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Phenotypic and genotypic characterization of peptidoglycan hydrolases of *Lactobacillus sakei*

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ABSTRACT

Lactobacillus sakei, a lactic acid bacterium naturally found in fresh meat and sea products, is considered to be one of the most important bacterial species involved in meat fermentation and bio-preservation. Several enzymes of *Lb. sakei* species contributing to microbial safeguarding and organoleptic properties of fermented-meat were studied. However, the specific autolytic mechanisms and associated enzymes involved in *Lb. sakei* are not well understood. The autolytic phenotype of 22 *Lb. sakei* strains isolated from Tunisian meat and seafood products was evaluated under starvation conditions, at pH 6.5 and 8.5, and in the presence of different carbon sources. A higher autolytic rate was observed when cells were grown in the presence of glucose and incubated at pH 6.5. Almost all strains showed high resistance to mutanolysin, indicating a minor role of muramidases in *Lb. sakei* cell lysis. Using *Micrococcus lysodeikticus* cells as a substrate in activity gels zymogram, peptidoglycan hydrolase (PGH) patterns for all strains was characterized by two lytic bands of ~80 (B1) and ~70 kDa (B2), except for strain BMG.167 which harbored two activity signals at a lower MW. Lytic activity was retained in high salt and in acid/basic conditions and was active toward cells of *Lb. sakei*, *Listeria monocytogenes*, *Listeria ivanovii* and *Listeria innocua*. Analysis of five putative PGH genes found in the *Lb.* sakei 23 K model strain genome, indicated that one gene, *lsa*1437, could encode a PGH (N-acetylmuramoyl-L-alanine amidase) containing B1 and B2 as isoforms. According to this hypothesis, strain BMG.167 showed an allelic version of *lsa*1437 gene deleted of one of the five LysM domains, leading to a reduction in the MW of lytic bands and the high autolytic rate of this strain. Characterization of autolytic phenotype of *Lb. sakei* should expand the knowledge of their role in fermentation processes where they represent the dominant species.

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Introduction

Lactobacillus sakei is a psychotrophic lactic acid bacterium naturally found on fresh meat and fish, and is considered to be one of the most important bacterial species involved in meat fermentation and bio-preservation [1–3]. *Lb. sakei* influences

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color, flavor and texture of products during meat processing [4–6]. Although autolysis of lactic acid bacteria such as *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus* by peptidoglycan hydrolases (PGHs) has been shown to play an important role in the organoleptic properties of fermented dairy products [7,8], the specific autolytic mechanisms and associated enzymes involved in *Lb. sakei* are not well understood. Improved understanding may help to expand our knowledge of their role in fermentation processes and their survival during the processing of meat products.

Peptidoglycan is the major component of bacterial cell wall which determines cell shape and provides resistance to internal osmotic pressure [8]. The peptidoglycan network consists of glycan strands composed of alternating *N*-acetylglucosaminyl and *N*-acetylmuramyl residues cross-linked by short peptides of various compositions [9]. Peptidoglycan hydrolases are defined as endogenous enzymes capable of cleaving covalent bonds in polymeric peptidoglycan and/or in its soluble fragments [10,11]. Four types of PGHs are known to alter the three-dimensional network of the cell wall and most often several with various specificities coexist in the cell [9]: (i) β-N-acetylglucosaminidases (hydrolyze the β1–4 bonds between the alternating N-acetylmuramic acid and the N-acetylglucosamine residues of the glycan chain, (ii) β-N-acetylmuramidases (hydrolyze the N-acetylmuramyl, 1,4-β-N-acetylglucosamine bonds, (iii) N-acetylmuramoyl-L-alanine amidase or amidase (hydrolyze the bond between the glycan chain and the peptide side chain and (iv) peptidases (cleave peptide bonds in either the peptide side chain or in the cross-bridge peptides). Many bacteria possess multiple hydrolases that appear to have redundant roles including regulation of cell wall synthesis, turnover of peptidoglycan during growth, separation of daughter cells during cell division, and autolysis [9,12,13]. Prophage-encoded lysins in the genome can also exert a weakening effect on the cell wall, leading to enhanced autolysis [14]. Most of *Lb. sakei* autolytic enzymes predicted from the genome sequence predicted to possess an N-acetylmuramoyl alanine amidase activity [3]. Peptidoglycan amidases are in particular responsible for the geometry of cell division. Mutant lacking N-acetylmuramoyl-L-alanine amidase has multiple, misplaced, and sometimes curved septa that do not bisect daughter cells equally [15].

A wide variety of enzymes with peptidoglycan hydrolytic activities have been identified based on digestion of the intact peptidoglycan macromolecule. Zymography is the most commonly used detection method, involving the separation of proteins from cell extracts by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using gels containing peptidoglycan, followed by *in situ* enzyme renaturation [16].

Autolytic systems of several Gram-positive low G + C bacteria have been studied such as those found in *Staphylococcus aureus* [17,18], *Bacillus subtilis* [19], *Bacillus thuringiensis* [20] *Pediococcus* spp. [21], *L. lactis* [22], *Enterococcus faecalis* [23] and *Lactobacillus pentosus* [24]. Several studies relate to autolysin genes in lactic acid bacteria particularly *E. faecalis* [25], *Enterococcus hirae* [26] and *Lc. lactis* [27]. Typically, PGH contains the tandem repeated sequences at or near the N- or C-terminal end of the amino acid sequence, known as the lysin motif (LysM domains), which are thought to direct the binding of the enzyme to the cell wall in Gram-positive bacteria [28–30].

The aim of the present study was to investigate the autolytic phenotype, the PGH activities and the putative PGH candidate genes of a nonredundant collection of 22 *Lb. sakei* strains, isolated from fresh meat, traditional salted meat and seafood products [31]. The results of this work should provide further information, needed to select the most suitable strains for use as starters in the production of fermented meat products where these species occur as primary or secondary bacterial population.

Material and methods

Bacterial strains and growth conditions

Lb. sakei strains used in the present study were isolated from traditionally prepared Tunisian meat and fish products [31]. The reference strains *Lb. sakei* 23 K (INRA Jouy-en-Josa, Paris) [32,3], and *Lc. lactis* IL1403 (INRA, Jouy-en-Josas, Paris) [33], were used as controls for autolytic experiments. *Lb. sakei* and *Lc. lactis* strains were grown at 30 °C in MRS broth (Scharlau Chemie, Barcelona, Spain) [34]. Stock cultures for long term maintenance were stored at −80 °C, in 20% (v/v) glycerol (Scharlau).

Autolysis of whole cells in buffer solution

Autolysis rates of *Lb. sakei* strains were evaluated as described by Ostlie et al. [35] with some modifications. In order to investigate the effect of carbon sources on autolysis rate, bacterial strains were grown until late exponential growth phase (OD_{600} = 0.8–1.0) in reconstituted MRS culture medium containing (liter^{-1}) 10 g polypeptone (Difco Laboratories Inc., Detroit, MI), 8.0 g beef extract (Difco), 5 g yeast extract (Difco), 2 g K_2HPO_4, 2 g diammonium citrate, 0.1 g $MnSO_4$ (Merck Darmstadt, Germany), 0.05 g $MgSO_4$ (Merck), 0.1% (vol/vol) Tween 80 (Merck), and 1% (w/v) of glucose, ribose or fructose (Merck). Harvested cells were washed twice in deionized water and suspended in potassium phosphate buffer (50 mM, pH 6.5) or in Tris–HCl (Sigma) buffer (50 mM, pH 8.5) at an initial OD_{600} of 0.6–0.8. Samples were incubated at 30 °C and autolysis was monitored by measuring the decrease in OD_{600} after 72 h. The extent of autolysis was expressed as the percentage decrease of the optical density [14].

Mutanolysin sensitivity

Mutanolysin sensitivity was evaluated on *Lb. sakei* cells as described by Ouzari et al. [22]. Cells were harvested in late exponential phase (OD_{600} = 0.8–1.0) and suspended to an OD_{600} of approximately 0.5, using MES (2-*N*-Morpholinoethansulfonic acid) (Sigma) buffer (50 mM, pH 6.0) supplemented with 1 mM $MgCl_2$ (Scharlau). A volume of 3 ml aliquot of cell suspension was then equilibrated at 37 °C after which the OD_{600} was measured. Subsequently, 5 µl of mutanolysin solution (Sigma) (150 U ml^{-1}) prepared in TES buffer (N-Tris-hydroxymethyl-2-aminoethansulfonic acid) (Sigma) (50 mM, pH 7.0), 1 mM $MgCl_2$ (Scharlau), was added to the cell suspension. The mixture was incubated at 37 °C for 20 min and the OD_{600} was then measured. The mutanolysin activity was expressed as the percentage decrease of the OD_{600}.

Detection of PGH activity

Whole cell extracts were prepared based on the approach of Cibik et al. [24]. Briefly, 5 ml of *Lb. sakei* cultures (grown 8, 24, 48, or 72 h) was used. After centrifugation at 5000 g for 5 min, the pellet was resuspended in SDS-extraction solution [10 mM Tris–HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM NaCl, SDS 2% (w/v) (Scharlau)]. The obtained suspension was vigorously mixed, boiled for 5 min and centrifuged at 12,000*g* for 20 min. The supernatant (containing cell-protein extract) was stored at −20 °C until used. Electrophoresis, renaturing and staining were performed as described by Cibik et al. [24]. Sample preparation was carried out by mixing 40 µl of whole-cell SDS extract with 30 µl of Laemmli loading buffer (Laemmli 1970) [0.5 M Tris–HCl, pH 6.8; 10% (w/v) SDS; 25% (v/v) glycerol; 5% (v/v) ß-mercaptoethanol (Sigma); and 0.5% (w/v) bromophenol blue (Scharlau)]. Mixtures were boiled for 3 min before loading onto 12% (w/v) polyacrylamide gels containing 0.2% (w/v) autoclaved cells of *Micrococcus lysodeikticus* ATCC 4698 (Sigma), used as a reference target cells for PGHs enzymes. Activity spectrum of PGHs of 22 *Lb. sakei* was determined as described by Mora et al. [21] against vegetative cells of the most common food-borne pathogens bacteria, including *Listeria monocytogenes* MACa1 (DSMZ), *Staphylococcus aureus* (ATCC 25923), *Listeria ivanovii* BUG 469 (INRA, Jouy-en-Josas, Paris) and *Listeria innocua* (INRA, Jouy-en-Josas, Paris). The two last strains were investigated as a possible useful as nonpathogenic model. Furthermore, *Lb. sakei* 23 K strain was also used as a target in order to determine the intra-specificity of PGH activities.

The effect of different chemicals on PGH activity was evaluated according to the method of Raddadi et al. [36]. Gel slices were incubated in renaturation buffer containing the following compounds: NaCl (Scharlau) (1%, 2%, 4%, 6% and 8%), $MgCl_2$ (Scharlau) (10 mM), $MnCl_2$ (Scharlau) (10 mM), $CaCl_2$ (Scharlau) (10 mM), and EDTA (Scharlau) (10 mM). The effect of pH was also evaluated using gel renaturation solution buffered with 10 mM sodium acetate (pH 5.0) or Tris–HCl (pH 6.0, 7.0, 8.0 and 9.0). To estimate the molecular mass of PGH bands, duplicate samples containing parietal extracts of two selected strains BMG.136 and BMG.167, representing the two PGH profiles of *Lb. sakei* collection, and *Lc. Lactis* IL1403, a reference strain used as a control characterized by a major activity band of 45 kDa [22], were loaded simultaneously in two gels within the same electrophoresis system. The first gel, containing *M. lysodeikticus* cells was revealed by the renaturing method described above. The second gel, containing the protein molecular weight marker (Fermentas GmbH, St. Leon-Rot, Germany): β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.05 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease Bsp98I (25 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa), was stained with Coomassie blue (Scharlau) according to the standard method of Laemmli [37].

DNA manipulation and sequencing

Chromosomal DNA was extracted from 22 *Lb. sakei* strains [31] and *Lb. sakei* 23 K [32,3],used as a positive control, according to the method described by Anderson and Mac Kay [38]. The quantification and the degree of DNA purity were determined spectrophotometrically using Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, NC). Primers (BioFaster, Germany) (Table 1), corresponding to the five putative PGH candidate genes *lsa*0862, *lsa*1437, *lsa*1558, *lsa*1581, and *lsa*1788, were designed using the free online software "Primer3" (http://simgene.com/Primer3) after in silico analysis of *Lb. sakei* 23 k genome [3]. Polymerase chain reactions (PCRs) were performed on a MJ Research PTC-200 thermocycler. The 50 µl PCR reaction mixture contained PCR buffer 1× (Fermentas), $MgCl_2$ 1.5 mM, 0.2 mM of each dNTP, 0.5 µM of each primer, 1 µg of chromosomal DNA, and 1 U of Taq DNA polymerase (Fermentas). Amplification conditions were 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min [39]. PCR products (aliquots of 5–10 µL) were first checked electrophoretically in 0.8–1% (w/v) agarose gel (Agarose, DNA grade, Electran) in Tris–borate-EDTA (TBE) 0.5× buffer at 100 V for 30 min. Gel was stained with ethidium bromide (5 mg/mL) and the DNA bands were visualized under ultraviolet illumination at 254 nm. A 100 bp DNA Ladder was used as a molecular mass marker (GeneRuler 100 bp, 0.5 mL/mL, Fermentas). PCR products were then cleaned from residual primers using a QIAquick PCR purification kit (Qiagen, Courtaboeuf, France). DNA concentrations were quantified using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, NC). For DNA sequencing, 10 µl of PCR products was first treated with 0.1 U of Shrimp alkaline phosphatase (New England Biolabs Inc, Ipswich, UK) and 1 U of exonuclease I (Biolabs) in alkaline buffer solution (20 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$) for 1 h at 37 °C, followed by 10 min inactivation at 94 °C, and then sequenced in both strands using forward and reverse primers (Table 1). Sequencing was conducted by Sanger sequencing service (Eurofins MWG operon, Ebersberg, Germany) using big-dye terminator chemistry. Unidirectional DNA sequences were checked with CHROMAS-LITE software (Technelysium Pty. Ltd., South Brisbane, Australia). The gene sequences were assembled using DNAstar software (DNASTAR Inc., WI). Sequence similarity searches were performed using the online sequence analysis resources "BLAST" (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

Autolytic phenotype of Lb. sakei and mutanolysin sensitivity

The autolysis rates of *Lb. sakei* strains were evaluated with different carbon sources (glucose, fructose and ribose) after three days of incubation, and at pH 6.5 and 8.5. The incubation time of 72 h was chosen because no significant variation in lyses rate was observed upon further incubation (data not shown). Under starvation conditions, autolysis levels appeared as strain dependent characteristics influenced by the carbon source and the buffered solution composition (Fig. 1A and B). Ten out of twenty-three strains had the highest autolysis rate when cells were grown in glucose and incubated at pH 6.5 (Fig. 1A). Other ten strains showed the highest autolytic rate in fructose and only three strains showed the autolytic phenotype when

Table 1 List of oligonucleotides used in gene PCR detection. Target genes and their assigned functions are indicated.

Gene name Locus_tag	Primer name	Sequence 5′ to 3′	Assigned function	Theoretical protein size (kDa)/CDS length (bp)	Predicted size of the mature proteins (kDa)	Expected size of PCR product (bp)
*lsa*1558	LSA1558-F	GTCCTGCTGGGCGTTTTATT	Putative extracellular N-acetylmuramoyl-L-alanine amidase precursor	25.4/660	18.3	530
	LSA1558-R	CCGGATAATTAGGATCCGTTG				
*lsa*1437	LSA1437-F	CCAGGATAGATGAAGTTATTACGG	N-acetylmuramoyl-L-alanine amidase precursor	71.7/2007	66	668
	LSA1437-R	TTAAATCGCCTTATCCAACA				
*lsa*0862	LSA0862-F	GCGTTCGTTATCACGAAGTA	N-acetylmuramoyl-L-alanine amidase precursor	48/1323	43.8	1600
	LSA0862-R	AAGCAAACGTCGTTAATGTG				
*lsa*1581	OLS1806-F	TGTTTTATTAGTTAATAGTT	Teichoic acid-binding N-acetylmuramoyl L-alanine amidase	76.5/2085	76.5	1689
	OLS1807-R	GGATAGTTATTTTTTTGGTG				
*lsa*1788	LSA1788-F	GCCGTAACGCACACTATTAT	Phage-related 1,4-beta-N-acetyl muramidase	27.6/759	22.8	660
	LSA1788-R	CGAGTACGACCTAATTCGG				

they were grown on ribose at pH 6.5 (Fig. 1A). At pH 8.5, *Lb. sakei* strains were clustered in three groups composed of 6, 9 and 8 strains showing respectively the highest autolytic phenotype when cultured on glucose, fructose and ribose (Fig. 1B). These results led to hypothesize that for some strains, the carbon source might have a significant effect on the autolytic phenotype. More generally, all the tested strains showed autolytic rate variation according to carbon source ranging between 0.7% and 15% for the majority of strains and reaching approximately 25% for strains BMG.167 and BMG.115 at pH 6.5, and over 50% for *Lb. sakei* BMG.148 and 23 K at pH 8.5. Integrity of the cell wall structure and osmotic sensitivity of *Lb. sakei* strains were examined by evaluating the mutanolysin sensitivity of cells harvested in exponential growth phase. The results indicated a high resistance to mutanolysin for most strains, with activity values ranging from 0.6% to 13.9%, and no correlation between enzyme sensitivity and autolytic rate of cultures grown in glucose at pH 6.5 (Fig. 1A). However, strain BMG.167 was observed to have the highest autolytic rate (64%) and showed a high level of sensitivity (31.3%) to the enzyme (Fig. 1A).

Zymogram analysis of PGH patterns

The peptidoglycan hydrolase activity of whole cell protein extracts was examined by renaturing SDS–PAGE containing *M. lysodeikticus* cells as substrate. The PGH content was first evaluated according to the growth phase with strain *Lb. sakei* 23 K. The most intense and clearest lytic bands were detected using cultures incubated for 24 and 48 h (data not shown). The PGH profile was thus determined for all strains after 48 h incubation. Except from BMG.167, all the *Lb. sakei* strains showed a similar pattern profile (P1) of two large proteins of approximately 70 and 80 kDa. The 70 kDa PGH (B2) was characterized by an intense band detectable after 2–3 h of renaturation, whereas the 80 kDa PGH (B1) protein was less intense and appeared in gels after only after 16 h of renaturation. This latter band showed also some variations in intensity among tested strains and between extracts. Two representative strains (BMG.136 and BMG.120) showing the PGH profile P1 are illustrated in the Fig. 2A. The second profile P2, solely detected with the strain BMG.167, was characterized by two PGH bands with lower molecular weights of 60 and 70 kDa for thick and thin bands respectively (Fig. 2A). A few very weak secondary bands of molecular weight ranging between 45 and 80 kDa were occasionally observed and were not considered further.

The effect of pH (5.0, 6.0, 7.0, 8.0 and 9.0), sodium chloride (1%, 2%, 4%, 6% and 8%) and three chemical compounds ($MgCl_2$ 10 mM, $MnCl_2$ 10 mM, $CaCl_2$ 10 mM) on lytic activity were studied in strain 23 K by incubating gel slices under different conditions during the renaturation procedure. Under the modified conditions, the lytic bands appeared after 48 h incubation in different renaturing buffers, rather than 2 or 16 h in the standard conditions. A slight decrease in band intensity was observed in the presence of $MgCl_2$, $MnCl_2$, $CaCl_2$ and EDTA, while no significant effect of NaCl was noticed. Finally, the optimum pH for lytic activity was found between 6 and 8 (data not shown).

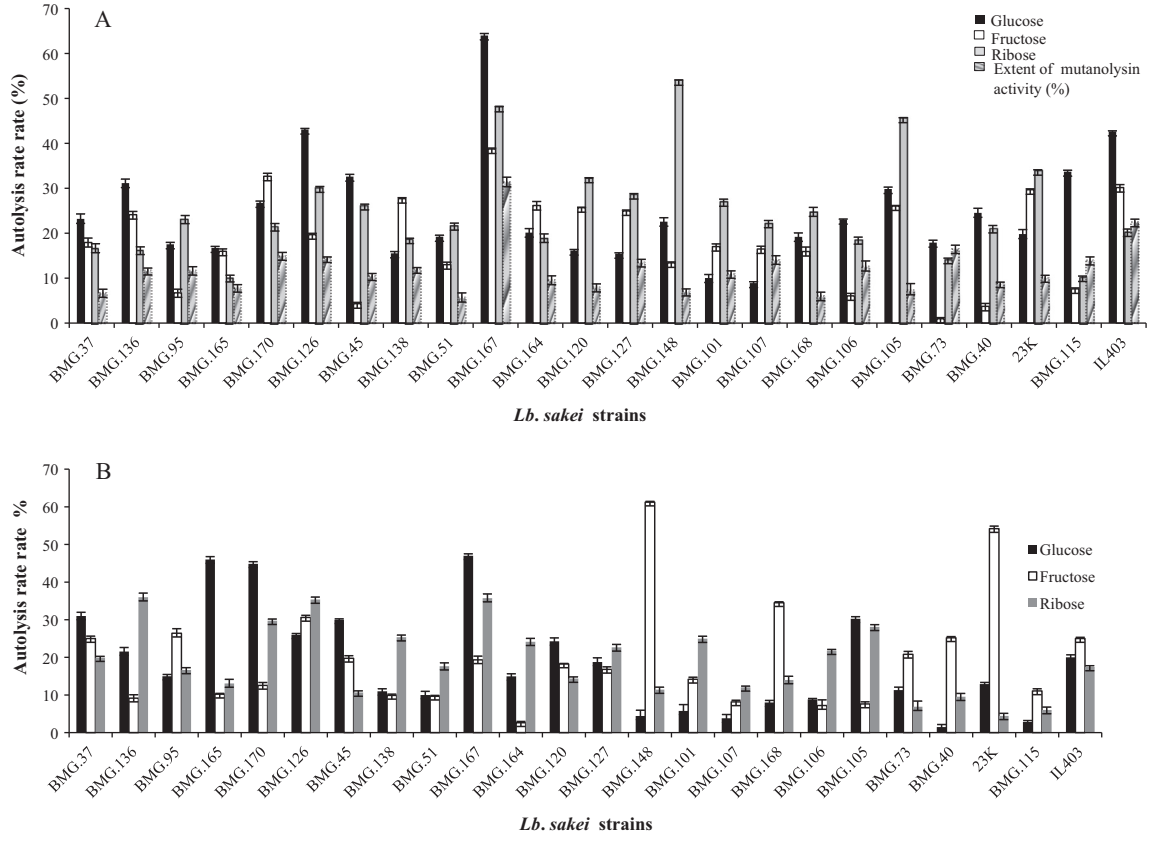

Fig. 1 Extent of autolysis of *Lb. sakei* strains after 72 h of incubation at 30 °C. Bacteria were grown in MRS broth containing 1% (w/v) glucose (black bars), fructose (white bars) or ribose (gray bars), and resuspended in: (A): Potassium phosphate buffer (50 mM, pH 6.5) and (B): Tris–HCl buffer (50 mM, pH 8.5). Mutanolysin activities were evaluated on the same bacterial cells grown in MRS broth containing 1% (w/v) glucose (black bars) and resuspended in phosphate buffer (50 mM, pH 6.5) of the (A). The extent of mutanolysin sensitivity is represented by degraded bars (A). The 24th strain in the figures corresponds to the reference strain *Lc. lactis* IL403, used as a control. The average of three replicates is indicated for all strains.

Spectrum of PGH specificity

The activity spectrum of PGH of all *Lb. sakei* strains was evaluated against cells of lactic acid bacteria and other species. No notable lytic bands were visualized when *S. aureus* was used as a substrate. The same profile, initially detected with *M. lysodeikticus* and showing the common B1 and the B2 bands, was also observed toward cells of *Lb. sakei*, *L. innocua*, *L. ivannovii* and *L. monocytogenes*.

Characterization of PGH candidate genes

In silico analysis of the genome of *Lb. sakei* 23 K (GenBank accession number CR936503) for putative PGH encoding genes revealed the presence of five candidates, encoded by *lsa*0862, *lsa*1437, *lsa*1558, *lsa*1581, and *lsa*1788. For two candidates, the theoretical size of the encoded proteins (LSA1437, 71 kDa and LSA1581, 76 kDA) was compatible with the experimentally observed size of the major PGH band B2 (70 kDa). The presence of these two genes, corresponding to an N-acetylmuramoyl-L-alanine amidase and a teichoic acid-binding N-acetylmuramoyl L-alanine amidase, was examined by PCR in the 22 *Lb. sakei* strains of the collection. In addition, since it is known that *Lb. sakei* exhibits high genomic diversity, as within strains, as evidenced by PCR analysis of several genes [39], the presence of the three other PGHs encoding candidate genes was also investigated. PCR results are presented in Table 2. The results indicated that *lsa*0862 and *lsa*1558 were present in all strains. The amplification of an internal fragment of *lsa*1581 could be detected from only four out of the twenty-two strains, indicating the absence of correlation with the rate of measured autolysis. Moreover, the gene *lsa*1788 was detected in ten out of twenty-two strains and no correlation with the PGH band patterns could be established. In contrast, for *lsa*1437 we observed that all strains, except BMG.167, showed a band of the expected size (with regard to the difference between theoretical molecular weight and that observed in the denaturant conditions). Interestingly, a PCR fragment was present in BMG.167 that was smaller than observed in other isolates. The *lsa*1437 gene fragment of BMG.167 was sequenced (GenBank accession number GQ847621) and we deduced a short internal deletion of part of the gene corresponding to the first out of five C-terminal LysM domains. This deletion was correlated with a reduction of 132 bp in the coding sequence, as it was revealed by the size difference of the PCR fragment related to the strain BMG.167.

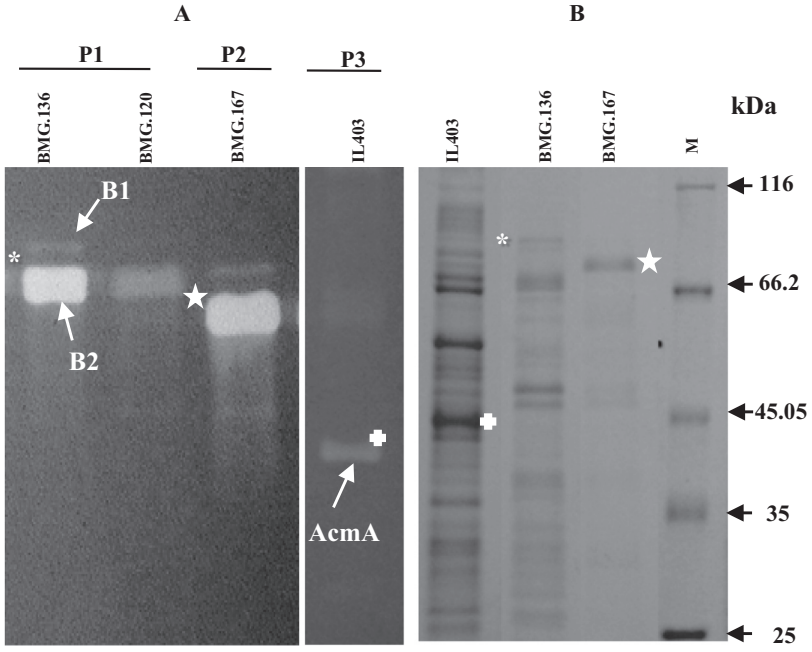

Fig. 2 Evaluation and molecular weight assessment of PGH profiles of *Lb. sakei* strains were performed simultaneously onto two gels: (A) Peptidoglycan hydrolase profiles (P1 and P2) of whole cell SDS extracts of *Lb. sakei* collection evaluated by renaturing SDS–PAGE containing 0.2% (w/v) cells of M. lysodeikticus. Activity was revealed after 18 h of renaturation in standard buffer 25 mM Tris–HCl, pH 7.0, containing 1% (v/v) Triton X-100. Profile P1 (*), characterized by two bands B1 (80 kDa) and B2 (70 kDa), is represented by two strains BMG.136 and BMG.120. Profile P2 (★), characterized by two bands with lower MW of 70 and 60 kDa, is represented by the unique strain BMG.167. Profile P3 (●) represents the known PGH AcmA (45 kDa) of *Lc. lactis* IL403 included as a control. (B) The SDS–PAGE fingerprinting gel of whole cell-SDS extracts proteins patterns after Coomassie blue staining of *Lb. sakei* strains (BMG.136 BMG.167), *Lc. lactis* IL403 and the molecular weight marker (Fermentas).

Table 2 PCR detection of five putative PGH encoding genes.

Strain	PGH genes amplification[a]				
	*lsa*1558 (25.4 kDa)	*lsa*1581 (76.5 kDa)	*lsa*1437 (71.7 kDa)	*lsa*1788 (27.6 kDa)	*lsa*0862 (48 kDa)
BMG.106	+	+	+	+	+
BMG.37	+	–	+	+	+
BMG.73	+	–	+	+	+
BMG.170	+	–	+	+	+
BMG.51	+	–	+	+	+
BMG.148	+	–	+	+	+
BMG.168	+	–	+	+	+
BMG.167	+	–	+ (R)	+	+
BMG.164	+	+	+	+	+
BMG.126	+	–	+	–	+
BMG.127	+	–	+	–	+
BMG.101	+	–	+	–	+
BMG.105	+	–	+	–	+
BMG.95	+	–	+	–	+
BMG.115	+	–	+	–	+
BMG.107	+	+	+	–	+
BMG.40	+	–	+	–	+
BMG.45	+	–	+	–	+
BMG.136	+	–	+	–	+
BMG.138	+	–	+	–	+
BMG.120	+	–	+	–	+
23 K	+	+	+	+	+

[a] PGH putative genes and the predicted molecular weight of the corresponding proteins, based on the 23 K genome sequence. + indicates when putative candidate genes were detected by PCR amplification. – indicates no PCR amplification. (R): a reduced molecular weight of about 132 bp in the PCR fragment.

Discussion

The present study evaluated the autolytic phenotype of a collection of *Lb. sakei* strains, isolated from meat and sea products. The highest level of autolysis was observed for most strains, when cells were grown on glucose and incubated in potassium buffer (50 mM, pH 6.5). These results correlated with those reported for other lactic acid bacteria such as *Lc. lactis* [40,41], *Leuconostoc* [42], and *S. thermophilus* [43], and supporting the hypothesis of a weaker cell wall structure in cultures grown in the presence of glucose. The difference in lysis after growth on different carbon sources could be explained by reduced binding of PGH, containing LysM domains (that specifically bind N-acetylglucosaminidase) due to modification of the cell wall as reported by Buist et al. [30] and Steen et al. [29,44]. The mutanolysin test carried out in MES buffer indicated that most strains possessed high resistance to the muramidase. This indicates that no correlation exists between autolysis and mutanolysin sensitivity. These results were in accordance with data described for *Bacillus* species [20,36] but differ from those reported for lactic acid bacteria such as *Lc. lactis* [22] and *Pediococcus pentosaceus* [21]; and this can be explained by differences in the cell wall sensitivity to lytic enzymes that could be related to different modifications of the peptidoglycan, such as deacetylation [45,46,14]. In addition, according to the predicted putative PGH types that are mostly N-acetyl muramoyl L-alanine amidase [3], muramidase-like enzymes could have a minor role in autolysis functions compared to the case of *Lc. lactis* [28,47].

The obtained lytic profile of *Lb. sakei* strains, using *M. lysodeikticus* as substrate, was previously reported by Lortal et al. [48], showing the B1 and B2 lytic bands of approximately 80 kDa and 70 kDa respectively. The exception was strain BMG.167, in which both bands had slightly smaller molecular weights (of approximately 70 and 60 kDa for thin and thick bands, respectively). With regard to PCR analysis of the five putative PGH genes of *Lb. sakei*, this strain also showed a smaller PCR product corresponding to the *lsa*1437 gene. This variation reflected a deletion in the first LysM domain of the C-terminal part of the translated peptide. The LysM domain was originally identified in enzymes that degrade bacterial cell wall components, but it is also found in many other bacterial proteins [49,31]. It is composed of a lysine motif of approximately 44 residues and may have a general peptidoglycan binding function. As previously described for *Lc. lactis* and *E. faecalis* [50,51], the deletion of one lysM domain does not lead to the loss of PGH activity, however does affect its intensity. Interestingly, analysis of *Lb. sakei* autolytic activities demonstrated that, for strain BMG.167 which contained the lysM deletion, a relatively high level of autolysis is obtained after growth on different carbon sources and incubation at different levels of pH. In this case, the loss of the lysM domain for the amidase LSA1437 is proposed as beneficial for PGH activity, cell wall binding and/or autolysis. Based on the predicted molecular weight of the putative PGH calculated from the 23 K genome, comparison with the experimentally derived PGH profiles was performed. Although, the masses of proteins LSA1437 and LSA1581 are comparable, LSA1581 does not contain a signal peptide according to signal IP prediction (Table 1). This suggests that the protein is not secreted. Moreover, the results of PCR demonstrated that the gene *lsa*1581 is present only in four strains however *lsa*1437 gene and the corresponding PGH are present in all strains. It can therefore be postulated that *lsa*1437 encodes the protein B2, as characterized by strong lytic activity associated with a protein of 70 kDa. However, it could also encode the B1 band, as both have a diminished size in strain BMG.167. In addition, the protein B1 showed variability in both presence and intensity, and this could be attributed to the precursor molecule of B2, as demonstrated for the thin band accompanying the AcmA of *Lc. lactis* [28].

When varying the cell wall substrate by including cells of different bacteria in the gel, the main PGH bands, initially revealed with *M.* lysodeikticus were also visualized using cells of *L. ivanovii*, *L. innocua*, *L. monocytogenes* and *Lb. sakei*. With the exception of the *lsa*1437 gene, which was attributed to be the main autolytic band, no other correlations were found between the presence of other putative PGH encoding genes and the lytic bands detected in the zymogram analysis. As previously observed for *Lc. lactis* [52], these results could be attributed to renaturing SDS–PAGE method used to reveal PHG profiles and they do not necessarily reflect the actual number of enzymes produced by bacteria because of possible irreversible denaturation, and to the stringent substrate specificity of some enzymes. Moreover, as previously reported for many Gram-positive bacteria [53–57,21], partial proteolytic degradation may produce fragments that retain enzymatic activity.

The effect of different renaturing conditions on autolytic activity bands was evaluated using renaturing buffers of different compositions and at different levels of pH. Peptidoglycan hydrolase activity was observed, although at a slightly reduced level in high salt concentrations and in acid/basic conditions. This observation has also been reported for other species of lactic acid bacteria, such as *P. pentosaceus* [21], *Lc. lactis* [22], as well as for some propionibacteria [35], indicating that the detected PGH is active in stressful conditions, low temperature and/or high salt concentration, such as those encountered in meat environments [58,59,3,52].

Lb. sakei, commonly presents as a part of the adventitious microbiota or as a starter culture, plays a crucial role during meat fermentation, due to the proteolytic enzymes contributing to the increase of small peptides and free amino acids known to be precursors of volatile compounds [4–6]. In this context, PGHs can play an important role in the releasing of the bacterial cytoplasmic content which is rich in proteolytic enzymes involved in the development of organoleptic properties. In the present work, BMG.167 showing a high autolytic rate constitutes therefore a promising candidate for further application as starter culture for meat fermentation. Furthermore, the ability of *Lb. sakei* PGHs to hydrolyze the cell wall of food-borne pathogenic bacterial species and the stability of *Lb. sakei* PGH activity in different environmental conditions could play an important role in bacterial biocontrol, for example, after the fermentation and during the drying step of fermented sausage production, when pH is low and NaCl concentration is increasing.

Conclusions

Evaluation of autolytic properties of *Lb. sakei* strains showed a high degree of diversity among isolates. The higher autolytic

rate was observed when cells were grown in the presence of glucose, at pH 6.5. The PGH band pattern was determined by renaturing SDS–PAGE on whole cell samples. In the majority of strains two PGH bands with a MW of about 70 kDa and 80 kDa respectively were detected, with some strain-dependent variations in the band intensity. One strain showed a different pattern with 2 PGH bands of 60 and 70 kDa. Lytic activity was retained in high salt and in acid/basic conditions and was active toward cells of *Lb. sakei*, *L. monocytogenes*, *L. ivanovii* and *L. innocua*. The presence and size of putative PGH genes was verified by PCR in the *Lb. sakei* collection and we found that the gene *lsa1437* showed a clear correlation between PGH profiles and PCR results.

The ability of *Lb. sakei* PGHs to hydrolyze the cell wall of food-associated spoilage and pathogenic bacterial species, could be used in combination with the selection of *Lb. sakei* bacteriocin producing strains in order to select starter cultures effective in the biopreservation of fermented meat quality.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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