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CHARACTERISATION OF NATURAL ISOLATE *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* BGIS29, A STRAIN PRODUCING BACTERIOCIN IS29 AND CELL WALL-ASSOCIATED PROTEINASE

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Abstract - Strain *Lactococcus lactis* subsp. *lactis* BGIS29, a natural isolate from homemade cheese produces the bacteriocin IS29 and the PI-type proteinase. The results obtained by biochemical characterisation of bacteriocin IS29 suggest that it belongs to class II of bacteriocins. The DNA-DNA hybridisation and PFGE analysis showed that the genes for proteinase and bacteriocin production are located on separate genetic elements. Production of the proteinase of BGIS29 and the bacteriocin IS29 depend on the concentration of casitone in medium. Higher concentrations of casitone expressed an inhibitory effect on the proteinase activity. In contrast, production of bacteriocin IS29 was more pronounced in medium containing high casitone concentrations.

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INTRODUCTION

Lactic acid bacteria (LAB) are used in the production of a wide variety of dairy products such as cheeses and yoghurts. Several metabolic properties of lactic acid bacteria serve special functions, which directly or indirectly have the impact on the processes such as flavour development and ripening of dairy products. An important property of the LAB is their potential to produce a number of different substances with an antimicrobial activity, which can be used as biopreservatives. Antagonism of LAB in fermented food has been associated with the major end products of their metabolism, such as lactic acid, acetic acid, diacetyl, hydrogen peroxide, and other substances including enzymes, defective phages, lytic agents and bacteriocins (Lindgren and Dobrogosz 1990). In the last several years bacteriocins produced by LAB were intensively investigated (Klaenhammer 1988; De Vuyst 1993; Klaenhammer 1993; Nes *et al.* 1996). Bacteriocins are a subgroup of the antimicrobial peptides defined originally as proteinaceus compounds that kill closely-related bacteria (Tagg *et al.* 1976). Bacteriocins can be classified into three groups (for review see Nes *et al.* 1996).

In the most thoroughly characterised bacteriocin-producing strains studied so far, the genes specifying lactococcin production, immunity and secretion are plasmid located (Van Belkum *et al.* 1989; Holo *et al.* 1991; Van Belkum *et al.* 1992). The works of Van Belkum *et al.* (1989, 1992) revealed that one *Lactococcus lactis* strain could produce more than one bacteriocin. This strain produces three plasmid-encoded bacteriocins, two one-peptide bacteriocins and one two-peptide bacteriocin. These authors determined the nucleotide sequences of three different bacteriocin determinants from the plasmid p9B4-6, specifying a low (lactococcin M), high (lactococcin A) and intermediate (lactococcin B) bacteriocin activities. Each bacteriocin has its own specific counterpart protein conferring immunity, which is expressed in parallel with the bacteriocin. In all bacteriocin operons defined so far, potential immunity genes have been identified next to and downstream of the bacteriocin structural genes. For example, a promoter located immediately upstream of the *lcnA* drives the expression of both lactococcin A activity and immunity (Van Belkum *et al.* 1989). The immunity protein is functionally expressed in the absence of transport and processing in bacteria (Holo *et al.* 1991; Venema *et al.* 1994). Moreover, it was shown that two additional genes were required for bacteriocin activity.

These genes, designated as *lcnC* and *lcnD*, are plasmid-located in an operon immediately upstream of the lactococcin structural and immunity genes (V a n B e l k u m *et al.* 1989). In contrast to the plasmid-encoded secretion genes close to the lactococcin A, B and M operons described above, the *L. lactis* IL1403 strain carries genes for LcnC and LcnD on its chromosome (V a n B e l k u m *et al.* 1989; H o l o *et al.* 1991; V a n B e l k u m *et al.* 1992).

Another important property of the LAB is the potential for casein hydrolysis. The degradation of caseins plays a crucial role in development of the texture and the flavour. It has been well-established that degradation of caseins is initiated by the action of a single cell wall-associated extracellular proteinase, PrtP. Biochemical and genetic aspects of lactococcal proteolytic system have been extensively studied (K u n j i *et al.* 1996). Considering their caseinolytic specificities, lactococcal cell wall–associated proteinases have been divided into two groups. The PI–type proteinases exemplified by *Lactococcus lactis* subsp. *cremoris* HP, predominantly hydrolyse β–casein. The PIII–type proteinases, detected in *Lactococcus lactis* subsp. *cremoris* AM1 degrade the α_{S1}– and κ–caseins in addition to β–casein (V i s s e r *et al.* 1986; K o k and V e n e m a 1988). Recently, a medium–dependent regulation of *prt* genes expression was observed (M a r u g g *et al.* 1995). Expression was repressed at increased nitrogen concentrations or by the addition of specific dipeptides to the growth medium.

Here we report the characterisation of the natural isolate from homemade cheese producing the lactococcin B-like bacteriocin IS29 and the PI-type proteinase. Our results show the reciprocal influence of casitone on the proteinase and the bacteriocin production.

MATERIAL AND METHODS

Bacterial strains and growth conditions. Lactococcal strains used in this study are listed in Table 1. The strains were grown in M17 broth (Merck GmbH Darmstadt, Germany) containing 0.5% glucose (GM17) at 30°C. For the test of proteolytic activity, the cells were grown on milk-citrate agar (MCA) plates containing 4.4% reconstituted non-fat skim milk (RSM), 0.8% sodium citrate, 0.1% yeast extract, 0.5% glucose and 1.5% agar. In addition, chemically defined medium (CDM) was used: 2*g* $(NH_4)_2SO_4$, 6*g* Na_2HPO_4, 3*g* KH_2PO_4, 0.011 *g* Na_2SO_4, 1 *g* NaCl, dissolved in 200 mL of water and called solution A. Solution B contains 0.2*g* $MgCl_2$, 0.01*g* $CaCl_2$, 0.0006 *g* $FeCl_3x7H_2O$, dissolved in 800 mL water. A and B are mixed after autoclaving by pouring A into B, and glucose 5*g* L^{-1}, vitamins, sodium acetate 2*g* L^{-1}, and asparagine 80*mg* L^{-1} were added. The final concentration of the added vitamins was 0.1 *mg* L^{-1} for biotin, 1*mg* L^{-1} for folic acid, riboflavin, nicotinic acid, thiamine, and pantothenic acid, and 2*mg* L^{-1} for pyridoxal (D i c k e l y *et al.* 1995). This medium modified by replacement of casamino acids with casitone was used for testing proteinase and bacteriocin production of lactococcal strains. Agar plates were prepared by adding agar (1.5%, w/v) (Difco) to each broth when used as a solid medium.

Bacteriocin detection and activity assay. For bacteriocin detection, soft GM17 agar (0.7%) containing different indicator strains, was overlaid on GM17 plates (T e r z a g h i and S a n d i n e 1975). Wells were made as described previously (K o j i ć *et al.* 1991a). Aliquots (50 μL) of supernatant of overnight cultures (16 h) were poured in the wells. To confirm that a bacteriocin-like substance was produced, a crystal of pronase E was placed close to the edge of the well containing the bacteriocin sample. The plates were incubated overnight at 30°C. The appearance of 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 of bacteriocin was defined as the reciprocal of the highest dilution yielding a zone of growth inhibition on the indicator lawn. The sensitivity of bacteriocin IS29 to various enzymes (proteolytic enzymes, α-amylase, lysozyme, catalase, DNase, and RNase A) has been tested by enzymatic treatment of the filtered supernatant (0.45 μm Millipore filter) of an overnight culture. Incubation of reaction mixtures containing 1 and 0.5 *mg* mL^{-1} and of controls (buffers without enzymes) lasted for 1 *h* at 37°C. Retention of bacteriocin activity in treated samples was assayed as described above.

Kinetics of bacteriocin production. To remove bacteriocin from 1 mL of overnight culture (16 *h*), the cells were collected and washed twice with GM17 broth (1 mL) by centrifugation. Pelleted cells were resuspended in 1 mL of GM17 and new culture was started by inoculation of GM17 broth with approx. 10^5cells *per* mL. The culture was incubated at 30°C and the samples were taken at hourly intervals to determine CFU *per* mL and bacteriocin production (AU). Determination of the kinetics of bacteriocin production was done in duplicate; variation of AU values was less than 5%.

Assay of proteinase activity. Proteolytic activities of lactococcal strains were assayed as described previously (K o j i ć *et al.* 1991b). For this purpose, *L. lactis*

Table 1. Bacterial strains and plasmids.

Bacterial strain	Description*	Source or references
Lactococcus lactis subsp. *lactis*		
BGIS29	Natural isolate from soft home-made cheese, Bac^+, Bac^{Im}, Prt^+	Laboratory collection
NCDO712	Prt^+, Lac^+	Gasson, 1983
MG1363	Plasmid-free NCDO712-derivative, Prt^-	Gasson, 1983
Nb10-3	BGIS29 derivative, Bac^-, Bac^{Im}, Prt^+	This work
Nb10-2	BGIS29 derivative, Bac^-, Bac^S, Prt^+	This work
Nb10-5	BGIS29 derivative, Bac^-, Bac^S, Prt^+	This work
IL1403/pMB553	Em^r, specifying lactococcin A	van Belkum *et al.*, (1989)
IL1403/pMB580	Em^r, specifying lactococcin B	van Belkum *et al.*, (1992)
IL1403/pMB225	Em^r, specifying lactococcin M/N	vanBelkum *et al.*, (1989)
BGMN1-5	Natural isolate producing bacteriocin 501 and bacteriocin 513, Prt^+, Clu^+	Gajić *et al.*, (1999)
BGMN1-501	Derivative of BGMN1-5 producing bacteriocin 501, Prt^+, Clu^+	Gajić *et al.*, (1999)
BGMN1-513	Derivative of BGMN1-5 producing bacteriocin 513, Prt^-, Clu^-	Gajić *et al.*, (1999)
NP45	Nisin-producer	Laboratory collection
Plasmids		
pMB580	Em^r, pGKV210 derivative, containing *lcnB* and *lciB* genes, 5.6 kb	van Belkum *et al.*, (1992)

Bac^+-bacteriocin producer; Bac^--bacteriocin non-producer; Bac^S-bacteriocin sensitive; Bac^{Im}-immunity to bacteriocin; Prt^+-proteinase producer; Prt^--proteinase non-producer; Em^r-erythromycin resistance.

subsp. *lactis* BGIS29 was grown either on GM17 plates, or on MCA, or on CDM plates supplemented with either 0.1%, 0.5%, 1.0% or 2.0% of casitone (Difco) for 48 *h* at 30°C prior to cell collection. Collected fresh cells of BGIS29 (15 *mg;* approx. density of 10^{10} cells *per* mL) were resuspended in one of the following buffers (100 mM Na-acetate pH 5.2, pH 6.2, pH 7.2, or pH 8.2, 100 mM NH_4–acetate pH 6.2, or pH 7.2, 100 mM Na-phosphate pH 6.2, or pH 7.2). The cell suspension was mixed with the substrate (5 *mg* mL^{-1} of β–casein, Sigma Chemie GmbH, Deisenhofen, Germany) dissolved in the same buffer at a 1:1 volume ratio. After incubation (3 *h* at 30°C) the cells were pelleted by centrifugation (5 min at 12000xg), the clear supernatant was taken and the samples for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) were prepared. Analysis of casein hydrolysis was done by SDS–PAGE by loading 15% (w/v) acrylamide gel with the prepared samples. The gels were run on vertical slab electrophoresis cells (Bethesda Research Laboratories, Life Technologies, Inc., Baithersburg, Md., USA) for 16 *h* at 10 mA constant current, stained with Coomassie brilliant blue G250 (SERVA, Heidelberg, Germany) and destained in a methanol (20%) and acetic acid (7%) mixture.

Plasmid curing. Plasmid curing was achieved as described previously (Gajić *et al.* 1999) by growing the cells in the presence of novobiocin at sublethal temperature. Prewarmed GM17 broth (41°C) containing

novobiocin (10 μg mL^{-1}) was inoculated with 10^3 cells *per* mL. After the incubation for 2 *h*, the cells were collected by centrifugation and resuspended in the same volume of prewarmed GM17 broth containing novobiocin to avoid a bacteriocin-killing-effect towards cured (Bac^-, Bac^s) cells. This procedure was repeated five times, and then the aliquots (0.1 mL) were plated onto GM17 agar plates that were incubated at 30°C for 48 *h*. To detect Bac^-, Bac^s derivatives, master plates were made in duplicate. One of the plates was overlaid with GM17 soft agar containing indicator cells (strain NS1) and incubated overnight at 30°C. Colonies that did not inhibit the indicator strain were taken from the original master plate and rechecked for their Bac^-, Bac^s phenotype by using them as indicator strains. In addition, *L. lactis* subsp. *cremoris* NS1 was used as a control indicator strain.

DNA methods, restriction-enzyme analysis and pulse fields gel electrophoresis. Plasmids from lactococci were isolated by the method after B o j o v i ć *et al.* (1991). Restriction enzymes, supplied by Pharmacia (Pharmacia LKB Biotechnology, USA) were used according to the manufacturer's instructions. Pulse field gel electrophoresis (PFGE) was performed with a 2015 Pulsafor unit (LKB Instruments, Broma, Sweden), equipped with a hexagonal electrode. Agarose gels (1.2 %, w/v) were run for 15 *h* in TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) at 8°C and 300 V. The pulse time was 8 sec during the electrophoresis. All other recombinant DNA techniques were used as described by S a m b r o o k *et al.* (1989).

DNA/DNA hybridisation. The mix of proteinase gene probes Q1, Q6, and Q92 originating from *L. lactis* subsp. *cremoris* Wg2 was used. The probes were labelled by using the BioNick Labelling System (GIBCO-BRL). Hybridisation experiments were carried out essentially as described by K o j i ć *et al.* (1991b) at 45 °C.

PCR analysis. Total DNA from *L. lactis* subsp. *lactis* BGIS29 was used as a template for PCR amplification with primers specific for 5-end of the *lcnB* gene – HE36 (5AAAACTGCAGTAAGGAGATTATTATGA-AAAATCAATTAAA-3) and for 3-end of *lcnB* gene–HE13 (5-CGGGATCCCCCATCCTTCTGCCAT TACACC-3), originating from the plasmid p9B4-6 of strain *L. lactis* subsp. *cremoris* 9B4 (V a n B e l k u m *et al.* 1989). PCR amplification was performed in 30 successive cycles of melting DNA at 95°C for 5 min in the first cycle and 1 min in all other cycles, annealing at 50°C for 2 min, and elongation at 72°C for 2 min in the first 29 cycles and for 10 min in the last cycle.

RESULTS

Strain characterisation of Lactococcus lactis subsp. lactis BGIS29. The strain BGIS29 was originally isolated from soft homemade cheese by using standard microbiological procedures for the detection of lactic acid bacteria. Analysis of the plasmid content revealed that it has several plasmids. The BGIS29 produces bacteriocin with a narrow antibacterial spectrum. In addition, the strain BGIS29 produces an extracellular, cell wall–associated proteinase, which according to the substrate specificity, belongs to the PI–type of lactococcal proteinases.

Bacteriocin production. The bacteriocin production of BGIS29 in GM17 media was dependent on the growth phase. It was not possible to detect the bacteriocin activity during the first 4 *h* of the BGIS29 growth. Production of bacteriocin IS29 by BGIS29 reached a plateau after 10 *h* and production continued till 12 *h* of incubation (Fig. 1). Bacteriocin production in different growth media was also examined. When BGIS29 was grown in GM17 broth, production of bacteriocin IS29 was very moderate. However, a considerable increase in bacteriocin IS29 production, as judged by the agar-well diffusion assay, was observed when the strain was grown in a chemically defined medium (CDM) containing the casitone. The amount of bacteriocin IS29 was directly dependent on the concentration of casitone present in CDM. Maximal bacteriocin IS29 activity was obtained in CDM containing 4% casitone (Fig. 2).

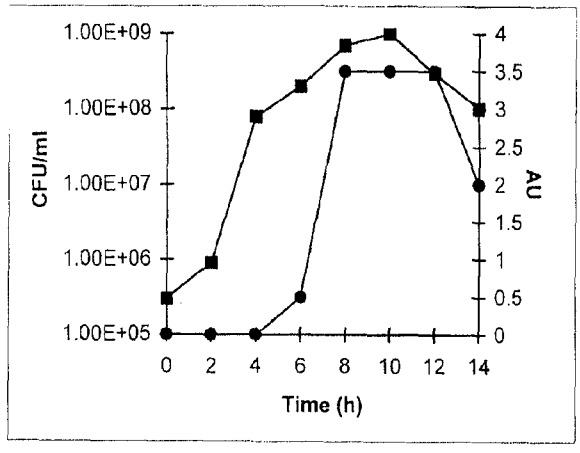

Fig. 1. Correlation between the growth and bacteriocin production of *L. lactis* subsp. *lactis* BGIS29. ■ - CFU/mL; ● - arbitrary units (AU).

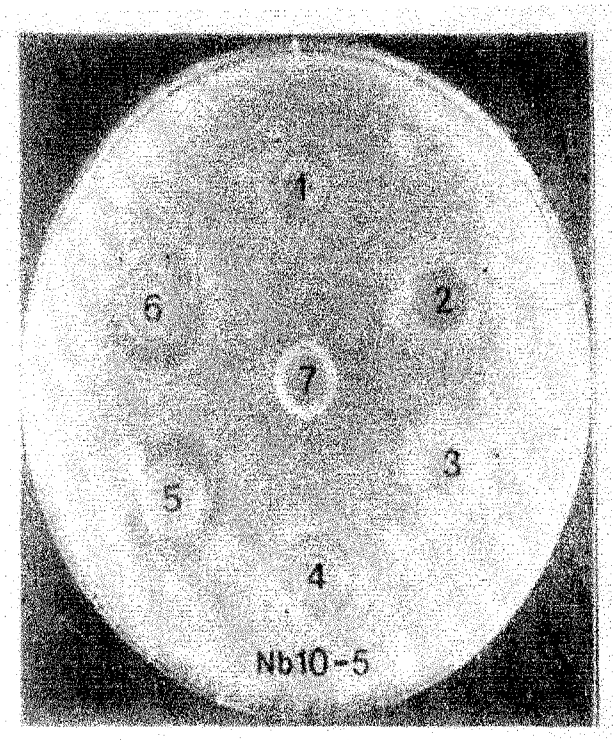

Fig. 2. Agar plate indicating bacteriocin activity of *L. lactis* subsp. *lactis* BGIS29. Non-bacteriocin producing derivative Nb10-5 was used as an indicator strain.

7 - sample of spent broth of non-bacteriocin producing derivative Nb10-5.

1 - bacteriocin sample prepared from GM17 broth; 2,3,4,5,6 bacteriocin samples prepared from CDM containing 0.1%, 0.5%, 1%, 2% and 4% casitone, respectively. Black spot-location of pronase E.

Biochemical characterisation of bacteriocin IS29. When present in a cell-free supernatant of the producer strain bacteriocin IS29 retained the activity within the pH range of 2 to 10. Maximal activity was observed at pH 2. Bacteriocin IS29 kept the activity for 60 min at pH 2, and after that period the activity was completely lost. Activity of the bacteriocin sample could be restored within 15 min by raising pH to 7. Antimicrobial activity of bacteriocin IS29 was not affected by treatment at 100°C for 2 *h*. When it was autoclaved at 121°C for 20 min, it retained only about 25% of the original activity, while autoclaving for one hour completely abolished antimicrobial activity. The inhibitory action of bacteriocin IS29 was not affected by treatment with α-amylase, catalase, DNase I, RNase A or lysozyme but was completely destroyed by proteolytic enzymes such as pepsin, trypsin, chymotripsin, pronase E and proteinase K. The results obtained by biochemical characterisation of bacteriocin IS29 showed that it belongs to class II bacteriocins defined as a group of small, heat stable nonlantibiotics.

Antimicrobial spectrum and cross-immunity between bacteriocin producing strains. The bacteriocin IS29 did not inhibit the growth of either Gram-positive bacteria, such as *Lactobacillus*, *Leuconostoc*, *Bacillus*, *Enterococcus* or *Staphylococcus* or several Gram-negative bacteria tested (*E. coli* C600, *Salmonella thyphimurium* LT2, *and Pseudomonas* sp.). A difference in the antimicrobial activity on lactococci was recorded. The bacteriocin inhibited the growth of *L. lactis* subsp. *cremoris* NS1, *L. lactis* subsp. *lactis* biovar. diacetylactis S50 and *L. lactis* subsp. *lactis* IL1403, *L. lactis* subsp. *lactis* NCDO712, its plasmid-free derivative MG1363, and the nisin-producer *L. lactis* subsp. *lactis* NP45. The strain *L. lactis* subsp. *lactis* BGMN1-5, producing bacteriocins 501 and 513, and its derivative BGMN1-501, producing only bacteriocin 501, expressed no effect on the growth of BGIS29 and *vice versa*. The BGMN1-5 derivative BGMN1–513, producing only bacteriocin MN513, expressed no effect on the growth of BGIS29 while BGIS29 showed an inhibitory effect on the growth of BGMN1–513. Strain BGIS29 inhibited the growth of *L. lactis* subsp. *lactis* IL1403 harbouring plasmids pMB553, pMB580 or pMB225 that specify production of lactococcin A, B and M/N, respectively. However, none of the lactococcin producers inhibited of the growth of the strain BGIS29. Reciprocal inhibitory activity of nisin producer *L. lactis* subsp. *lactis* NP45 and strain BGIS29 was observed (Fig. 3).

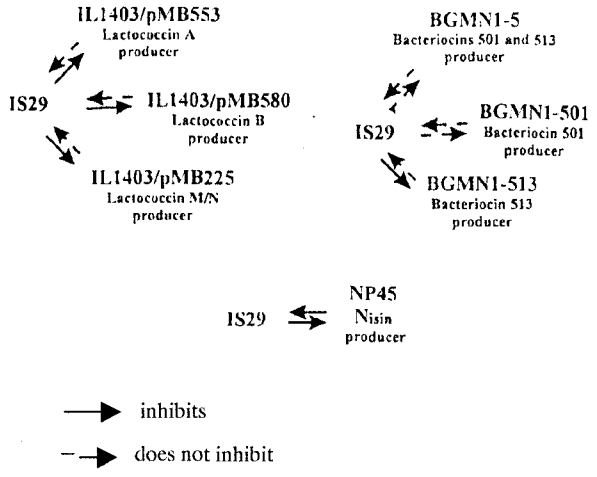

Fig. 3. Schematic presentation of cross-immunity between various bacteriocin producers

Proteinase activity. The ability of *Lactococcus lactis* subsp. *lactis* BGIS29 to hydrolyse α_{s1}-, β-, and κ-casein was tested after induction of proteinase on MCA plates. Proteinase-positive *Lactococcus lactis* subsp. *lactis* NCDO712 and proteinase-negative derivative of this strain, *Lactococcus lactis* subsp. *lactis* MG1363, were used as controls. It was found that the BGIS29 proteinase degrades only β–casein in a manner similar to that found in *L. lactis* subsp. *lactis* NCDO712 when the whole cells were used in the test. The comparison of the kinetics of β–casein degradation by the BGIS29 and NCDO712 proteinases when the same number of cells was used in the experiments showed that the kinetics of both strains was similar. Total degradation of β–casein by the either BGIS29 or NCDO712 proteinases was obtained after 3 *h* when bacteria were grown on MCA plates, and after 7 *h* when GM17 plates were used. The BGIS29 proteinase is a serine–type proteinase, since its activity is inhibited by PMSF.

Influence of growth medium on proteinase activity. The influence of the growth medium on β-casein hydrolysis by the BGIS29 proteinase was tested. For that purpose, the cells were grown on either CDM containing casitone, GM17, or MCA plates. Their proteolytic activities were determined by following β–casein hydrolysis in 0.1M NH_4–acetate buffer (pH 6.2). The results showed that β–casein hydrolysis by the strain BGIS29 depends on the growth medium. The highest β–casein hydrolysis was obtained when the cells were grown on CDM plates supplemented with 0.1% casitone or on MCA plates. Somewhat, lower proteinase activity was recorded when the cells were grown on CDM plates supplemented with 0.5%, or 2.0% of casitone. The lowest level of the BGIS29 proteinase activity was observed when the cells were grown on CDM plates supplemented with 1.0% of casitone or GM17 plates (Fig. 4). The influence of casitone on the regulation of proteinase activity in the strain BGIS29 was also tested by scoring β-casein hydrolysis in different buffers (100 mM Na-acetate pH 6.2, or pH 7.2, 100 mM NH_4–acetate pH 7.2, 100 mM Na-phosphate pH 6.2) (data not shown). It appeared that in the presence of 0.1% of casitone the BGIS29 proteinase activity was the most efficient regardless of the type or the strength of buffer used. Interestingly, in the presence of 0.5% or 1.0% of casitone the BGIS29 proteinase was more or less inhibited depending on the type of buffer used. In all cases the BGIS29 proteinase was active in the presence of 2.0% casitone, less then in the presence of 0.1% but more than in the presence of 0.5% or 1.0% casitone.

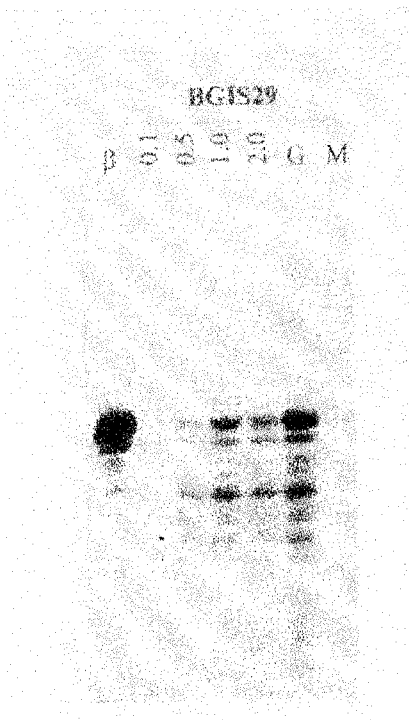

Fig. 4. The influence of growth medium on proteolytic activity of *L. lactis* subsp. *lactis* BGIS29. β = β–casein substrate; 0.1, 0.5, 1.0 and 2.0 = the whole cells collected from CDM plates containing 0.1%, 0.5%, 1% and 2% casitone, respectively; G = GM17 plates; M = MCA plates.

Determination of the genes coding bacteriocin and proteinase production. To examine possible plasmid localisation of the genes coding for bacteriocin and proteinase production, plasmid curing was performed. Two distinct bacteriocin phenotypes were obtained. One derivative, Nb10-3 did not produce babacteriocin IS29

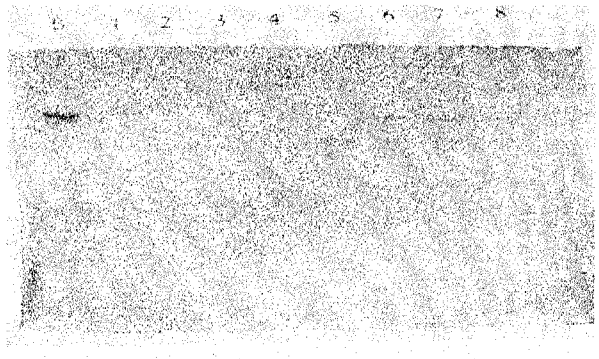

Fig. 5. The influence of growth medium on the proteolytic activity of *L. lactis* subsp. *lactis* BGIS29. β = β–casein substrate; 1. and 5. BGIS29; 2. and 6. Nb10-3; 3. and 7. Nb10-5; 4. and 8. Nb10-2; 1. - 4. the whole cells collected from CDM plates containing 0.5% casitone; 5. - 8. = the whole cells collected from GM17 plates.

but retained immunity to it and two derivatives, Nb10-2 and Nb10-5 did not produce bacteriocin IS29 and were sensitive to the bacteriocin. All derivatives retained ability to produce proteinase indicating that the genes governing bacteriocin and proteinase production are located on different genetic elements. Interestingly, the proteinase activity of the bacteriocin producing strain BGIS29 was higher then proteinase activity of its derivatives (Fig. 5). There were no visible differences between plasmid profiles of the wild type BGIS29 and its derivatives Nb10-3 and Nb10-5. Pulse field gel electrophoresis (PFGE) of either *Bgl*II, *Cla*I, *Not*I, *Pvu*II or *Sac*I-digested total DNA was performed to elucidate differences between the original strain and its derivatives. Comparing the number of the DNA fragments obtained by digestion of total DNA isolated *in-situ* and separated by PFGE of the strain BGIS29 and its derivative Nb10-5, showed that there is a difference in a number of the DNA fragments. One fragment in the total DNA of Nb10-5 digested with *Sac*I restriction enzyme was missing indicating that the genes for bacteriocin production and immunity are located on a large plasmid of at least 70 kb (Fig. 6). Differences in the position of the fragments on PFGE were also observed between the BGIS29 and its derivative Nb10-3, suggesting that possible rearrangement on the chromosomal DNA has occurred. Preliminary results of the hybridisation analysis of total DNA from BGIS29 with DNA fragments originating either from *lcnA*, *lcnB* or *lcnM/N* genes showed that bacteriocin IS29 is most likely lactococcin B like (data not shown). To prove the results obtained by hybridisation analysis, PCR amplification with primers originating from *lcnB* gene HE36 and HE13 was performed. As templates for the PCR amplification total DNA from either BGIS29 or its derivatives Nb10-3 or Nb10-5 were used. The plasmid pMB580 containing *lcnB* gene served as a positive control. The PCR products were obtained when total DNA from BGIS29 and its derivative Nb10-3 were used as templates, but not when total DNA from Nb10-5 was used (Fig. 7). The results suggest that the genes encoding bacteriocin production and immunity in the Nb10-3 derivative are intact and that the absence of bacteriocin production in this strain is due to the rearrangement in the genes for transport that could be located on the chromosome.

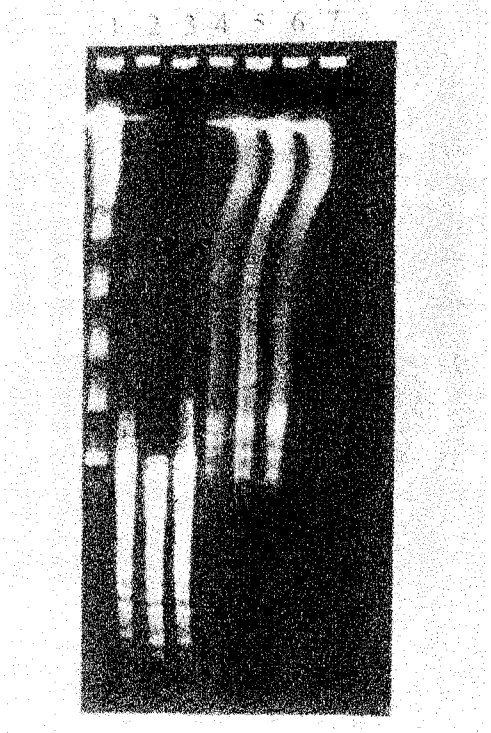

Fig. 6. Pulse-field gel electrophoresis of *Pvu*II (2-4) or *Sac*I (5-7) - digested total DNA. 2 and 5 = BGIS29; 3 and 6 = Nb10-5; 4 and 7 = Nb10-3; 1 - λ concatamers

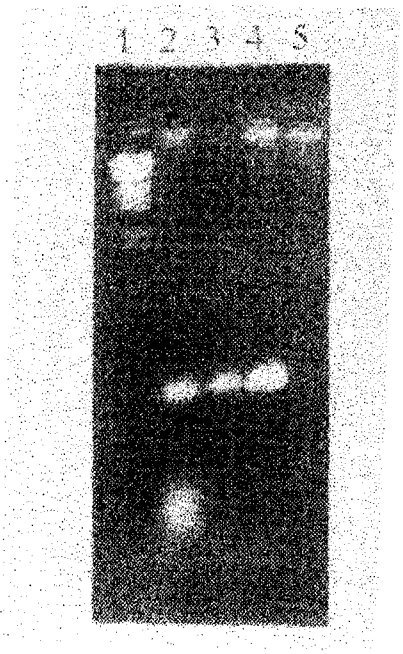

Fig. 7. PCR amplification with HE36 and HE13 primers. 1. λ DNA digested with *Hin*dIII and *Eco*RI; PCR performed with plasmid pMB580 (2), total DNA from BGIS29 (3), Nb10-3 (4), or Nb10-5 (5).

DNA of the strain BGIS29, and its derivatives Nb10-5 and Nb10-3 were tested by hybridisation with mix of the proteinase probes Q1, Q6, and Q92 (originating from the *prtP* and *prtM* genes of *L. lactis* subsp.

cremoris Wg2). PFGE of *Bam*HI- and *Kpn*I-digested total DNA was performed. Hybridisation signals were detected with DNA isolated from either BGIS29 or its derivatives (Fig. 8). These results highly correlated with the findings dealing with the proteolytic activity assay suggesting that bacteriocin IS29 and proteinase production are not linked to the same genetic element.

DISCUSSION

Lactic acid bacteria (LAB) are of eminent economic importance because of their widespread use in food and feed fermentations. Several metabolic properties of LAB serve special functions, which directly or indirectly have an impact on processes such as flavour development and ripening of dairy products. They also have an potential to produce a number of different su-

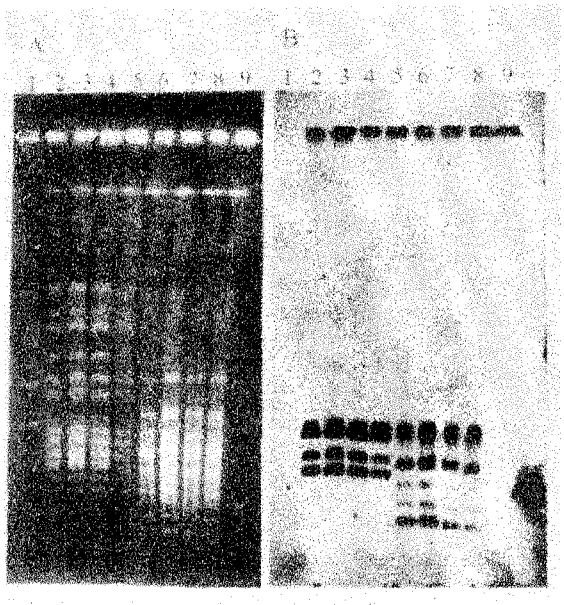

Fig. 8. Pulse-field gel electrophoresis of *Bam*HI- (2 - 5) and *Kpn*I- (6 - 9) digested total DNA (A) and hybridisation with lactococcal proteinase gene probe Q6 (B). 1.λ, 2. and 6. BGIS29, 3. and 7. Nb10-2, 4. and 8. Nb10-3, 5. and 9. Nb10-5.

substances with antimicrobial activity, which can be used as biopreservatives. Factors that have contributed to the increase in applied research on bacteriocins and bacteriocinogenic LAB are its approval as a GRAS (generally regarded as safe), concern about the safety of traditional preservatives and realisation that bacteriocinogenic strains have been isolated from food and, therefore, can be considered as safe. Strain *Lactococcus lactis* subsp. *lactis* BGIS29 characterised in this paper, is a natural isolate from homemade cheese. This strain produces the bacteriocin IS29 and the PI-type proteinase.

Bacteriocin IS29 conforms to the general characteristics of bacteriocins: it is proteinaceous in nature, has a narrow-antimicrobial-spectrum and a bactericidal mode of action (T a g g z *et al.* 1976). Bacteriocin IS29 produced by *L. lactis* subsp. *lactis* BGIS29 is sensitive to the action of various proteinases. This bacteriocin is a relatively heat-stable protein and is active within a broad pH range. Bacteriocin IS29 belongs to the narrow-antimicrobial-spectrum-bacteriocins as it exclusively inhibits the growth of lactococci. Similar results were obtained in the analysis of bacteriocins such as bacteriocin 501, lactococcin A, lactocin S or carnocin (H o l o *et al.* 1991; M ø r t v e d t *et al.* 1991; S t o f f e l s *et al.* 1992; G a j i ć *et al.* 1999). According to these characteristics, bacteriocin IS29 could be classified into class II bacteriocins (N e s *et al.* 1996).

Detectable production of the bacteriocin IS29 commenced after 4 *h* of the cells growth, *i.e.* during the logarithmic phase of the cell growth. Maximal synthesis of the bacteriocin IS29 was recorded at the early stationary phase of growth. Similarly, lactococcin B was synthesised by *L. lactis* IL1403 (pMB580) during logarithmic growth reaching also maximal production at the early stationary phase (V e n e m a *et al.* 1997).

It has been reported that the level of lactococcin B and pediocin PA-1 production is dependent on the growth medium. The highest production of lactococcin B was found in double M17 medium containing glucose. Analysis of growth medium-dependent bacteriocin IS29 production revealed that much more bacteriocin was synthesised when BGIS29 was grown in CDM containing high casitone concentrations.

Since it is known that there is a medium-dependent regulation of the proteinase (M a r u g g *et al.* 1995.), the influence of casitone on β-casein hydrolysis by the proteinase of the strain BGIS29 was also studied. The results showed that β-casein hydrolysis by this strain depended on casitone concentration in CDM and that higher casitone concentrations expressed an inhibitory effect on the proteinase activity.

From the genetic point of view, it appeared that the genes encoding proteinase and bacteriocin production are located on the separate genetic elements. This conclusion can be drawn out from PFGE analysis. In addition, the PFGE and PCR experiments suggest possible rearrangement on the chromosomal DNA in the genes for transport of bacteriocin in the Nb10-3 derivative that could explain the absence of bacteriocin production in this strain.

In general, it is known that the synthesis of proteinase is repressed when the growth medium contains casitone (M a r u g g *et al.* 1995), but also the expression of the peptide transport systems of *L. lactis* is affected by the composition of the growth medium (K u n j i *et al.* 1996). Medium-dependent bacteriocin production has also been observed. Taking all results together, it could be inferred that the medium-dependent regulation of the proteinase activity and bacteriocin production in *L. lactis* subsp. *lactis* BGIS29 occurred. Strains producing bacteriocins and proteinases are of potential interest to the food industry because of their ability to inhibit food-related pathogens. Bearing in mind the role of proteolysis in the cheese ripening this proteinase-producing strain could be used as a source of proteinase for starter cultures construction.

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КАРАКТЕРИЗАЦИЈА ПРИРОДНОГ ИЗОЛАТА *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* BGIS29, ПРОИЗВОЂАЧА БАКТЕРИОЦИНА IS29 И ПРОТЕИНАЗЕ ВЕЗАНЕ ЗА ЋЕЛИЈСКИ ЗИД

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Сој *Lactococcus lactis* subsp. *lactis* BGIS29 је природни изолат из сира произведеног у домаћинству који производи бактериоцин IS29 и протеиназу PI-типа. Резултати добијени биохемијском карактеризацијом бактериоцина IS29 сугеришу да он припада класи II бактериоцина. ДНК-ДНК хибридизација и PFGE анализа су показали да су гени за протеиназу и производњу бактериоцина лоцирани на различитим генетичким елементима. Производња протеиназе соја BGIS29 и бактериоцина IS29 зависе од концентрације казитона у медијуму за раст бактерија. Веће концентрације казитона имају инхибиторни ефекат на активност протеиназе. Насупрот томе, производња бактериоцина IS29 је израженија у медијуму који садржи веће концентрације казитона.