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CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF NATURAL ISOLATE *LACTOCOCCUS LACTIS SUBSP. LACTIS* BGSM1-19

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The strain Lactococcus lactis subsp. lactis BGSM1-19, isolated from traditionally homemade white cheese, produces two bacteriocins: lactococcin B-like bacteriocin named bacteriocin BacSMa and bacteriocin BacSMb which have shown similarity with lacticin RM. Plasmid curing resulted in a low yield (0.33%) of BacSMa$^-$ BacSMa^s and BacSMa$^-$ BacSMa^s, BacSMb$^-$, BacSMb^s derivatives. The bacteriocin biosynthesis was observed in the logarithmic phase of growth and the production plateau was reached after 8 h of incubation at 30^{o}C, when the culture entered the early stationary phase. Biochemical characterization showed that strain BGSM1-19 retained antimicrobial activity within the pH range of 1 to 12 or after treatment at 100^{o}C for 15 min. However, bacteriocin activity was completely lost after treatment with different proteolytic enzymes. The strain BGSM1-19 contains five plasmids. Plasmid curing indicated that genes coding for bacteriocins synthesis and immunity seem to be located on plasmids. BGSM1-19 exhibited antimicrobial activity against some pathogenic bacteria such as Salmonella paratyphi, Micrococcus flavus, Pseudomonas aeruginosa and Staphylococcus aureus.

Keywords: Lactococcus, bacteriocin, antimicrobial activity, Staphylococcus aureus, Micrococcus flavus

INTRODUCTION

Lactic acid bacteria (LAB) are used as starter cultures for a wide variety of fermented dairy, meat and vegetable products. LAB produce a number of antimicrobial substances that might be of importance for food and feed fermentation and preservation. Because of their metabolic properties, LAB significantly contribute to flavor development and ripening of fermented products. An important property of LAB is their capacity to produce a number of different substances with antimicrobial activity, making them suitable as natural food preservatives. Antimicrobial effect of LAB in fermented foods has been associated with the major metabolic end products, such as lactic acid, acetic acid, diacetyl, hydrogen peroxide, and other substances including enzymes, defective phages

and lytic agents (Lindgren and Dobrogosz, 1990). Apart from the metabolic products, many strains of LAB produce antimicrobial substances known as bacteriocins. Among other antimicrobial peptides, bacteriocins were originally defined as the proteinaceous compounds that inhibit growth of closely related bacteria (Tagg *et al*.,1976).

During the last two decades the bacteriocins produced by LAB were intensively investigated (Klaenhammer, 1988; Klaenhammer, 1993). According to their chemical, structural and functional properties bacteriocins could be classified into three main groups (for review see Nes *et al.,* 1996). Since some of them affect growth of food spoilage bacteria, they have become the topic of many investigations concerning food preservation and safety. The use of bacteriocins or bacteriocin-producing LAB strains in food production might be useful for the improvement of particular technological processes.

Since most LAB bacteriocins have a narrow antibacterial spectrum, several bacteriocins with a wider antibacterial spectrum have been also described, such as nisin, the bacteriocin from the lantibiotic group, produced by lactococci (Hurst, 1981; Klaenhammer, 1988; Diep and Nes, 2002). These antimicrobial peptides can inhibit the growth of many Gram-positive pathogenic and food spoilage bacteria, as well as growth of some Gram-negative species (Blackburn *et al*., 1989; Stevens *et al.,* 1991; Cintas *et al.,* 1995; Arihara *et al.,* 1996; Cardi, 2002).

In the process of cheese manufacturing, a population of adventitious microflora, known as non-starter lactic acid bacteria (NSLAB), usually can proliferate during the ripening period and become the dominant cheese flora, significantly contributing to texture, flavour and the quality of the product. These bacteria are present mostly in homemade cheeses and other dairy products, manufactured in a traditional way without the use of commercial starter cultures. Many of these strains represent the local, geographically specific microflora and it is most likely that differences between certain types of cheeses arise from the presence of NSLAB. The exact role of NSLAB strains in flavour development is still not completely clear, but they certainly have an influence on the process of cheese ripening, mainly due to their proteolytic and lipolytic activity.

In our previous work, we have isolated LAB from different traditionally homemade cheeses, manufactured without the use of starter cultures, and tested these isolates for production of antimicrobial compounds. The purpose of this study was to analyze the bacteriocin production in the natural isolate *Lactococcus lactis subsp*. *lactis* BGSM1-19. Besides testing the antimicrobial activity of strain BGSM1-19, especially against food spoilage and pathogen microorganisms, it was found that it produces two bacteriocins, designated as BacSMa and SMb.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions. *Lactococcus lactis* strains used in this study (Table 1) were grown in M17 medium (Merck GmbH Darmstadt, Germany) (Terzaghi and Sandine, 1975) supplemented with 0.5%, w/v glucose (GM17 broth) and incubated at 30^{o}C. The other indicator strains were cultivated in the following media: *Staphylococcus aureus* on Baird parker agar (Torlak,

Belgrade, Serbia), *Salmonella paratyphi* and *Salmonella enteridis* on Wilson-Blair agar (Torlak), *Bacillus subtilis* on Columbia agar with addition of 5% of horse blood (Torlak), *Streptococcus faecalis* on blood agar with triptone – peptone (15 g/L), extract of bovine heart (3 g/L), NaCl (5g/L) with addition of 7% of sheep blood (Torlak). *E. coli* and *Pseudomonas aeruginosa* strains were grown in LB medium at 37°C. Other pathogenic strains were cultured on Mueller-Hinton agar (Torlak) (Ericson and Sherris, 1971). Antimicrobial activity was defined by a clear zone of inhibition in the indicator set around the producer. To each of the above a medium agar (2%, w/v) (Torlak) was added when used as a solid medium. The plates were incubated overnight at 30°C or 37°C depending on the strain.

Table1. The bacterial strains and plasmids used in this study

Strains or plasmids	Description	Source or reference
Strains *Lactococcus lactis subsp. lactis*		
BGSM1-19	Natural isolate producing bacteriocin SMa and SMb	current study
BGSM1-191	Cured derivative producing bacteriocin SMb	current study
BGSM1-192	Cured derivative of BGSM1-19, Bac^-, Bac^s	current study
BGSM1-193	Cured, plasmid free derivative, Bac^-, Bac^s	current study
BGMN1-596	Prt^-, Bac^s, plasmid free derivative of BGMN1-5	(Gajic *et al.,*1999)
NP45	Nisin producer	lab. collection
IL1403/pMB553	Em^r, specifying lactococcin A	(van Belkum *et al.,* 1992)
IL1403/pMB580	Em^r, specifying lactococcin B	(van Belkum *et al.,* 1992)
IL1403/pMB225	Em^r, specifying lactococcin M/N	(van Belkum *et al.,* 1992)
E. coli DH5a	Δ*lacU196*(ø80 *lacZ*ΔM15)	(Hanahan, 1983)
Plasmids pUC19	Ap^r, ColE1 replicon	(Yanisch-Perron *et al*., 1985)
pSM500	Ap^r, pUC19 carrying 500 bp *Hha*I fragment containing part of *bac gene*	current study

Bac^- – bacteriocin non-producer; Bac^s – sensitivity to bacteriocin; Ap^r – resistance to ampicillin; Em^r – resistance to erythromycin

Bacteriocin detection and activity assay. For detection of bacteriocin activity, an agar-well diffusion assay was used (Tagg and McGiven, 1971). Soft GM17 (0.7%, w/v), containing the indicator strain *L. lactis subsp*. *lactis* BGMN1-596 was overlaid onto GM17 plates. For strains other then lactococci given in Table 2, corresponding soft agars and plates were used. Wells were made in the lawn of hardened soft agars. Aliquots (50 μL) of supernatant of overnight cultures (16 h) were poured in the wells. To confirm the production of a substance of proteinaceous nature, a crystal of pronase E was placed close to the edge of

bacteriocin containing well. The plates were incubated overnight at 30°C or 37°C depending on the indicator strain used. A clear zone of inhibition around the well, but not in the vicinity of the pronase E crystal, was taken as a positive signal for bacteriocin production. One arbitrary unit (AU) of bacteriocin was defined as the reciprocal of the highest dilution yielding a zone of growth inhibition on the indicator lawn (Mayr-Harting *et al*., 1972).

Kinetics of bacteriocins production. The cells from 1 mL of overnight culture were collected, and washed twice with GM17 broth by centrifugation. Pelleted cells were resuspended in 1 mL of fresh GM17 and thus used as the inoculum for a new culture in GM17 broth with approximately 10^5 cells per ml. The culture was incubated at 30°C and samples taken every hour to determine bacteriocin production AU/mL) and CFU/mL. To quantify the yield of bacteriocin production supernatants were serially diluted in GM17 broth before loading 50 μL of each dilution onto indicator strain BGMN1-596. Determination of the kinetics of bacteriocin production was done in duplicate, variation of AU values being less than 5%.

Effect of cations and enzymes on bacteriocin activity. To test the effect on bacteriocin activity, the following enzymes (final concentration: 1 mg/mL) were used: pronase E (Sigma) and proteinase K (Sigma) in 10 mM Tris-HCl (pH 8); trypsin (Calbiochem) in 50 mM Tris-HCl (pH 8); lysozyme (Sigma), DNase I (Sigma) and RNase A (Sigma) in 50 mM Na-phosphate (pH 6.5) and lipase (Sigma) in 50 mM Na-phosphate (pH 6.5). Reaction mixtures were incubated at 37°C for 1 h. The remaining bacteriocin activity was tested by the agar-well diffusion assay. Enzyme free buffers, incubated at 30°C for 1 h, were used as controls. Remaining bacteriocin activity was tested by the agar-well diffusion assay.

Thermal and pH stability of bacteriocin. Cell-free culture supernatant of a bacteriocin producer BGSM1-19 was incubated for 15 min at temperatures ranging from 40°C to up to 100°C with 10 degrees increments. After heating treatment, the samples were cooled spontaneuously to room temperature and the remaining bacteriocin activities were determined. To determine the effect of pH on bacteriocin activity, the pH of bacteriocin samples was adjusted stepwise from 1 to 12, in steps of one pH unit, by using 1M HCl or 1M NaOH. Samples were incubated for 1 h at 30°C and bacteriocin activity was determined. Supernatant of the bacteriocin non-producer strain *L. lactis subsp*. *lactis* BGMN1-596 was pre-treated in the same way as bacteriocin samples and used as a control to eliminate the effect of pH alone on the growth of the indicator strain. In each case the bacteriocin activity was determined by the agar-well diffusion assay.

Assay for bacteriocin activity in SDS-PAGE. The strain BGSM1-19 was grown on GM17 plates for 48 h at 30°C prior to cell collection. Cell films (500 mg) were collected from the surface of GM17 plates with a glass spreader, resuspended in 1 mL of buffer (100 mM Na-phosphate, pH 7.2), and vigorously agitated. Cell suspensions were incubated for 15 min at room temperature and cells pelleted by centrifugation (5 min at 13000 rpm). The clear supernatant that contained bacteriocin was concentrated (10x) and mixed with the same volume of 2x

concentrated sample buffer (125 mM Tris-HCl buffer, pH 6.8, 10 mM EDTA, 0.1% SDS, 25% glycerol and 0.07% Brom phenol blue). The prepared samples were analyzed on SDS PAGE. "Rainbow^{TM}" (Amersham International, Buckinghamshire, UK) was used as the protein standard. After electrophoresis the gel was divided into two parts. One part was stained with Coomassie brilliant blue G250 (SERVA, Heidelberg, Germany) and distained in a methanol (20%) and acetic acid (7%) mixture to determine the molecular size. To detect the bacteriocin activity, the other part of the gel was pre-treated with isopropanol (20%) and acetic acid (10%) mixture and subsequently washed first with Tween 80 (Sigma) (0.5%) and then in water for 24 h, as described previously (Bhunia and Johnson, 1992). After washing, the gel was placed on a sterile plate and overlaid with GM17 soft agar (0.7%, w/v) containing 100 μL of 10^{-2} diluted overnight culture of the indicator strain BGMN1-596. The plate was incubated overnight at 30°C and the appearance of inhibition zones was examined.

Plasmid curing and isolation from lactococci. Plasmid curing was achieved by growth of cells in the presence of novobiocin at sublethal temperatures. Prewarmed GM17 broth (40°C) containing novobiocin (10 μg/mL) was inoculated with 10^3 cells per mL. After incubation at 40°C for 2h cells were collected by centrifugation and resuspended in the same volume of fresh prewarmed GM17 broth containing novobiocin to avoid the bacteriocin-killing effect on cured cells (Bac^s). The same procedure was repeated six times, and then cell-aliquots (0.1 mL) were plated onto GM17 agar plates which were incubated at 30°C for 48h. After that, plates were overlaid with GM17 soft agar containing the indicator strain *L. lactis subsp*. *lactis* BGMN1-596 and incubated overnight at 30°C. Colonies which failed to inhibit the indicator strain were purified and re-checked for Bac^- phenotype. Also, the Bac^- derivatives were used as indicator culture in bacteriocin tests to check sensitivity to bacteriocin.

Plasmid isolation from lactococci was performed by using the method described by Anderson and McKay (Anderson and McKay, 1983).

RESULTS

Characterization of Lactococcus lactis subsp. lactis BGSM1-19

Lactococcus lactis subsp. *lactis* BGSM1-19 strain was isolated from semi-hard homemade cheese using standard microbiological procedures for detection and isolation of lactic acid bacteria. Analyzed cheese was produced in a traditional way in the village Zabrdje, located close to the seacoast at the bay Boka Kotorska, Montenegro. The manufacturing of the cheese was performed without the addition of any commercial starter cultures. The isolate was identified on the basis of their sugar fermentation patterns, according to the API50 CHL system (bioMerieux, Marcy l'Etoile, France), and other classical microbiological techniques. The identity of the isolate was confirmed by $(GTG)_5$-PCR and was identified at the subspecies level as *Lactococcus lactis subsp*. *lactis* (data not shown).

Bacteriocin production

Bacteriocin production of isolate BGSM1-19 was tested in agar-well diffusion assay. Production of bacteriocin in GM17 broth was dependent on growth phase (Fig.1). It was not possible to detect bacteriocin activity during the first 5 h of incubation. Production of bacteriocin by BGSM1-19 reached a plateau after 8 h and maximum production (8 AU) continued until 9 h of incubation. Afterwards, production of bacteriocin decreased from 8 AU to 1 AU in 20 h old cultures (Fig.1).

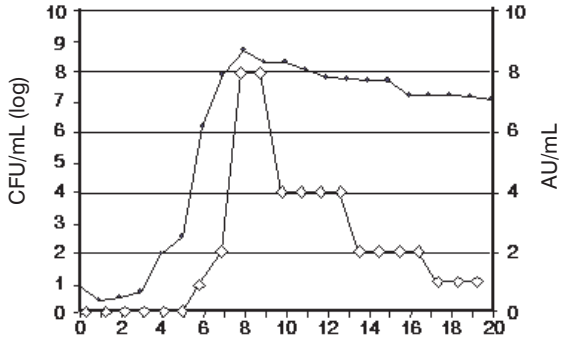

Figure 1. Kinetics of bacteriocin production during the growth of *L. lactis subsp. lactis* BGSM1-19 in GM17 broth at 30°C. Growth of BGSM1-19 was determined by the plate count method (CFU/mL, •) and bacteriocin concentration is expressed as arbitrary units per mL (AU/mL, ◊).

Linkage between bacteriocin production and plasmids

The strain *L. lactis subsp*. *lactis* BGSM1-19 harbors five plasmids. To check whether the genes coding for bacteriocin synthesis and immunity are plasmid located the curing of plasmids was performed. Two distinct bacteriocin phenotypes were obtained leading to the conclusion that BGSM1-19 most probably produces two different bacteriocins. The difference in bacteriocin phenotypes of plasmid cured derivatives was determined by using agar-well diffusion assay and each derivative was used either as an indicator strain or as potential bacteriocin producer. One derivative, BGSM1-191, had lost the ability to produce one of bacteriocins, named BacSMa and was BacSMa sensitive, retaining the production of another bacteriocin, designated as BacSMb. However, the second group of derivatives, named BGSM1-192 and BGSM1-193 did not produce any of the two bacteriocins and were sensitive to both bacteriocins (Fig. 2A). In addition, the plasmid profile analysis revealed that these two derivatives also lost some plasmids (derivative BGSM1-192 lost two plasmid bends, derivative BGSM1-193 lost all five plasmids) (Fig. 2B). These results indicated that the genes coding for bacteriocins SMa and SMb synthesis and immunity are

located on plasmids. The derivatives BGSM1-191 and BGSM1-193 were chosen for further analysis.

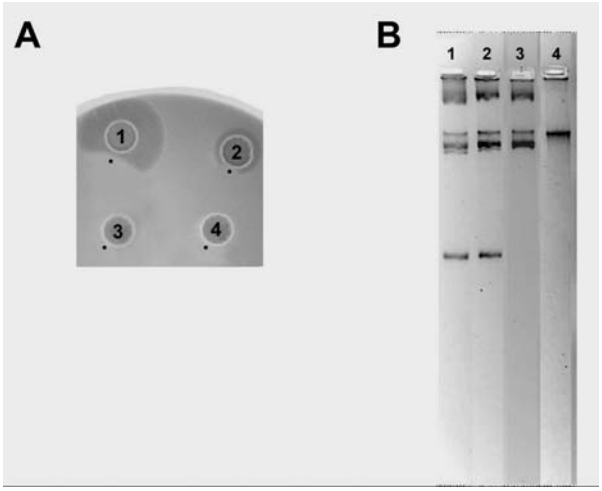

Figure 2. Agar plate indicating bacteriocin activity of *L. lactis subsp*. *lactis* BGSM1-19 (1); BGSM1-191 (2); BGSM1-192 (3) and BGSM1-193 (4). Indicator strain was *L. lactis subsp. lactis* BGMN1-596. Black spot – location of pronase E (A).
Agar gel electrophoresis of plasmids isolated from *L. lactis subsp*. *lactis* BGSM1-19 (1); BGSM1-191 (2); BGSM1-192 (3) and BGSM1-192 (4) (B).

Biochemical characterization of bacteriocins from BGSM1-19

Both bacteriocins are relatively temperature stable molecules. The antimicrobial activity was not affected by the treatment at 100^{o}C for 15 minutes. Bacteriocins, present in a cell-free supernatant of the producer strain, retained antimicrobial activity within the pH range between 1 to 12. Maximum activity was observed between pH 5 and 7.

The inhibitory action on the growth of sensitive indicator cells was not affected by the treatment with catalase, DNase I, RNase A or lysozyme but was abolished by the treatment proteolytic enzymes such as pepsin, trypsin, pronase E and proteinase K. These results strongly suggest that both molecules are of proteinaceous nature.

Estimation of molecular size of bacteriocins

Bacteriocin activities of isolate BGSM1-19 and derivative BGSM1-191 were also visualized by SDS-PAGE (Fig. 3). The activities of bacteriocins were visualized on the gel after SDS-PAGE and one zone of inhibition was detected on the gel. Its position agrees with the expected migration of a protein of approximately 7 kDa.

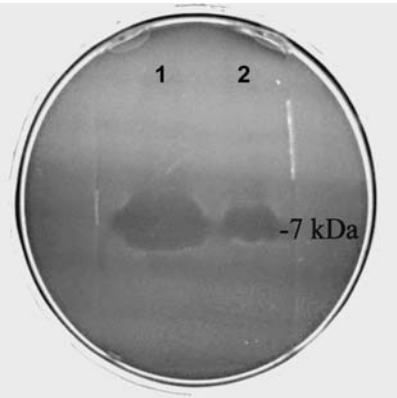

Figure 3. Analysis of bacteriocin activity on SDS-PAGE. The gel was overlaid with GM17 medium containing BMNI-56 as an indicator strain. Lane 1 - BGSMI-19: Lane 2 - BGSMI-191

Antimicrobial spectrum of strain BGSM1-19

The antimicrobial activity of *L. lactis subsp*. *lactis* BGSM1-19 against different pathogenic microorganisms was also tested. Obtained results showed that antimicrobial spectrum of this isolate includes various microorganisms such as *Salmonella*, *Micrococcus*, *Staphylococcus* and *Pseudomonas* (Table 2).

Table 2. Antimicrobial activity of *L. lactis subsp. lactis* BGSM1-19

Indicator strain	BGSM1-19
Salmonella paratyphi A	++
Salmonella enteridis ATCC3 1806	–
Moraxella 162	–
Micrococcus flavus ATCC 10240	+++
Esherichia coli (clinical isolate)	–
Pseudomonas aeruginosa ATCC29212	+
Streptococcus faecalis ATCC29212	–
Staphilococcus aureus ATCC 25923	+
Enterococcus faecalis ATCC 628	–
Bacillus subtilis ATCC 8633	–
Staphylococcus aureus MR427	–

–, no zone of inhibition; +, zone < 2 mm; ++, zone between 2 and 4 mm; +++, zone > 4 mm

Antimicrobial activity and cross-immunity between bacteriocin producing strains

Antimicrobial activity and cross-immunity of bacteriocin producers BGSM1-19, BGSM1-191 and plasmid free derivative BGSM1-193 were tested against different lactococcal strains: *L. lactis subsp. lactis* BGMN1-5 (bac501 and bac 513 producer), *L. lactis subsp. lactis* NP45 (nisin producer) and *L. lactis subsp. lactis* IL1403 harboring plasmids pMB553, pMB580 and pMB225 that specify the production of lactococcin A, lactococcin B and lactococcin M/N, respectively (Fig. 4). However, *L. lactis subsp. lactis* IL1403 harboring plasmid pMB580 which is responsible for production of lactococcin B, did not inhibit the growth of BGSM1-19 isolate and, on the other hand, partially inhibits the growth of derivative BGSM1-191. These results indicated that derivative BGSM1-191 produces lactococcin B like substance.

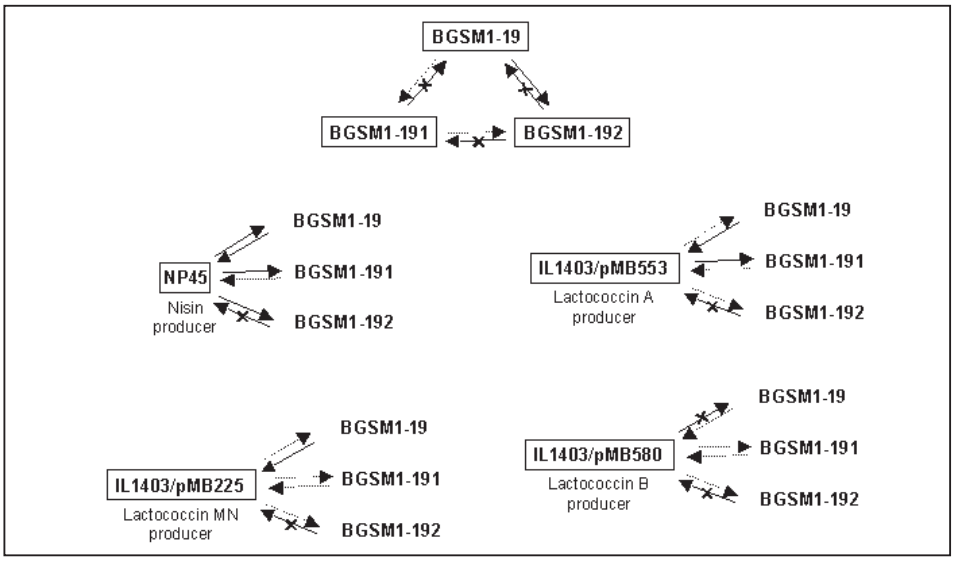

Figure 4. Schematic presentation of cross-immunity between BGSM1-19 and its derivatives, as well as various bacteriocin producers. → – full inhibition; ⇢ – partial inhibition; ↛ – no inhibition

During the test for antimicrobial activity, the relationship between BGSM1-19 and its derivatives BGSM1-191 and BGSM1-193 was also studied (Fig. 4). The results obtained from cross-immunity tests revealed that the parental strain BGSM1-19 expressed a lower inhibitory effect (2 mm zone of inhibition) on derivative BGSM1-191 and a full inhibitory effect (7 mm zone of inhibition) on derivative BGSM1-193. From the results of antimicrobial activity and cross-immunity experiments it can be concluded that isolate BGSM1-19 produced two bacteriocins, and that one of them (bactreiocin SMa) belongs to the group of lactococcin B-like bacteriocins.

Analysis of pSM1 plasmid

The plasmid pSM1 is the smallest plasmid from strains BGSM1-19, responsible for the production and immunity of SMb bacteriocin. In order to determine *bac* genes of pSM1, *Hha*I fragments of plasmid were cloned into pUC19 plasmid. Competent *E. coli* DH5α cells were transformed with these constructs and the construct pSM500 was sequenced. Computer analysis revealed that one 500bp *Hha*I fragment shares the 100% identity with *lacA,* structural gene for bacteriocin lacticin RM. This gene is located on pHU1 plasmid from *Lactococcus lactis subsp. lactis* EZ26 (Yarmus *et al*., 2000). These results indicate very high structural similarity between lacticin RM and bacteriocin SMb.

DISCUSSION

Among the bacteriocins produced by strains of *Lactococcus lactis,* nisin is the best characterized, and until now the only one that is widely commercially used. It is known that nisin inhibits the growth of the majority of Gram-positive bacteria including a great number of lactic acid bacteria. Besides, all *Lactobacillus* strains tested so far were nisin sensitive. Moreover, the growth of *E. coli* and other Gram-negative bacteria could be inhibited by nisin, but only if their membranes are damaged (Stevens *et al*., 1991). Most of the other bacteriocins from lactococci belong to class II, small (<10 kDa) heat-stable unmodified membrane active peptides. More than 50 bacteriocins of this class have been isolated from LAB and characterized so far. Class II bacteriocins from lactococci in general show a high level of heat stability, retaining the activity after treatment at 121^{o}C, like diacetin (Ali *et al*., 1995), lactococcin R (Yildirim and Johnson, 1998) and lactocin G13 (Janes *et al.,* 1999). Antimicrobial spectrum of lactococcal bacteriocins can be much wider compared to those produced by lactobacilli, and some of them showed capacity to inhibit the growth of *Listeria* species, which makes them particularly interesting for potential use as natural preservatives. Antilisterial effect was observed in lactococcin MMFII (Ferchichi *et al*., 2001), as well as in lactococcin R (Yildirim and Johnson, 1998) and lactocin G13 (Janes *et al.,* 1999).

Strain *Lactococcus lactis subsp. lactis* BGSM1-19 is a natural isolate from homemade cheese, producing bacteriocins BacSMa and BacSMb. BGSM-19 exhibits antibacterial activity against various pathogenic microorganisms (*Bacillus, Streptococcus, Staphylococcus*) including Gram-negative bacteria *Salmonella* and *Pseudomonas.* Bacteriocins BacSMa and BacSMb are a heat-stable proteins and are active within a broad pH range. Broad pH range activity of bacteriocins was, among others, reported for nisin (Jack *et al*., 1995), bacteriocin S50 (Kojic *et al*., 1991) and bacteriocin 501 (Gajic *et al.,* 1999). Bacteriocins produced by some other species of LAB, like for example plantaricin UG1 (Enan *et al*., 1996) and bacteriocin produced by *Lactobacillus acidophilus* 30SC (Oh *et al.,* 2000), are active only within a narrow pH range and their activities drastically decrease with pH changes.

The results obtained in the cross-resistance tests suggested that BacSMa belongs to the lactococcin B-like bacteriocins. Also, the zones of inhibition obtained on SDS PAGE can be addressed to the activity of BacSMa, since its estimated size corresponds to that of lactococcin B (van Belkum *et al*.,1992). The gene for bacteriocin BacSMb is, on the other hand, identical with the gene encoding for bacteriocin lacticin RM (Yarmus *et al*., 2000) on the nucleotide sequence level. Putative structural gene for the lacticin RM encodes the protein of 134 amino acids, and is located on a plasmid of a size similar to pSM1, the plasmid associated with the production of BacSMb. Except for the sequence of the genes responsible for biosynthesis and immunity for lacticin RM, other data concerning the size and biochemical properties of the active molecule are not available in the literature. In order to elucidate these properties for SMb and lacticin RM, further experiments related to the processing and transport of bacteriocins are required. However, being the natural isolate that produces two bacteriocins with relatively wide antimicrobial spectrum, strain *Lactococcus lactis subsp. lactis* BGSM1-19 could be a good candidate for the construction of new starter cultures, based on the strains that represent the microflora specific for a particular region.

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REFERENCES

1. *Ali D, Lacroix C, Thuault D, Bourgeois CM, Simard RE,* 1995, Characterization of diacetin B, a bacteriocin from *Lactococcus lactis subsp lactis* bv. diacetylactis UL720, *Can J Microbiol,* 41, 832-41.
2. *Anderson DG, McKay LL,* 1983, Simple and rapid method for isolating large plasmid DNA from lactic streptococci, *Appl Environ Microbiol,* 46, 549-52.
3. *Arihara K, Ogihara S, Mukai T, Itoh M, Kondo Y,* 1996, Salivacin 140, a novel bacteriocin from *Lactobacillus salivarius subsp. salicinus* T140 active against pathogenic bacteria, *Lett Appl Microbio,* 22, 420-4.
4. *Bhunia AK, Johnson MG,* 1992, A modified method to directly detect in SDS-PAGE the bacteriocin of *Pediococcus acidilactici*, *Lett Appl Microbiol,* 15, 5-7.
5. *Blackburn P, Polak J, Gusik SA, Rubing SD,* 1989, Nisin composition for use as enhanced broad range bactericides. International Patent Application publication WO 89112399.
6. *Cardi A,* 2002, Selection of *Escherichia coli*-inhibiting strains of *Lactobacillus paracasei subsp. paracasei, J Ind Microbiol Bioteh,* 29, 303-8.
7. *Cintas LM, Rodriguez JM, Fernandez MF, Sletten K, Nes IF, Hernandez PE, Holo H,* 1995, Isolation and characterisation of pediocin L50, a new bacteriocin from *Pediococcus acidilacti* with a broad inhibitory spectrum, *Appl Environ Microbiol,* 61, 2643-8.

8. *Diep DB, Nes IF, 2002,* Ribosomally synthesised antibacterial peptides in Gram positive bacteria, *Curr Drug Targets,* 3, 107-22.
9. *Enan G, el Essawy AA, Uyttendaele M, Debevere J,* 1996, Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization, production and bacterial action of plantaricin UG1, *Int J Food Microbiol,* 30, 189-215.
10. *Ericson HM, Sherris JC,* 1971, Sensitivity testing, *Acta Path Microbiol Scan. Section B Supp,* No 217.
11. *Ferchichi M, Frere J, Mabrouk K, Manai M,* 2001, Lactococin MMFII, a novel class IIa bacteriocin produced by *Lactococcus lactis* MMFII, isolated from a Tunisian dairy product, *FEMS Microbiol Lett,* 205, 49-55.
12. *Gajic O, Kojic M, Banina A, Topisirovic L,* 1999, Characterisation of natural isolate *Lactococcus lactis subsp. lactis* BGMN1-5, a strain producing two bacteriocins, cell wall-associated proteinase and showing clumping phenotype, *Arch Biol Sci,* 51, 69-78.
13. *Hanahan D,* 1983, Studies on transformation of *Escherichia coli* with plasmids, *J Mol Biol,* 166, 557-80.
14. *Hurst A,* 1981, Nisin, *Adv Appl Microbiol*, 27, 85-123.
15. *Jack RW, Tagg JR, Ray B,* 1995, Bacteriocins of Gram-positive bacteria, *Microbiol Rev,* 59, 171-200.
16. *Janes ME, Nannapneni R, Johnson MG,* 1999, Identification and characterization of two bacteriocin-producing bacteria isolated from garlic and ginger root, *J Food Prot,* 62, 899-904.
17. *Klaenhammer TR,* 1988, Bacteriocins of lactic acid bacteria, *Biochimie,* 70, 337-49.
18. *Klaenhammer TR,* 1993, Genetics of bacteriocins produced by lactic acid bacteria, *FEMS Microbiol Rev,* 12, 39-86.
19. *Kojic M, Svircevic J, Banina A, Topisirovic L,* 1991, Bacteriocin producing strain of *Lactococcus lactis subsp. lactis* biovar. diacetylactis S50, *Appl Environ Microbiol* 57, 1835-37.
20. *Lindgren SE, Dobrogosz WJ,* 1990, Antagonistic activities of lactic acid bacteria in food and feed fermentations, *FEMS Microbiol Rev,* 87, 149-64.
21. *Mayr-Harting A, Hedges AJ, Berkeley RCW,* 1972, Methods for studying-bacteriocins, p. 315-422. *In* Norris JR, Ribbons DW, editors, *Methods in microbiology,* vol. 7a. Academic Press, New York.
22. *Nes IF, Diep DB, Havårstein LS, Brurberg MB, EijsinkV, Holo H,* 1996, Biosynthesis of bacteriocins in lactic acid bacteria, p. 17-32. *In* G. Venema JH, Huis Veld J, Hugenholtz J, editors, *Lactic Acid Bacteria*: Genetics, Metabolism and Applications, *Kluwer Academic Publishers*, The Netherlands.
23. *Oh S, Kim SH, Worobo RW,* 2000, Characterization and Purification of a Bacteriocin Produced by a Potential Probiotic Culture, *Lactobacillus acidophilus* 30SC, *J Dairy Sci,* 83, 2747-52.
24. *Stevens KA, SheldonBW, Klapes NA, Klaenhammer TR*, 1991, Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria, *Appl Environ Microbiol,* 57, 3613-15.
25. *Tagg JR, Dajan AS, Wannamaker LW*, 1976, Bacteriocins of Gram-positive bacteria, *Bacteriol Rev,* 40, 722-56.
26. *Tagg JR, McGiven AR*, 1971, Assay system for bacteriocins, *Appl Microbiol,* 21, 943.
27. *Terzaghi BE, Sandine WE*, 1975, Improved medium for lactic streptococci and their bacteriophages, *App Microbiol,* 29, 807-13.
28. *van Belkum MJ, Kok J, Venema G*, 1992, Cloning, sequencing and expression in *Echerichia coli* of *lcnB*, a thrid bacteriocin determiner from the lactococal bacteriocin plasmid p9B4-6, *Appl Environ Microbiol,* 58, 572-7.
29. *Yanisch-Perron C, Vieira J, Messing J*, 1985, Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors, *Gene*, 33, 103-19.
30. *Yarmus M, Mett A, Shapira R*, 2000, Cloning and expresion of the gene involved in the production of immunity against the bacteriocin lacticin RM, *Biochim et Biophys Acta*, 1490, 279-90.
31. *Yildirim Z, Johnson MG,* 1998, Detection and characterization of a bactriocin produced by *Lactococcus lactis subsp. cremoris* R isolated from radish, *Lett Appl Microbiol,* 26, 297-304.

KARAKTERIZACIJA I ANTIMIKROBNA AKTIVNOST PRIRODNOG IZOLATA *LACTOCOCCUS LACTIS SUBSP. LACTIS* BGSM1-19

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SADRŽAJ

Soj *Lactococcus lactis subsp. lactis* BGSM1-19, izolovan iz sira tradicionalno proizvedenog u domaćinstvu, sintetiše dva bakteriocina: bakteriocin BacSMa koji pripada grupi laktokokcina B i bakteriocin BacSMb koji pokazuje visoku homologiju sa lakticinom RM. Čišćenjem plazmida, sa relativno niskim prinosom (0,33%), dobijene su dve grupe derivata: BacSMa^{-} BacSMa^{s} derivat i BacSMa^{-} BacSMa^{s}, BacSMb^{-}, BacSMb^{s}. Sinteza bakteriocina je ispitivana tokom logaritamske faze rasta pri čemu je utvrđen maksimum proizvodnje u kulturi staroj 8 sati gajenoj na temperaturi od 30^{o}C, što odgovara ranoj stacionarnoj fazi rasta. Biohemijska karakterizacija je ukazala da soj BGSM1-19 zadržava antimikrobnu aktivnost u opsegu pH vrednosti od 1 do 12 kao i posle tretmana na 100 ^{o}C u trajanju od 15 minuta. Utvrđeno je da se antimikrobna aktivnost potpuno gubi nakon tretmana različitim proteolitičkim enzimima. Soj BGSM1-19 poseduje pet plazmida. U eksperimentima čišćenja od plazmida utvrđeno je da se geni za sintezu i imunost na bakteriocine nalaze na plazmidima. Pored toga, BGSM1-19 pokazuje antimikrobnu aktivnost na ispitivane patogene bakterije kao što su *Salmonella paratyphi, Micrococcus flavus, Pseudomonas aeruginosa* i *Staphylococcus aureus*.