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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 2480 (B1) and 2470 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, lsa1437, 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 lsa1437 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 psychrotrophic 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...
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-acetyltalosaminyl 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 bonds in polymeric peptidoglycan and/or in its soluble fragments [4–6]. Although autolysis of lactic acid bacteria such as 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-Josas, Paris) [32, 33], 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 (OD600 = 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 K2HPO4, 2 g diammonium citrate, 0.1 g MnSO4 (Merck Darmstadt, Germany), 0.05 g MgSO4 (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 OD600 of 0.6–0.8. Samples were incubated at 30°C and autolysis was monitored by measuring the decrease in OD600 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 (OD600 = 0.8–1.0) and suspended to an OD600 of approximately 0.5, using MES (2-N-Morpholinoethanesulfonic acid) (Sigma) buffer (50 mM, pH 6.0) supplemented with 1 mM MgCl2 (Scharlau). A volume of 3 ml aliquot of cell suspension was then equilibrated at 37°C after which the OD600 was measured. Subsequently, 5 µl of mutanolysin solution (Sigma) (150 U ml−1) prepared in TES buffer (N-Tris-hydroxyethyl-2-aminoethanesulfonic acid) (Sigma) (50 mM, pH 7.0), 1 mM MgCl2 (Scharlau), was added to the cell suspension. The mixture was incubated at 37°C for 20 min and the OD600 was then measured. The mutanolysin activity was expressed as the percentage decrease of the OD600.
Peptidoglycan hydrolases of Lactobacillus sakei

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) β-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) autolysin of Micrococcus lysodeikticus ATCC 4698 (Sigma), as used as a reference target cells for PGH 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%), MgCl2 (Scharlau) (10 mM), MnCl2 (Scharlau) (10 mM), CaCl2 (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 IL.1403, 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 Bsp9I (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 [32,33] used as a positive control, according to the method described by Anderson and MacKay [38]. The quantification and the degree of DNA purity were determined spectrophotometrically using Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, NC). Primers (BioFastar, Germany) (Table 1), corresponding to the five putative PGH candidate genes lsa0862, lsa1437, lsa1558, lsa1581, and lsa1788, 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), MgCl2 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 2 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, Courtabœuf, 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 1 (Biolabs) in alkaline buffer solution (20 mM Tris–HCl pH 8.0, 10 mM MgCl2) 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

http://dx.doi.org/10.1016/j.jare.2015.04.004
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 (MgCl₂ 10 mM, MnCl₂ 10 mM, CaCl₂ 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 MgCl₂, MnCl₂, CaCl₂ 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).

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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Locus_tag</th>
<th>Primer name 1</th>
<th>Sequence 5’ to 3’</th>
<th>Assigned function</th>
<th>Theoretical protein size</th>
<th>Predicted size of mature protein (kDa)</th>
<th>Expected size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lsa1558</td>
<td>LSA1558</td>
<td>LSA1558-F</td>
<td>GTCCTGCTGGGCGTTTTATT</td>
<td>Putative extracellular N-acetylmuramoyl-L-alanine amidase precursor</td>
<td>25,4600</td>
<td>18.3</td>
<td>500</td>
</tr>
<tr>
<td>lsa1437</td>
<td>LSA1437</td>
<td>LSA1437-F</td>
<td>CGGAGAATGAAGAATACGACG</td>
<td>N-acetylmuramoyl-L-alanine amidase precursor</td>
<td>71,72007</td>
<td>43.8</td>
<td>1600</td>
</tr>
<tr>
<td>lsa0862</td>
<td>LSA0862</td>
<td>LSA0862-F</td>
<td>GCTGCTGGTGTCAGAAGAATA</td>
<td>N-acetylmuramoyl-L-alanine amidase precursor</td>
<td>40,12113</td>
<td>16089</td>
<td>1689</td>
</tr>
<tr>
<td>lsa1581</td>
<td>LSA1581</td>
<td>LSA1581-F</td>
<td>TTAAATCGCCTTATCCAACA</td>
<td>Teichoic acid-binding N-acetylmuramoyl-L-alanine amidase</td>
<td>76,52085</td>
<td>66.5</td>
<td>660</td>
</tr>
<tr>
<td>lsa1788</td>
<td>LSA1788</td>
<td>LSA1788-F</td>
<td>GCCGTAACGCACACTATTAT</td>
<td>Phage-related 1,4-beta-N-acetyl muramidase</td>
<td>27,6759</td>
<td>22.8</td>
<td>300</td>
</tr>
</tbody>
</table>

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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 lsa0862, lsa1437, lsa1558, lsa1581, and lsa1788. 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 lsa0862 and lsa1558 were present in all strains. The amplification of an internal fragment of lsa1581 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 lsa1788 was detected in ten out of twenty-two strains and no correlation with the PGH band patterns could be established. In contrast, for lsa1437 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 lsa1437 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PGH genes amplification^a^</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>lsa1558</em> (25.4 kDa)</td>
</tr>
<tr>
<td>BMG.106</td>
<td>+</td>
</tr>
<tr>
<td>BMG.37</td>
<td>+</td>
</tr>
<tr>
<td>BMG.73</td>
<td>+</td>
</tr>
<tr>
<td>BMG.170</td>
<td>+</td>
</tr>
<tr>
<td>BMG.51</td>
<td>+</td>
</tr>
<tr>
<td>BMG.148</td>
<td>+</td>
</tr>
<tr>
<td>BMG.168</td>
<td>+</td>
</tr>
<tr>
<td>BMG.167</td>
<td>+</td>
</tr>
<tr>
<td>BMG.164</td>
<td>+</td>
</tr>
<tr>
<td>BMG.126</td>
<td>+</td>
</tr>
<tr>
<td>BMG.127</td>
<td>+</td>
</tr>
<tr>
<td>BMG.101</td>
<td>+</td>
</tr>
<tr>
<td>BMG.105</td>
<td>+</td>
</tr>
<tr>
<td>BMG.95</td>
<td>+</td>
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<tr>
<td>BMG.115</td>
<td>+</td>
</tr>
<tr>
<td>BMG.107</td>
<td>+</td>
</tr>
<tr>
<td>BMG.40</td>
<td>+</td>
</tr>
<tr>
<td>BMG.45</td>
<td>+</td>
</tr>
<tr>
<td>BMG.136</td>
<td>+</td>
</tr>
<tr>
<td>BMG.138</td>
<td>+</td>
</tr>
<tr>
<td>BMG.120</td>
<td>+</td>
</tr>
<tr>
<td>23 K</td>
<td>+</td>
</tr>
</tbody>
</table>

^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 explained 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 \textit{Lb. sakei}, \textit{L. monocytogenes}, \textit{L. ivanovii} and \textit{L. innocua}. The presence and size of putative PGH genes was verified by PCR in the \textit{Lb. sakei} collection and we found that the gene \textit{Isa1437} showed a clear correlation between PGH profiles and PCR results.

The ability of \textit{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 \textit{Lb. sakei} bacteriocin producing strains in order to select starter cultures effective in the biopreservation of fermented meat quality.

Compliance with Ethics Requirements

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

Conflict of interest

The authors have declared no conflict of interest.

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References

Peptidoglycan hydrolases of *Lactobacillus sakei*


