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The presence of *prtP* proteinase gene in natural isolate *Lactobacillus plantarum* BGSJ3–18

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Abstract

Aims: The study of proteolytic activity and examination of proteinase gene region organization in proteolytically active *Lactobacillus plantarum* strains from different natural sources.

Methods and Results: A set of 37 lactobacilli was distinguished by using multiplex PCR assay. Results showed that 34 strains were *Lact. plantarum* and three of them were *Lact. paraplantarum*. The examination of proteolytic activity revealed that 28 *Lact. plantarum* and two *Lact. paraplantarum* hydrolyse β-casein. Further analyses of all proteolytically active *Lact. plantarum* with primers specific for different types of CEPs demonstrated that strain BGSJ3–18 has *prtP* catalytic domain as well as *prtP–prtM* intergenic region showing more than 95% sequence identity with the same regions present in *Lact. paracasei*, *Lact. casei* and *L. lactis*. No presence of *prtB*, *prtH* or *prtR* proteinase genes was detected in any of tested *Lact. plantarum* strains.

Conclusions: One out of 28 analysed *Lact. plantarum* strains harbours the *prtP*-like gene. The other proteolytically active *Lact. plantarum* probably possesses a different type of extracellular proteinase(s).

Significance and Impact of the Study: It is the first report about the presence of the *prtP*–like gene in *Lact. plantarum*, which illustrates the mobility of this gene and its presence in different species.

Introduction

Lactic acid bacteria (LAB) are heterogeneous group of mesophilic or thermophilic Gram-positive microaerophilic bacteria with fermentative metabolism. The genus *Lactobacillus* represents the most numerous and diverse group among LAB, with many species that inhabit different ecological niches such as plant surfaces and silage, fermented milk products, meat products, fermented vegetables, fruits, as well as gastrointestinal or urogenital tract of humans and animals. Because LAB grow in various protein-rich media, their survival depends on the efficient proteolytic system. As well as other LAB, lactobacilli have complex nutritional requirements and multiple amino acid auxotrophies (Kandler and Weiss 1986). Essentially, cell envelope proteinases (CEPs) play a crucial role in hydrolysis of proteins into oligopeptides in media in which LAB grow. Oligopeptides are transported further on into bacterial cell by specific transport system and degraded to free amino acids by a range of peptidases (Kok and de Vos 1994; Kunji *et al.* 1996).

Proteinase PrtP was the first CEP characterized in different strains of *Lactococcus lactis*. In addition, the PrtP proteinase-encoding DNA regions of *Lactococcus lactis* ssp. *cremoris* Wg2 as well as *Lactococcus lactis* ssp. *cremoris* SK11 have been cloned and sequenced (Kok and de Vos 1994). In both lactococci, the proteinase gene (*prtP*) is associated with the upstream-located and divergently transcribed *prtM* gene encoding the PrtM protein involved in the maturation of PrtP proteinase (Kunji *et al.* 1996). The *prtP* gene has been also identified in *Lactobacillus paracasei* ssp. *paracasei* BGHN14 (Kojic *et al.* 1991) and cloned and sequenced from *Lact. paracasei* ssp. *paracasei* NCDO 151 (Holck and Naes 1992). Regardless of the origin, the *prtP* genes-encoded proteinases show more than 95% sequence identity. In all strains studied so far, the *prtM–prtP* proteinase intergenic gene region is located on plasmids of different sizes in lactococci

(Kok and de Vos 1994), whereas in *Lact. paracasei* ssp. *paracasei* strains, this region is chromosomally located (Kojic *et al.* 1991, 1995).

Three markedly distinctive genes encoding CEPs, designated *prtB*, *prtH* and *prtR*, have been cloned and sequenced from *Lact. delbrueckii* susbp. *bulgaricus* NCDO 1489, *Lact. helveticus* CNRZ32 and *Lact. rhamnosus* BGT10, respectively. Additionally, the *prtM* gene is not recognized in these lactobacilli (Gilbert *et al.* 1996; Pederson *et al.* 1999; Pastar *et al.* 2003). All types of LAB proteinases, PrtB, PrtH, PrtR and PrtP belong to subtilases, a superfamily of subtilisin-like serine proteases (Siezen and Leunissen 1997). Prediction of CEPs structure revealed that these proteinases consist of various functional domains (Siezen 1999). The composition of domains in PrtP, PrtB, PrtH and PrtR proteinases varied, but all contain the catalytic serine protease domain showing sequence homology to the active site of subtilases.

The purpose of this study was to examine the type of *prt* gene present in proteolytically active *Lact. plantarum* isolated from different natural sources. Our investigations indicate that a *prtP*-like proteinase gene is present in *Lact. plantarum* BGSJ3–18. Moreover, results showed that organization of the proteinase gene region in this strain appears to be similar to that of *Lact. paracasei* ssp. *paracasei*, *Lact. casei* and *L. lactis.*

Materials and methods

Bacterial strains, media and culture conditions

The bacterial strains used in this study are listed in Table 1. *Lactobacillus* strains were grown in MRS broth (Merck GmbH, Darmstadt, Germany) at 30°C. *Lactococcus* strains used in this study were grown in M17 medium (Merck) supplemented with glucose (0·5%, w/v) (GM17 broth). Agar plates were prepared by adding agar (1·5%, w/v) (Torlak, Belgrade, Serbia) to each broth when used as a solid medium. For testing of proteolytic activity, *Lactobacillus* and *Lactococcus* cells were grown on milk-citrate agar (MCA) plates containing 4·4% reconstituted nonfat skim milk (RSM), 0·8% Na_3-citrate, 0·1% yeast extract, 0·5% glucose and 1·5% agar (w/v) in distilled water.

Proteinase activity assay

Proteolytic activities of *Lactobacillus* and *Lactococcus* strains were tested as described previously (Kojic *et al.* 1991) with modifications. Strain *Lactococcus lactis* ssp. *cremoris* MG1363 was used as a proteinase-negative control. The strains were grown on MCA plates for 48 h at 30°C prior to cells collection. Collected fresh cells (10 mg, approximate density 10^{10} cells ml^{-1}) were resuspended in 100 mmol Na-phosphate buffer (pH 6·5). The cell suspension was mixed with substrate and dissolved in the same buffer at a 1 : 1 volume ratio. The substrate used for the proteolytic assay was β-casein fraction (5 mg ml^{-1}), (Sigma Chemie GmbH, Deisenhofen, Germany). After incubation at 30°C for 3 h, cells were pelleted by centrifugation (5 min at 12 000 ***g***), and the supernatant was then used for preparation of samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Samples for analysis by SDS-PAGE were prepared by heating (100°C for 2 min) with an equal volume of 125 mmol Tris-HCl buffer (pH 6·8) containing 4% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) β-mercaptoethanol and 0·07% (w/v) bromophenol blue. Analysis of β-casein hydrolysis was performed on SDS-PAGE by loading 15% (w/v) acrylamide gel with samples obtained after substrate hydrolysis. Gels were run on vertical slab electrophoresis cells for 20 h at 10 mA constant current, stained with Coomassie brilliant blue R250 (Serva, Heidelberg, Germany) and destained in a mixture of methanol (20%) and acetic acid (10%) in distilled water.

DNA manipulation procedures

The list of primers used in this work is presented in Table 2. For species determination, PCR with *Lactobacillus* genus-specific primers U968 and L1401 and a multiplex PCR assay with the *recA* gene based-specific primers plantF, paraF, pentF and pREV were performed (Torriani *et al.* 2001).

One set of primers (Table 2) specific for different types of CEPs was designed to target the conserved region surrounding the active site of proteinases. In addition, primers PrtP700/PrtM700 were designed to target *prtP*–*prtM* intergenic region. Genosys (Sigma) synthesized primers.

Specific primers were used for amplifying chromosomal DNA isolated from *Lactobacillus* and *Lactococcus* strains (Table 1) by following the procedure described previously (Hopwood *et al.* 1985). DNA amplifications were carried out singly or in a multiplex PCR. The PCR amplifications were performed by using the GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) and *Taq* polymerase (Pharmacia, Vienna, Austria). All PCR amplifications were carried out in tubes containing 25 μl reaction mixture composed of 1× Taq buffer, 1 U Taq polymerase, 1·5 mmol $MgCl_2$, 200 μmol dNTPs each and 1·5 μmol primer each. PCR amplification conditions were as follows: initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C, 1 min; annealing at 53–56°C, depending on mT of primers (1 min); elongation at 72°C (1·5 min) and final extension at 72°C for 7 min. The multiplex PCR assay was performed as previously described (Torriani *et al.* 2001).

Table 1 Bacterial strains used in this study

Bacterial strain	Description*	Source or references
Lactococcus lactis ssp. *cremoris*		
MG1363	Prt^-, plasmid-free derivative of NCDO 712	Gasson 1983
Wg2	$PrtP^+$	Kok *et al.* 1988
Lactobacillus paracasei ssp. *paracasei*		
BGHN14	Natural isolate from home-made cheese, $PrtP^+$	Kojic *et al.* 1991
Lactobacillus plantarum		
B-4496	Pickled cabbage	NRRL collection
BGSJ1–8	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGSJ1–10	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGSJ3–18	Natural isolate from home-made cheese, $PrtP^+$	Laboratory collection
BGBUK2–5	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGBUK2–6	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGBUK2–14	Natural isolate from home-made cheese, Prt^-	Laboratory collection
BGGA8	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGMI-1	Natural isolate from home-made cheese, Prt^-	Laboratory collection
BGPIR13	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGCH2P	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGZB19	Natural isolate from home-made cheese, Prt^-	Laboratory collection
BGHV52	Human vaginal isolate, Prt^+	Laboratory collection
BGHV43	Human vaginal isolate, Prt^+	Laboratory collection
BGHV54	Human vaginal isolate, Prt^+	Laboratory collection
BGHO10	Human oral isolate, Prt^+	Laboratory collection
A112	Isolate from plant material, Prt^+	Vujcic and Topisirovic 1993
BGFD50	Natural isolate from home-made cheese, Prt^-	Laboratory collection
BGCG31	Natural isolate from home-made cheese, Prt^-	Laboratory collection
BGPV2–45a	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGHN40	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGGJ8–26	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGGJ5–6	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGGJ5–22	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGGJ6–16	Natural isolate from home-made cheese, Prt^+	Laboratory collection
LMG9205	Fermented corn product, Prt^+	BCCM/LMG collection
LMG9206	Human isolate, dental caries, Prt^-	BCCM/LMG collection
LMG9208	Sauerkraut, Prt^+	BCCM/LMG collection
LMG9212	Italian human, saliva, Prt^+	BCCM/LMG collection
LMG18021	Isolate from milk, Prt^+	BCCM/LMG collection
LMG18023	Natural isolate from Mish cheese, Prt^+	BCCM/LMG collection
LMG18024	Isolate from buffalo milk, Prt^+	BCCM/LMG collection
LMG18027	Isolate from Laban Rayeb, Prt^+	BCCM/LMG collection
LMG18053	Fermented food from cassava, Prt^+	BCCM/LMG collection
LMG11475	Cheese showing rusty spot, Prt^+	BCCM/LMG collection
Lactobacillus paraplantarum		
B-23115	Isolate from beer	NRRL collection
BGKP15	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGGJ6–5	Natural isolate from home-made cheese, Prt^+	Laboratory collection
BGGJ6–40	Natural isolate from home-made cheese, Prt^-	Laboratory collection
Lactobacillus pentosus		
B-227		NRRL collection
Lactobacillus helveticus		
CNRZ32	$PrtH^+$	Pederson *et al.* 1999
Lactobacillus rhamnosus		
BGT10	Human vaginal isolate, $PrtR^+$	Pastar *et al.* 2003
Lactobacillus delbrueckii ssp. *bulgaricus*		
BGPF1	Yoghurt, $PrtB^+$	Fira *et al.* 2001

*Prt^+, proteolytically active; Prt^-, proteolytically inactive.
BCCM/LMG – Laboratorium voon Microbiologie, Univerasitet Gent, Gent, Belgium.
NRRL – Agricultural Research Service Culture collection, Peoria, IL, USA.

Table 2 List of primers used in this study

Primers	Primer sequence	Locus	PCR amplicon length
PrtP700 PrtM700	5′-GCTTGAATTCGTTGTCGCTGCGGTTGT-3′ 5′-GCATGAATTCAATGCACGATAAATGAG-3′	Intergenic region of *prtP/prtM* genes – designed on the basis of *Lactococcus lactis* ssp. *cremoris* Wg2 *prtP* gene sequence	685 bp
P15C P06C	5′-AACCAAATCTGATGTTG-3′ 5′-TTTCAGCGGAAGCAACT-3′	Catalytic domain of *prtP* gene – designed on the basis of *L. lactis* ssp. *cremoris* Wg2 *prtP* gene sequence	560 bp
PRTB10 PRTB20	5′-GGTGTTGCTCCTGATGCCCAGC-3′ 5′-CCCCGTTTAACAACTGCAAGTT-3′	Catalytic domain of *prtB* gene – designed on the basis of *Lactobacillus delbrueckii* ssp. *bulgaricus* NCDO 1489 *prtB* gene sequence	597 bp
Jp23 Jp25	5′-GCTTGGATAGTAGCGTTAGC-3′ 5′-GGTGAACAAACTGAAGACG-3′	Catalytic domain of *prtH* gene – designed on the basis of *Lactobacillus helveticus* CNRZ32 *prtH* gene sequence	1034 bp
prti2 IP6Xba	5′-CAACACCGGGACCACGGTG-3′ 5′-CTGATCGTGGACGGTGTTGC-3′	Catalytic domain of *prtR* gene – designed on the basis of *Lactobacillus rhamnosus* T10 *prtR* gene sequence	1052 bp
U968 L1401	5′-AACGCGAAGAACCTTA-3′ 5′-GCGTGTGTACAAGACCC-3′	V6–V8 region of 16S rDNA	399 bp
pREV	5′-TCGGGATTACCAAACATCAC-3′	Partial *recA* gene	
paraF	5′-GTCACAGGCATTACGAAAAC-3′		107 bp
pentF	5′-CAGTGGCGCGGTTGATATC-3′		218 bp
plantF	5′-CCGTTTATGCGGAACACCTA-3′		318 bp

Annealing temperatures (mT): 56°C for primers PrtP700/PrtM700; PRTB10/PRTB20; Jp23/Jp25, 55°C for P15C/P06C; prti2/IP6Xba and 53°C for U968/L1401.

The PCR products obtained with U968/L1401, PrtP700/PrtM700 and P15C/P06C primers were purified by QIAquick PCR Purification KIT (Qiagen GmbH, Hilden, Germany) and sequenced by CRIBI-BMR servizio sequenziamento DNA (Universita di Padova, Italy). The BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=web&PAGE_TYPE=BlastHome) was used to determine the most related sequence relatives in the NCBI nucleotide sequence database.

Nucleotide sequence accession numbers

The nucleotide sequences of partial *prtP–prtM* intergenic spacer region and catalytic domain from *Lact. plantarum* BGSJ3–18 have been deposited in GenBank under accession numbers FM883699 and FM883700, respectively.

Results

Molecular determination and proteolytic activity of selected natural isolates

Molecular determination of 37 Lactobacillus strains isolated from natural sources such as cheeses, milk, human vaginal and oral mucosa, plant material (Table 1) was performed. Preliminary identification of isolates was carried out by sequencing of the 369 bp long PCR amplicon originated from the V6 to V8 region of 16S rDNA. Results show that all tested isolates belong to the *Lactobacillus plantarum/paraplantarum* group. To confirm species determination, a single-step identification multiplex PCR assay was performed. The strains *Lact. plantarum* B-4496, *Lact. pentosus* B-227 and *Lact. paraplantarum* B-23115 were used as a controls. Each PCR mixture was composed of three forward primers (plantF, paraF and pentF) and one reverse primer (pREV) (Table 2). The expected sizes of the PCR amplicons were 318, 218 and 107 bp for *Lact. plantarum*, *Lact. pentosus* and *Lact. paraplantarum*, respectively. The results indicated that 34 isolates belonged to *Lact. plantarum* group, while three of them, isolates BGKP15, BGGJ6–5 and BGGJ6–40, were identified as *Lact. paraplantarum* (Fig. 1).

The proteolytic activities of all 37 isolates were analysed by following the degradation of β-casein. Results revealed that 28 *Lact. plantarum* and two *Lact. paraplantarum* isolates showed significant proteolytic activity, similar to that of strains *Lact. paracasei* ssp. *paracasei* BGHN14 and

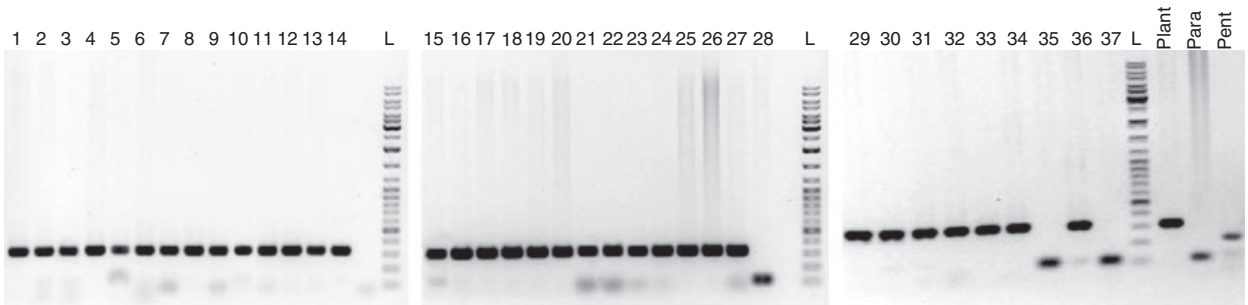

Figure 1 Amplification products obtained from the *recA* multiplex assay. 1 – LMG9205, 2 – LMG9206, 3 – LMG9208, 4 – LMG9212, 5 – LMG18021, 6 – LMG18023, 7 – LMG18024, 8 – LMG18027, 9 – LMG18053, 10 – LMG11475, 11 – BGSJ1–8, 12 – BGSJ1–10, 13 – BGSJ3–18, 14 – BGBUK2–5, 15 – BGBUK2–6, 16 – BGBUK2–14, 17 – BGGA8, 18 – BGMI-1, 19 – BGPIR13, 20 – BGCH2P, 21 – BGZB19, 22 – BGHV52Ta, 23 – BGHV43T, 24 – BGHV54T, 25 – BGHO10, 26 – BGA112, 27 – BGFD50, 28 – BGKP15, 29 – BGCG31, 30 – BGPV2–45a, 31 – BGHN40, 32 – BGGJ8–26, 33 – BGGJ5–6, 34 – BGGJ5–22, 35 – BGGJ6–5, 36 – BGGJ6–16, 37 – BGGJ6–40, plant – *Lactobacillus plantarum* B-4496, para-*Lactobacillus paraplantarum* B-23115, pent – *Lactobacillus pentosus* B-227, L – 'Ready-Load™' 100 bp DNA Ladder (Fermentas, Vilnius, Lithuania).

L. lactis ssp. *cremoris* Wg2, which were used as controls. However, proteolytic activity was not detected in six isolates of *Lact. plantarum* and one *Lact. paraplantarum* (data not shown). Among tested isolates, *Lact. plantarum* BGSJ3–18 showed the same level of activity compared to that of control strains (Fig. 2).

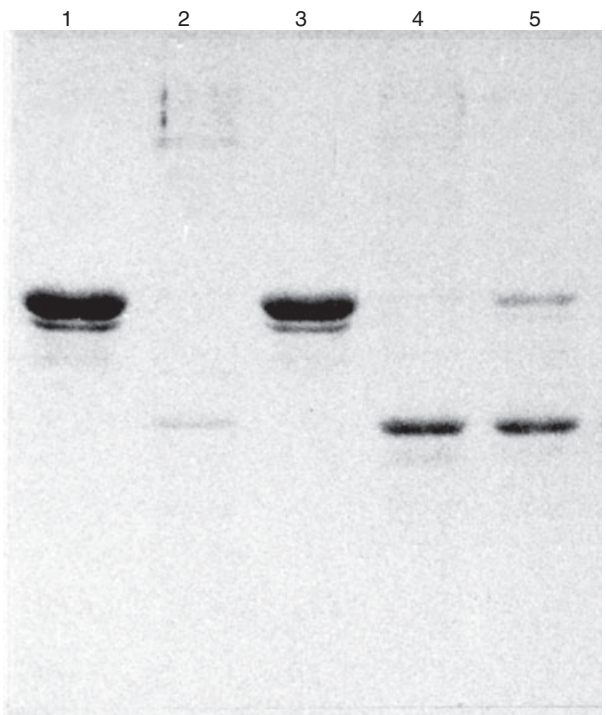

Figure 2 Proteolytic activity of whole cells towards β-casein. Lane 1, starting substrate (β-casein); Lane 2, *Lactococcus lactis* ssp. *cremoris* Wg2; Lane 3, *Lactococcus lactis* ssp. *cremoris* MG1363; Lane 4, *Lactobacillus paracasei* ssp. *paracasei* BGHN14; Lane 5, *Lactobacillus plantarum* BGSJ3–18.

Detection of the *prtP* gene in *Lact. plantarum* BGSJ3–18

The presence of proteinase genes in all *Lact. plantarum* isolates was analysed by PCR using primers specific for different types of CEPs (Table 2). When the pair of primers P15C/P06C that targets the active site of the PrtP proteinase was used, PCR amplicon of the expected size (560 bp) was obtained only with total DNA isolated from *Lact. plantarum* BGSJ3–18 (Fig. 3a). Nucleotide sequence

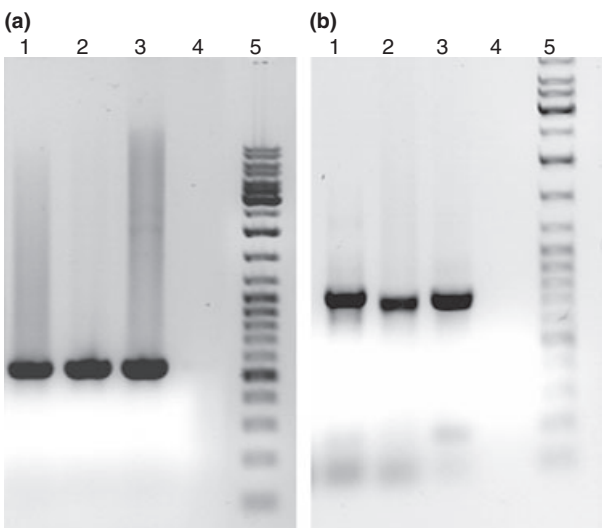

Figure 3 Detection of the *prtP* gene in *Lactobacillus plantarum* BGSJ3–18 by using PCR amplification with P15C/P06C set of primers (a) and with PrtP700/PrtM700 set of primers (b). Total DNA isolated from: Lane 1, *Lactococcus lactis* ssp. *cremoris* Wg2; Lane 2, *Lactobacillus paracasei* ssp. *paracasei* BGHN14; Lane 3, *Lactobacillus plantarum* BGSJ3–18; Lane 4, Negative control – PCR mixture without DNA; Lane 5, 'Ready-Load™' 100 bp DNA Ladder (Fermentas, Vilnius, Lithuania).

analysis of this fragment showed more than 98% of identity with the corresponding active site region from *Lact. casei* BL23, *Lact. casei* ATCC 334 and *Lact. paracasei* ssp. *paracasei* NCDO 151. Primers PrtP700/PrtM700, designed to amplify the *prtM–prtP* intergenic region, gave similar results (Fig. 3b). In this case, the PCR amplicon obtained with DNA isolated from *Lact. plantarum* BGSJ3–18 was of the same size (685 bp) as that corresponding to the intergenic region of *Lact. paracasei* ssp. *paracasei* BGHN14. Moreover, the PCR amplicon was slightly smaller than that obtained when DNA isolated from *Lact. lactis* ssp. *cremoris* Wg2 was used as a template. Nucleotide sequence analysis of the *prtM–prtP* intergenic region of *Lact. plantarum* BGSJ3–18 also revealed homology of 99% to the same region in the strain *Lact. casei* BL23, 98% to *Lact. casei* ATCC 334 and 97% to *Lact. paracasei* ssp. *paracasei* NCDO 151, indicating the presence of an identically organized proteinase region in the tested strain (Fig. 3). No PCR products were obtained with any other set of primers recognizing other *prt* genes present in LAB.

Discussion

Lactobacillus plantarum has the capacity to adapt to a variety of environmental conditions. Therefore, this species was detected in many fermented dairy, meat and plant products. In addition, in the last few years, some strains are characterized as probiotics (Vasiljevic and Shah 2008). The *Lact. plantarum* group are genotypically very closely related to *Lact. paraplantarum* and *Lact. pentosus,* and multiplex PCR analysis of partial *recA* gene with species-specific primers permitted a distinction among them (Torriani *et al.* 2001). Results confirmed that majority of the isolates tested in this study could be differentiated by using multiplex PCR analysis. Out of 37 tested isolates, 34 belong to *Lact. plantarum* group (92%), whereas three were recognized as *Lact. paraplantarum* (8%).

Cell envelope proteinases (CEPs) are present in lactococci and several species of lactobacilli, but also in some species of pathogenic streptococci such as *Streptococcus pyogenes* and *Strep. agalactiae* (Siezen 1999). Because in LAB these enzymes are part of proteolytic system that provides essential amino acids for growth, in pathogenic streptococci they most likely act as a virulence factors.

Among all CEPs in LAB, the *prtP* is the only one gene that has been detected in bacteria that belong to different species and even different genera. The *prtM–prtP* region was identified in all of the *Lactococcus lactis* proteinase genes studied so far (Kok and de Vos 1994; Kunji *et al.* 1996). The same proteinase gene region was also found in all Prt^+ strains of *Lact. paracasei* ssp. *paracasei* tested so far (Kojic *et al.* 1991, 1995; Holck and Naes 1992), showing 95% of overall identity with the lactococcal genes at the nucleotide sequence level. On the other hand, the mechanism of release and processing by autodigestion that is dependent on Ca^{++} ions and the PrtM protein was confirmed only in the case of PrtP proteinase (Haandrikman *et al.* 1991).

In this work, the presence of the *prtP*-like gene in one of natural isolates, *Lact. plantarum* BGSJ3–18, was detected. BGSJ3–18 has the same level of proteolytic activity compared to the other strains harbouring PrtP proteinase. Computer-assisted nucleotide sequence analysis of the *prtM–prtP* intergenic region and catalytic domain of the *prtP* gene in BGSJ3–18 showed more than 95% identity with the same genes in previously studied strains of *Lact. paracasei*, *Lact. casei* and *L. lactis.* The occurrence of PrtP proteinase in different species of LAB makes this enzyme different from other CEPs that seem to be species related. On the other hand, the *prtP* gene is the only one of all proteinase genes studied so far in LAB that is located on plasmids at least in lactococci (Kok and de Vos 1994). Therefore, one can speculate that the presence of PrtP in different LAB species could be the consequence of horizontal gene transfer among them. This transfer could be the result of concomitant presence of lactococci and particular species of lactobacilli in same ecological niches, because they represent normal microflora of many types of artisanal cheeses. Considering the proteolytic activity of other *Lact. plantarum* natural isolates described in this work and absence of homology with *prtP*, *prtB*, *prtH* and *prtR* genes indicate that they have extracellular proteinases of different type(s). Therefore, the biochemical and genetic characterization of proteinases from *Lact. plantarum* and their relatedness to the species will be elucidated in further experiments.

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