudomonas fluorescens*, *Salmonella enterica typhimurium*, *Escherichia coli* were grown in Tryptic Soy medium (Merck, Germany) at 37°C. Bacterial stocks were stored at −80°C in their respective broths supplemented with 20% glycerol.

2.2 Detection of antimicrobial activity

Antimicrobial activity was evaluated as in Schillinger and Lücke [12]. Bacteriocin-producing *L. lactis* subsp. *lactis* strains were grown overnight at 30°C in M17 broth. By using sterile toothpicks, the strains were transferred to glucose-M17 plates. After incubating overnight, lawn of 3 mL of soft glucose-M17 agar (0.5% agar) containing 100 µL of the indicator strain was poured on the surface. Colonies were examined for inhibition zones after overnight incubation.

2.3 Effect of heat, enzymes, and pH on bacteriocin activity

To determine the effect of pH on bacteriocin activity, cell-free culture supernatants (CFCS) of the isolates were adjusted to pH values between pH 2.0 and 11.0 by using 6 N NaOH or HCl. Samples were assayed for the activity after 24 h at 4°C. *L. lactis* subsp. *lactis* IL1403 was used as indicator strain for both of the strains. *L. lactis* subsp. *lactis* SIK83 and *L. lactis* JC17 were used as positive controls as being nisin and lacticin 481 producers, respectively. As a control for possible 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, CFCS were heated at 100°C for 5, 10, 15 and 20 min and at 121°C for 15 min. CFCS were also treated with the following enzymes at a final concentration of 1 mg/mL: trypsin (pH 7.0, Merck, Germany), α-chymotrypsin (pH 7.0, type II, Sigma, 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 [13]. After heat or enzyme treatment, the remaining bacteriocin activity was determined by well diffusion assay [12, 13]. 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 multiplied by a factor of 100 to obtain the AU/mL of the sample

2.4 Purification of bacteriocins and SDS-PAGE

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 by starting with 1 L of cell-free supernatant of 18–h culture of each organism in GM17 medium. Bacteriocins were precipitated from the supernatant by adding 400 g ammonium sulfate. The precipitate was collected by centrifugation at $10\,000 \times g$ for 30 min, and resuspended in 10 mmol/L sodium phosphate buffer (pH 5.0) and loaded on a column of SP-Sepharose (Amersham Pharmacia Biotech) which was equilibrated with 10 mmol/L sodium phosphate buffer (pH 5.0). Bacteriocins were eluted with 1 mL of 1 mol/L NaCl [14]. Subsequent SDS-PAGE analysis of bacteriocins was performed as described by Laemmli [15]. The gel was stained with Coomassie blue-R250.

2.5 DNA isolation

DNA was extracted according to a protocol adopted from Head *et al*. [16] and Utaker and Nes [17]. The cells from 1 to 5 mL of culture grown overnight were harvested by centrifugation and washed once with TE buffer. The pellet was resuspended in ca 100 µL lysis buffer containing 1% v/v Tween 80 and frozen in liquid nitrogen. After this, the samples were thawn in a boiling water bath. Freezing and thawing were repeated five times after which 1 to 5 µL of the sample was used as the template in PCR amplification.

2.6 Identification of the bacteriocin genes by PCR amplification

PCR amplification was carried out using the primers specific for nisin and lacticin 481 structural genes. For the amplification of nisin gene of OC1, forward (5′ AAGAATCTCTCATGAGT 3′) and reverse primers (5′ CCATGTCTGAACTAACA 3′) were used [18]. (5′ TGATTTCGAAGGTAAG 3′) and (5′ AAAGCTTTACCTGTACT 3′) were forward and

reverse primers, used respectively to amplify lacticin 481 gene of OC2 [19]. The PCR amplification was carried out in a 50-μL mixture in a DNA thermocycler (Techne, Cambridge, UK). The PCR conditions were 94°C for 45 s, 56°C for 45 s and 72°C for 1 min, for 30 cycles. Amplified fragments were visualized on 1% agarose gels by staining with ethidium bromide using MBI, Fermentas Generuler 100-bp DNA ladder-plus as the molecular weight standard.

2.7 Plasmid DNA isolation

Plasmid DNA was isolated from the lactococcal strains by the method of Anderson and McKay [20]. The plasmid DNA samples were subjected to electrophoresis in 0.7% agarose gels.

2.8 Plasmid curing

Plasmids were cured by using acriflavine (Sigma) as a curing agent as described by McKay *et al*. [21]. Two loopsful of a 24-h-old culture in lactic broth of each organism were inoculated into 2.0 mL of lactic broth containing 5 μg acriflavine. After incubation at 32°C, each culture was diluted and spread over the surface of the fast-slow differential agar [22, 23] for selection of colonies with different morphologies. Colonies were further screened for their bacteriocin production.

2.9 Conjugation

Conjugation procedure was adopted from Gasson and Davies [24, 25]. Recipient and donor strains were grown in M17 broth medium at 30°C for 18 h. For the recipient strain *L. lactis* subsp. *cremoris,* streptomycin (500 μg/mL) and rifampicin (50 μg/mL) were added to this medium. Two milliliters of the donor and 3 mL of the recipient culture (both 10^{-4} diluted) were mixed and the cells were collected on sterile membrane filters (0.45 μm, Sartorius, Germany). The filters containing the recipient and donor cells were placed on the M17 agar plates, and kept at 30°C for 18 h. The filters were then taken from the M17 agar plates and washed in 1 mL of sterile Ringer's solution to resuspend the cells. The serial dilutions were made (up to 10^{-8}) and from each dilution the aliquots were spread onto fast-slow differential agar plates containing the antibiotics and left for incubation at 30°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. In conjugation experiments, the filters were pretreated with 2 mL of DNase I (100 μg/mL, Sigma) and kept at 37°C for 15 min to prevent any transformation.

2.10 Determination of lactose fermentation and proteolytic activity

In the *Lactococcus* strains, lactose fermentation and proteolytic activity (Prt) were determined on fast-slow differential [22, 23] agar and Milk agar plates [26] by incubating the cells at 30°C for 48 h. At the end of the incubation, lactose fermentation (Lac) and Prt of colonies were defined according to their morphological and cultural properties [23].

3 Results and discussion

3.1 Detection of antimicrobial activity

The lactococcal isolates, *L. lactis* subsp. *lactis* OC1 and OC2, were found to show inhibitory activity against a broad range of bacteria including lactobacilli, lactococci and staphylococci (Table 1). In addition, they showed inhibitory activity against *Listeria innocua*, *Enterococcus feacalis* and

Table 1. Inhibitory spectrum of bacteriocin producing *Lactococcus lactis* strains

	Tested strains		Control strains	
Indicator strains	OC1	OC2	SIK83	JC17
Lactococcus lactis SIK-83	–[a)]	+[b)]	–	–
Lactobacillus sake NCDO2714	+	+	+	+
Lactococcus lactis IL1403	+	+	+	+
Enterococcus faecalis	+	+	+	–
Listeria innocua	+	+	+	+
Lactococcus lactis 105	+	–	+	–
*Lactococcus lactis*1	+	–	+	–
Lactococcus lactis T1	+	+	+	+
Lactococcus lactis 731	+	–	+	–
Lactococcus lactis 2	+	–	+	–
Lactococcus lactis 1	+	–	+	–
E. coli CFAI (ETEC)	–	–	–	–
Salmonella enterica typhimurium SL1344	–	–	–	–
Pseudomonas fluorescence P1	–	–	–	–
Lactobacillus plantarum	+	+	+	+
Lactococcus lactis JC17	+	–	+	–
Bacillus cereus FM1	–	–	+	–
Staphylococcus carnosus MC1B	+	+	+	–
Pediococcus pentosaceus FBB61.1	+	+	+	–
Staphylococcus pentosaceus FBB61.1	+	–	+	–
Enterococcus faecalis NCDO581	+	–	+	–
Staphylococcus aureus FRI100	+	–	+	–
L. cremoris 2132	+	+	+	+
Lactococcus lactis	–	+	–	+

a) +: Sensitive.
b) –: Resistant.

Pediococcus pentosaceus. No inhibitory activity could be detected against *Bacillus cereus* in the strains. While activity against *Staphylococcus aureus*, *E. faecalis* NCDO581 and lacticin 3147 producers was seen in OC1 strain, no activity was observed in the OC2 strain against these indicator organisms. When compared with the respective control strains (*L. lactis* SIK83: nisin producer, *L. lactis* JC17: lacticin 481 producer), OC1 and SIK83 showed similar inhibitory activity against indicator organisms except for *B. cereus* FM1 which was resistant to the bacteriocin produced by OC1, but sensitive to SIK83 (nisin producer-control). Likewise, OC2 and JC17 showed similar inhibitory activity against indicator organisms except for *L. lactis* SIK83, *E. faecalis* and *S. carnosus* MC1B, which were resistant to the bacteriocin produced by OC2, but sensitive to that produced by JC17.

These differences in the inhibitory spectra of bacteriocins of OC1 and OC2 may lead to a misinterpretation such that the bacteriocins produced by these strains are different from nisin and lacticin 481. In the literature, such differences in inhibitory spectra were generally attributed to other products of LAB such as organic acids, hydrogen peroxide, diacetyl and inhibitory enzymes [27 – 31].

Supernatants of both strains were next tested for their cross reactivity. For the strain OC1, there was no cross reactivity with nisin and for OC2 there was no cross reactivity with lacticin 481 (data not shown). 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. 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 [3, 30]. 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 so-called ‚immunity' proteins, LanI in one system [32 – 35], while the second constitutes specialized ABC-transport proteins, LanFEG [33, 36, 37].

3.2 Effects of heat, enzymes, and pH on bacteriocin activity

The effect of heat and enzymes on bacteriocins is shown in Table 2. The bacteriocins produced by the isolates were as heat-stable as the reference strains, showing some activity even after heating at 121°C for 15 min. Inhibitory compounds of both lactoccocal strains had the same enzyme inactivation pattern. They were inactivated by α-chymotrypsin and proteinase K, but not by trypsin, lipase, catalase or lysozyme. This further confirmed that the inhibitory compounds had a proteinaceous nature and were bacteriocins.

Table 2. Effect of pH, enzymes and heat treatment on bacteriocin activity against *Lactococcus lactis* subsp. *lactis* IL1403

	Bacteriocin activity (AU/mL)			
Treatment	OC1	OC2	SIK-83	JC17
Control	12 800	3200	12 800	3 200
α-Chymotrypsin	0	0	0	0
Trypsin	6400	3 200	6400	3 200
Proteinase K	0	0	0	0
α-Amylase	0	0	12 800	3 200
Lipase	12 800	3 200	12 800	3 200
Catalase	12 800	3 200	12 800	3 200
Lysozyme	12 800	3 200	12 800	3 200
100°C 5 min	12 800	3 200	12 800	3 200
100°C 10 min	12 800	3 200	12 800	3 200
100°C 15 min	12 800	3 200	12 800	3 200
100°C 20 min	6400	3 200	6400	3 200
121°C 15 min	3 200	1 600	3 200	1 600
pH				
2	25 600	3 200	25 600	3 200
3	25 600	3 200	25 600	3 200
4	25 600	3 200	25 600	3 200
5	25 600	6400	25 600	6400
6	12 800	6400	12 800	6400
7	12 800	6400	12 800	6400
8	6400	6400	12 800	6400
9	6400	3 200	6400	3 200
10	6400	3 200	6400	3 200
11	1 600	1 600	1 600	1 600

Tramer [38] reported that nisin retained its activity after autoclaving at 115.6°C at pH 2.0 but when autoclaved at pH 6.8 the activity decreased by 90%. In our study, the effects of heat and enzymes on the activity of nisin-like bacteriocin produced by the isolate was determined after neutralizing the culture supernatants, therefore our results are comparable to those obtained in the above-mentioned work. In our work, the extent of decrease of bacteriocin activity upon autoclaving at 121°C for 15 min was 75%. Bacteriocin produced by OC2 was relatively more heat resistant. Its biological activity was unchanged after 20 min at 100°C, but 50% of its activity was lost after 15 min at 121°C. Similar results were reported for lacticin 481 by Piard *et al*. [39].

The bacteriocins produced by the strains OC1 and OC2 were resistant to inactivation by trypsin, as was reported earlier for nisin and lacticin 481 [40, 41]. However, their activity disappeared upon treatment with α-chymotrypsin, as was also the case with nisin and lacticin 481 [6, 41].

One distinctive property 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 was not seen in the case of the bacteriocins from the reference strains. This indicated that these bacteriocins are active only when they form aggregates with carbohydrate residues. 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 [42]. 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 [42–44].

The pH stability of the OC1 and OC2 bacteriocins was identical to that of the nisin and lacticin 481 produced by *L. lactis* SIK83 and *L. lactis* JC17, used as reference strains, respectively. The OC1 was the most active at pH ≤ 5.0. Its 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 [45, 46]. It was reported earlier that the nisin activity decreased drastically and was lost at basic pH values between 8.0 and 11.0 [47], as confirmed in the present study. The 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 was still detectable.

3.3 Molecular weight determination

The purified bacteriocins were run on SDS-PAGE and their molecular weights were 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 and 3.4 kDa, respectively (Fig. 1). The purified controls, nisin and lacticin 481 were also 3.5 and 3.4 kDa in their size, respectively, as shown earlier [6, 39, 47–49], therefore the local isolates seemed to produce nisin and lacticin 481, respectively.

3.4 Amplification of bacteriocin genes by PCR

PCR has been a powerful tool to identify bacteriocins [18, 50–52]. In our study, PCR using the primers specific to *nis* gene and structural gene of lacticin 481 led to a fast identification of bacteriocins produced by OC1 and OC2. The PCR amplification using OC1 and OC2 DNA as the templates yielded 536- and 300-bp fragments, respectively (Fig. 2). The fragments were of expected sizes, as amplified also from the 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.

3.5 The genetic nature of some industrially important characteristics of *L. lactis* strains

In certain *L. lactis* strains, some industrially important properties such as sucrose utilization, nisin production and

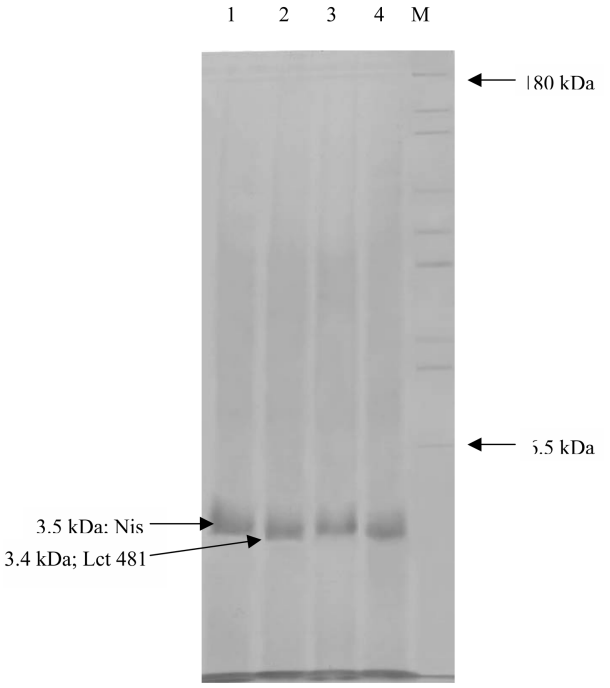

Figure 1. SDS-PAGE analysis of bacteriocins (shown by the arrows) from OC1 (lane 1), OC2 (lane 2), SIK83 (lane 3) and JC17 (lane 4). Marker lane contains proteins of 180, 116, 97, 58.1, 39.8, 29.0, 20.1, 4.3, 6.5 kDa, respectively.

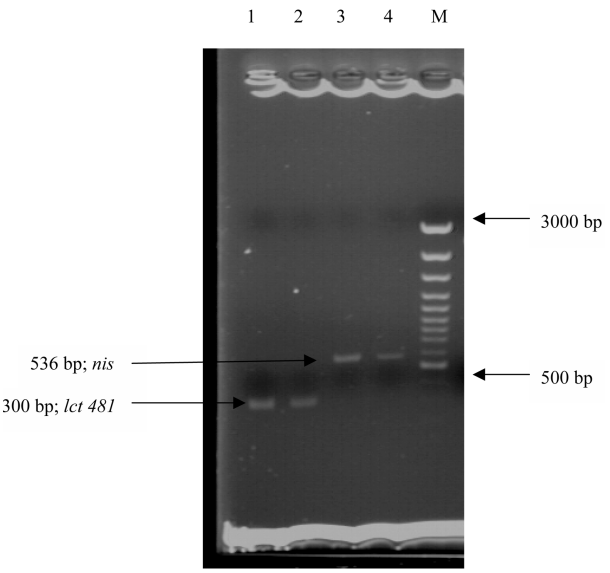

Figure 2. PCR amplification of bacteriocin gene fragments (shown by the arrows) from total genomic DNA of OC1 (lane 3), SIK83 (lane 4), OC2 (lane 1), JC17 (lane 2) strains. Nisin gene specific primers were used for OC1 and SIK83 total DNA and lacticin 481 gene specific primers were used for OC2 and JC17 total DNA. The marker DNA fragments were of 3000, 2000, 1500, 1200, 1030, 900, 800, 700, 600 and 500 bp, respectively.

proteolytic activity are encoded by the same plasmid [32, 43]. Similarly, lactose fermentation, lacticin 481 and immunity genes are also encoded by a specific plasmid [53]. Examination of plasmid contents of the strains OC1 and OC2 revealed that OC1 strain has nine distinct plasmids with MW varying between 2.0 and 39.7 kb and OC2 strain has also nine plasmids with MW varying between 2.0 and 23.3 kb (Fig. 3, Table 3). Our plasmid-curing attempt with OC1 strain resulted in the loss of 39.7-kb plasmid as well as the formation of mutant phenotypes. The cured derivatives were unable to ferment lactose and incapable producing of nisin and protease. 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 loss of the ability to ferment lactose and produce lacticin 481.

Table 3. Plasmid complements of OC1 and OC2 and their cured derivatives

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

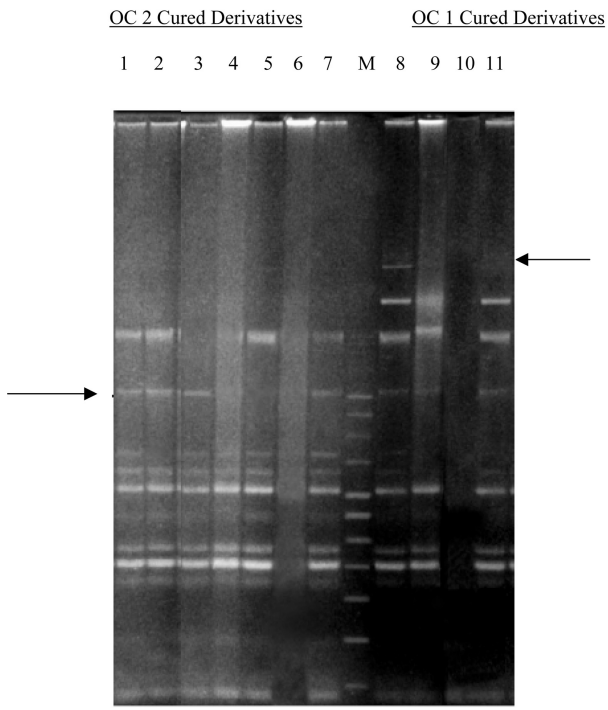

Figure 3. Plasmid contents of bacteriocin producing *L. lactis* subsp. *lactis* OC1 (lane 8) and OC2 (lane 7) strains and their cured derivatives which were incapable of producing bacteriocin for both OC1 (lanes 9 to 11) and OC2 (lanes 1 to 6). The arrows point to the missing plasmids in the cured derivatives.

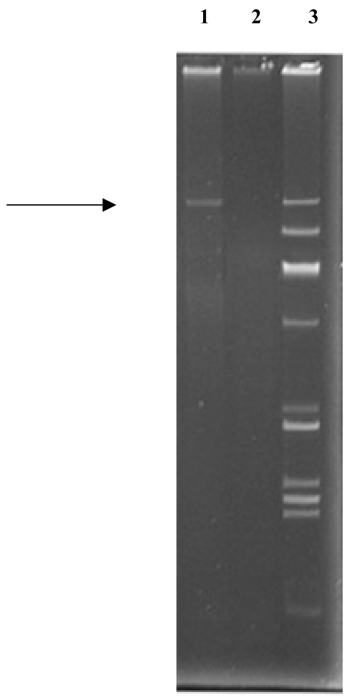

Figure 4. The result of conjugation (lane1) between *L. lactis* subsp. *lactis* OC1 (Lac$^+$, Prt$^+$, Str^s, Rif^s, Nis$^+$) as the donor strain (lane3) and *L. lactis* subsp. *cremoris* MG1363 (Lac$^-$, Prt$^-$, Str^r, Rif^r, Nis$^-$) as the recipient strain (lane 2). The arrow points to the 39.7-kb plasmid isolated from one of the transconjugants (Lac$^+$, Prt$^+$, Str^r, Rif^r, Nis$^+$, lane 1).

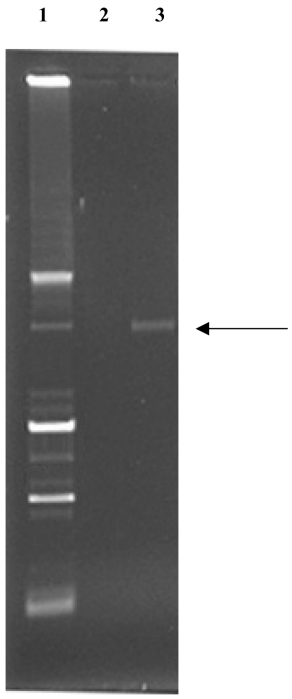

Figure 5. The result of conjugation between *L. lactis* subsp. *lactis* OC2 (Lac$^+$, Prt$^+$, Str^s, Rif^s, Lct481$^+$) as the donor strain (lane 1) and *L. lactis* subsp. *cremoris* MG1363 (Lac$^-$, Prt$^-$, Str^r, Rif^r, Lct481$^-$) as the recipient strain (lane 2). The arrow points to the 16.0-kb plasmid isolated from one of the transconjugants (Lac$^+$, Prt$^-$, Str^r, Rif^r, Lct481$^+$, lane 3).

To confirm the above-mentioned plasmid association of industrially important traits of *Lactococcus* isolates, conjugation trials were made. As a consequence of these trials, one plasmid from each isolate was found to be transferred to the recipient *L. 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. This proved that lactose fermentation ability, proteolytic activity and nisin production are encoded by the 39.7-kb plasmid in OC1 (Fig. 4). Similarly, the transconjugants of the cross between OC2 and MG1363 became lct481$^+$ and lac$^+$ via the transfer of the 16.0-kb plasmid, proving that lactose fermentation ability and lacticin 481 production characters are encoded by the 16.0-kb plasmid in OC2 (Fig. 5).

Various researchers have found that nisin genes are present on a number of conjugative plasmids [32, 54 – 56], usually a ~70-kb plasmid which also harbor the genes for sucrose utilization and integrate into the recipient chromosome following conjugal transfer [40]. In one publication [56], lacticin 481 gene cluster 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 16.0 kb. In our study, as the first time in the relevant literature, we showed that nisin production ability and lactose fermentation ability are encoded by the same plasmid. Lactose fermentation is much more important than sucrose fermentation in the 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 involvement, on the other hand is among the scopes of further studies with our isolates.

4 Concluding remarks

The results of the present study revealed that the bacteriocins produced by the *L. lactis* subsp. *lactis* strains OC1 and OC2 were nisin and lacticin 481, respectively. Because industrially important characteristics 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, providing developments in industrial starter cultures and bringing about an economical gain in fermentation industry. Further investigation of the phage resistance in these two strains will be of great importance for the determination of their 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 Turkish origin also offers reliability to their use in traditional fermented milk products in the country.

5 References

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