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Biochemical characterization of the major *N*-acetylmuramidase from *Lactobacillus buchneri*

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Abstract

Bacterial cell wall hydrolases are essential for peptidoglycan remodelling in regard to bacterial cell growth and division. In this study, peptidoglycan hydrolases (PGHs) of different *Lactobacillus buchneri* strains were investigated. First, the genome sequence of *L. buchneri* CD034 and *L. buchneri* NRRL B-30929 was analysed *in silico* for the presence of PGHs. Of 23 putative PGHs with different predicted hydrolytic specificities, the glycosyl hydrolase family 25 domain-containing homologues *LbGH25B* and *LbGH25N* from *L. buchneri* CD034 and NRRL B-30929, respectively, were selected and characterized in detail. Zymogram analysis confirmed hydrolysing activity on bacterial cell walls for both enzymes. Subsequent reversed-phase HPLC and MALDI-TOF MS analysis of the peptidoglycan breakdown products from *L. buchneri* strains CD034 and NRRL B-30929, and from *Lactobacillus rhamnosus* GG, which served as a reference, revealed that *LbGH25B* and *LbGH25N* have *N*-acetylmuramidase activity. Both enzymes were identified as cell wall-associated proteins by means of immunofluorescence microscopy and cellular fractionation, as well as by the ability of purified recombinant *LbGH25B* and *LbGH25N* to bind to *L. buchneri* cell walls *in vitro*. Moreover, similar secondary structures mainly composed of β -sheets and nearly identical thermal stabilities with T_m values around 49 °C were found for the two *N*-acetylmuramidases by far-UV circular dichroism spectroscopy. The functional and structural data obtained are discussed and compared to related PGHs. In this study, a major *N*-acetylmuramidase from *L. buchneri* was characterized in detail for the first time.

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INTRODUCTION

Bacterial peptidoglycan (PG) comprises a glycan backbone containing alternating β (1,4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues that are cross-linked by peptide chains of varying composition (Schleifer & Kandler, 1972; Vollmer *et al.*, 2008a).

Multilayered PG is the major cell wall component in Gram-positive bacteria; it is responsible for maintaining cell shape and morphology and confers resistance to internal turgor pressure (Delcour *et al.*, 1999).

The bacterial cell wall is continuously expanded and renewed (Vollmer *et al.*, 2008b). Bacterial PG hydrolases (PGHs) are produced by bacteria throughout their life cycle, and target either the PG sugar backbone or the peptide chains; they are involved in the regulation of cell wall growth, recycling and maturation of PG, and the separation of daughter cells (Vollmer *et al.*, 2008b). According to their cleavage specificities, PGHs can be divided into four distinct classes: (i) *N*-acetylmuramidases, (ii) *N*-acetylglucosaminidases, (iii) *N*-acetylmuramoyl-L-alanine amidases and (iv) peptidases (including carboxypeptidases and endopeptidases) (Layec *et al.*, 2008). Additional lytic transglycosylases may break the same bonds as muramidases, but generate anhydromuropeptides (Scheurwater *et al.*, 2008).

Lactobacilli are major components of both foods and the human and animal microbiota. Given their GRAS (generally regarded as safe) status, they are intensively exploited in applied research. Only a few lactobacillar PGHs have been investigated in detail. For the endopeptidases Msp1 from *Lactobacillus rhamnosus* GG (Claes *et al.*, 2012) and Lc-p75 from *Lactobacillus casei* BL23 (Regulski *et al.*, 2012) as well as for the glucosaminidase Acm2 from *Lactobacillus plantarum* WCFS1 (Rolain *et al.*, 2012), hydrolytic specificities have been experimentally proven; PGHs from *Lactobacillus buchneri* have not been studied up to now. *L. buchneri* is a facultative anaerobe that is of interest because of its property of efficient preservation of animal feed silage against aerobic spoilage. The obligatory heterofermentative nature and acid resistance makes this bacterial species well suited for application as a silage starter culture (Oude Elferink *et al.*, 2001; Danner *et al.*, 2003; Holzer *et al.*, 2003; Eikmeyer *et al.*, 2013). In the present study, the PGHs of *L. buchneri* CD034 (Heinl *et al.*, 2012), isolated from grass silage, and *L. buchneri* NRRL B-30929 (Liu *et al.*, 2011), isolated from an ethanol production plant, have been studied. *L. buchneri* possesses a typical Gram-positive cell wall profile with a thick PG of the A4 α chemotype with D-Ala⁴ $\rightarrow$ (D-Asp/D-Asn) $\rightarrow$ L-Lys³ interpeptide bridges (Schleifer & Kandler, 1972). As the outermost cell envelope decoration, *L. buchneri* possesses a self-assembling, 2D crystalline surface (S-) layer with oblique lattice symmetry (Möschl *et al.*, 1993; Anzengruber *et al.*, 2014). The *L. buchneri* S-layer protein of both strains is O-glycosylated at four serine residues within the motif Ser-Ala-Ser-Ser-Ala-Ser with, on average, seven Glc(α 1-6) residues each (Möschl *et al.*, 1993; Anzengruber *et al.*, 2014).

Motif search within the *L. buchneri* CD043 and NRRL B-30929 genomes revealed the presence of one extra protein each containing the above glycosylation motif. These are the glycosyl hydrolase family 25 (GH25) domain-containing proteins *LbGH25B*

(LBUCD034_0240) and *LbGH25N* (Lbuc_0200) from *L. buchneri* CD034 and *L. buchneri* NRRL B-30929, respectively, for which a database sequence similarity search predicted PG-hydrolysing capacity. According to MS evidence, indications for glucosylation of the two predicted PGHs were found (Anzengruber *et al.*, 2014). Similar to the S-layer glycoproteins SlpB and SlpN from *L. buchneri* CD034 and *L. buchneri* NRRL B-30929, respectively, the highly homologous enzymes *LbGH25B* and *LbGH25N* have a basic pI of 9.5.

In this study, we identified *in silico* putative PGHs of *L. buchneri* CD034 and their homologues in *L. buchneri* NRRL B-30929, and characterized in detail the GH25 domain-containing proteins *LbGH25B* and *LbGH25N*. This includes (i) biochemical analysis of cell wall hydrolysing activity and specificity, (ii) determination of the subcellular localization of the enzymes, including an *in vitro* binding study to lactobacillar cell walls, and (iii) experimental insight into structural properties and thermal stability of the PGHs.

METHODS

Bacterial strains and culture conditions

L. buchneri CD034 (Heinl *et al.*, 2012), *L. buchneri* NRRL B-30929 (Liu *et al.*, 2011), *L. rhamnosus* GG (Claes *et al.*, 2012) and *L. plantarum* CD033 (Spath *et al.*, 2012) were grown in De Man–Rogosa–Sharpe (MRS) broth (BD Difco) (De Man *et al.*, 1960) at 37 °C without shaking. *Escherichia coli* DH5 α cells (Life Technologies) and *E. coli* BL21 Star BL21(DE3) cells (Life Technologies) were cultivated at 37 °C and 200 r.p.m. in Luria–Bertani (LB) medium supplemented with 50 μ g kanamycin ml⁻¹.

Construction of expression plasmids

The coding sequences for *LbGH25B* (LBUCD034_0240) and *LbGH25N* (Lbuc_0200) devoid of the N-terminal signal peptide sequences (amino acids 1–29 of the pre-proteins) were PCR-amplified with primers (Life Technologies) *LbGH25B/N_F* (5'-AATCATCTAGAAATAATTTGTTTAACTTTAAGAAGGAGATATACCATGGCTTTGACGCCGTCCAGTACC-3', *Xba*I site underlined) and *LbGH25B/N_His_stop_R* (5'-AATCAAAGCTTTTAATGATGATGATGATGATGATTCAAATAACCGCGCCAAATC C-3', *Hind*III site underlined). Similarly, the coding sequences for the N-terminal GH25 domain-containing region (amino acids 30–235) and the C-terminal region (amino acids 236–380) of *LbGH25B* were PCR-amplified with primers *LbGH25B_F* and *GH25_His_stop_R* (5'-AATCAAAGCTTTTAATGATGATGATGATGATGGTAGAAGCTGTTGTTTAATTG-3', *Hind*III site underlined), and with *C_termius_LbGH25B_F* (5'-AATCATCTAGAAATAATTTGTTTAACTTTAAGAAGGAGATATACCATGTTGAACTCCGGTTCAGCGGC-3', *Xba*I site underlined) and *LbGH25B_His_stop_R*, respectively. PCR products were digested with *Xba*I and *Hind*III and ligated into pET28a+ expression vector (Novagen). For expression of the S-layer protein SlpB, plasmid pET28a-SlpB_{His6} was constructed as described by Anzengruber *et al.* (2014). The resulting recombinant plasmids were propagated in *E. coli* BL21 Star BL21(DE3) for production of hexahistidine-tagged proteins.

Production and purification of recombinant proteins

Transformed strains were grown in 400 ml LB broth supplemented with kanamycin ($50 \mu\text{g ml}^{-1}$) at 37°C and 200 r.p.m. At the mid-exponential growth phase (OD_{600} of ~ 0.6), protein expression was induced with 1 mM IPTG and cultivation was continued for 4 h. Cells were pelleted ($10\,000\text{ g}$, 20 min), resuspended in lysis buffer (50 mM sodium citrate buffer, pH 6.2, 0.1 % Triton X-100) and, after addition of lysozyme ($800 \mu\text{g ml}^{-1}$; Sigma-Aldrich) and benzonase (50 U ml^{-1} ; Sigma-Aldrich), incubated for 30 min at 37°C . Bacteria were lysed by ultrasonication (Branson sonifier, duty cycle 50 %; output 6) applying 10 cycles of 10 pulses with 30 s breaks, each, and inclusion bodies containing the recombinant proteins were pelleted. The proteins were extracted from the pellets with binding buffer [50 mM sodium citrate buffer, pH 5.5, 5 M guanidinium hydrochloride (GdHCl), 20 mM imidazol, 0.5 M NaCl] for 1 h at 4°C . The extracts were subjected to centrifugation and membrane-filtration ($0.45 \mu\text{m}$ pore size). The resulting protein samples were applied to a 1 ml HisTrap HP column (GE Healthcare) and recombinant proteins were recovered with elution buffer (50 mM sodium citrate buffer, pH 5.5, 5 M GdHCl, 1 M imidazol, 0.5 M NaCl). Recombinant His-tagged proteins were dialysed against 50 mM sodium citrate buffer, pH 5.5.

SDS-PAGE

SDS-PAGE was carried out on 10, 12 or 20 % slab gels in a Mini Protean electrophoresis apparatus (Bio-Rad) (Laemmli, 1970). Protein bands were visualized with colloidal Coomassie brilliant blue R-250 (CBB) and gels were imaged at 700 nm using the Odyssey imaging system (LI-COR).

Western immunoblotting

Polyclonal mouse antiserum was raised against purified recombinant *LbGH25N* (EF-BIO). Due to high amino acid homology (99 %) between *LbGH25N* and *LbGH25B*, the antiserum reacted with both proteins.

Proteins were transferred from the SDS-PAGE gel to a PVDF membrane (Bio-Rad) using a Mini Trans-Blot Cell (Bio-Rad). Muramidase-specific antiserum was used in combination with IR Dye 800CW goat anti-mouse antibody (LI-COR), and detection was performed at 800 nm using the Odyssey Infrared Imaging System.

Electronic circular dichroism (ECD) spectroscopy

Far-UV ECD spectra and temperature-mediated unfolding were recorded by ECD spectroscopy (Chirascan; Applied Photophysics). Prior to measurements the instrument was flushed with nitrogen at a flow rate of 5 l min^{-1} . An integrated Peltier element was used for temperature control. Far-UV ECD spectra using $1.9 \mu\text{M}$ *LbGH25B* or *LbGH25N* in 12.5 mM sodium citrate buffer, pH 5.5, were recorded between 195 and 260 nm at 25°C . The pathlength was 1 mm, spectral bandwidth was 1 nm and scan time per point was set to 10 s. Five scans were recorded for each protein and mean values were calculated. Secondary structure prediction was computed via the PSIPRED protein structure prediction server. Thermal unfolding was monitored at 222 nm between 20 and 95°C with stepwise increments of $1.0^\circ\text{C min}^{-1}$ using $7.5 \mu\text{M}$ *LbGH25B* or *LbGH25N* in 50 mM sodium citrate

buffer, pH 5.5. The pathlength was 1 mm, spectral bandwidth was 1 nm and scan time per point was set to 10 s. The fraction α of unfolded protein was calculated according to $\alpha = (\theta_N - \theta) / (\theta_N - \theta_U)$, with θ_N being the ellipticity (10^{-3} degrees) of the protein in the native folded state at 222 nm, θ the ellipticity at a defined temperature (T) and θ_U being the ellipticity of the completely unfolded state at 222 nm. Experiments were performed in duplicate and mean values and s_D of the melting temperature (T_m) and the van't Hoff enthalpy (H_m) were calculated.

Zymogram analysis

Cell wall hydrolysing activity of the PGHs was investigated by zymogram analysis (Lepeuple *et al.*, 1998). As putative substrates, cell walls of *L. buchneri* CD034, *L. buchneri* NRRL B-30929 and *E. coli* BL21 Star BL21(DE3) as well as whole cells from *L. rhamnosus* GG were used.

L. buchneri CD034 cell walls were isolated and purified according to published protocols (Schäffer *et al.*, 1999, 2000) with slight modifications. The high-molecular mass, polysaccharide-containing material obtained after lysozyme digestion and chromatography on Sephadex G-50 (2.5×120 cm, GE Healthcare) was further fractionated on a Sephacryl S-1000 column (XK16/40, GE Healthcare) at a flow rate of 1 ml min^{-1} with 50 mM NH_4HCO_3 as eluent. Carbohydrate-containing fractions were pooled and lyophilized.

L. buchneri NRRL B-30929 and *E. coli* BL21 Star BL21(DE3) cells were pelleted (10 000 g, 20 min), washed with 50 mM Tris/HCl, pH 7.2 (buffer A), and, subsequently, lysed by ultrasonication (duty cycle 50 %; output 8) applying six cycles of 10 pulses, with 30 s breaks, each. Cell wall fragments were sedimented (30 000 g, 15 min) and resuspended in buffer A, containing 1 % (v/v) Triton X-100. After three washing steps with buffer A, the cell walls were incubated in buffer A containing 5 M GdHCl at 4 °C for 30 min with shaking to remove the S-layer. The remaining pellet was washed twice with buffer A, boiled for 30 min in 1 % (w/v) SDS, and washed five times with MilliQ H_2O to remove SDS. Cell walls were further treated with 10 % (w/v) TCA at 4 °C for 24 h to remove exopolysaccharides and teichoic acids, washed twice with 0.25 M Tris/HCl, pH 8.0, and washed four times with MilliQ H_2O . The final pellet was kept at 4 °C. *L. rhamnosus* GG cells were autoclaved and treated with 10 % (w/v) TCA as described above.

Cells and cell wall preparations were co-polymerized at a concentration of 0.4 % (w/v) with SDS polyacrylamide gels to serve as enzyme substrates and zymogram analysis was performed as described by Claes *et al.* (2012). Bacterial cells were resuspended in zymogram sample buffer (62.5 mM Tris/HCl, pH 6.8; 2 % SDS; 0.01 % Bromophenol blue; 10 % glycerol), insoluble material was removed by centrifugation and soluble proteins were applied to zymogram gels. Recombinant LbGH25B and LbGH25N were also applied in purified form, as described above.

Determination of the hydrolytic specificity of LbGH25B and LbGH25N on PG

PG from *L. rhamnosus* GG, *L. buchneri* CD034 and *L. buchneri* NRRL B-30929 was prepared as described elsewhere (Claes *et al.*, 2012). Bacterial cells were incubated in 50

mM Tris/HCl, pH 7.2, containing 5 M GdHCl for 30 min at 20 °C with shaking to remove the S-layer. Nucleic acids were removed by treatment with benzonase (50 U ml⁻¹) in 20 mM Tris/HCl, pH 7.0, containing 1 mM MgCl₂, at 37 °C for 18 h. PG was then treated with 10 % TCA (w/v) at 4 °C for 24 h with shaking to remove exopolysaccharides and teichoic acids, washed twice with 50 mM Tris/HCl, pH 8.0, and washed twice with MilliQ H₂O. Subsequently, the material was incubated with 48 % (v/v) hydrofluoric acid at 4 °C to further eliminate residual exopolysaccharides and teichoic acids and washed as described above. The final pellet was freeze-dried and stored at -20 °C until further analysis.

The hydrolytic specificity of the enzymes was determined using PG from *L. buchneri* CD034, *L. buchneri* NRRL B-30929 and *L. rhamnosus* GG, respectively, as substrate. Purified PG (1 mg dry weight) was digested with either mutanolysin from *Streptomyces globisporus* (2500 U ml⁻¹, Sigma-Aldrich) and/or purified recombinant His₆-tagged LbGH25B (34 µg), LbGH25N (41 µg) or SlpB (14 µg) in 50 mM sodium citrate buffer, pH 5.5, containing 0.05 % (w/v) sodium azide, for 24 h at 37 °C with shaking. The resulting soluble muropeptides were reduced with sodium borohydride (Atrih *et al.*, 1999). Degradation products were separated by (reversed-phase) HPLC with an Agilent Infinity 1290 ultraHPLC system on a Nucleodur C18 Pyramid column (150 × 2 mm; particle size, 1.8 µm; Macherey-Nagel) at 50 °C using a methanol gradient in ammonium phosphate buffer (Courtin *et al.*, 2006). Muropeptides were analysed by MALDI-TOF MS using a Voyager-DE STR mass spectrometer (Applied Biosystems) as reported previously (Courtin *et al.*, 2006).

Subcellular fractionation of *L. buchneri* strains

Supernatant—*L. buchneri* CD034 and NRRL B-30929 were grown overnight in MRS broth at 37 °C without shaking. After centrifugation (10 000 g, 20 min), proteins were precipitated from the supernatant by incubation with 20 % (w/v) TCA at 4 °C for 30 min, sedimented proteins (9000 g, 20 min) were washed twice with ice-cold 100 % acetone and the final pellet was air-dried.

Cell wall—The cell pellet was washed with buffer A and, subsequently, lysed by ultrasonication (duty cycle 50 %; output 8) by applying six cycles of 10 pulses, with 30 s breaks, each. Cell wall fragments were precipitated at 30 000 g for 15 min, and the resulting pellet was resuspended in buffer A, containing 1 % (v/v) Triton X-100, and washed four times with buffer A.

Cytosol—Cytosolic proteins were obtained from the supernatant of the centrifugation step directly after ultrasonication during the cell wall isolation described above. Proteins were precipitated with TCA, washed twice with ice-cold acetone and air-dried.

The resulting pellets were resuspended in Laemmli buffer at a concentration of 0.1 mg ml⁻¹ (Laemmli, 1970) and run on SDS-PAGE gels.

In vitro protein-cell wall interaction studies—LbGH25B and LbGH25N were tested for their ability to interact with PG following a recently published protocol (Janesch *et al.*, 2013). Briefly, 1 µg of purified protein was incubated with 0.2 mg of lyophilized *L. buchneri*

NRRL B-30929 cell walls in 25 mM sodium citrate buffer, pH 5.5, in a total volume of 125 µl, for 1 h at 37 °C under gentle rotation. The mixture was centrifuged (16 100 g, 20 min), yielding a supernatant containing unbound protein and a pellet of insoluble cell wall components including attached protein. The pellet was washed twice to remove unbound proteins. Samples were investigated by SDS-PAGE followed by Western immunoblotting using muramidase-specific polyclonal antiserum. Mixtures without cell walls were used as control. The pellet and the supernatant fraction of the reactions as well as the controls were quantified using the Odyssey Infrared Imaging System (LI-COR). For comparability of datasets, the sum of the signals in the pellet fraction and the supernatant fraction of each reaction was set to 100 %. Experiments were performed in triplicate and SDS were calculated.

Immunofluorescence staining and microscopy—The cellular localization of LbGH25B and LbGH25N was determined by immunofluorescence. *L. buchneri* CD034, *L. buchneri* NRRL B-30929 and *L. plantarum* CD033 were grown until the late-exponential phase (OD_{600} of ~1.5). Cells were washed with PBS and adsorbed on a glass slide for 2 h. All procedures were performed at room temperature, if not stated otherwise. Cells were washed twice with PBS and fixed for 30 min at $-20\text{ }^{\circ}\text{C}$ in 70 % (v/v) ethanol in PBS. After two washing steps with PBS, cells were incubated in blocking buffer (10 %, w/v, BSA in PBS) for 1 h, followed by incubation in blocking buffer containing muramidase-specific polyclonal antiserum or pre-immune serum (1: 10 dilution) for 1 h, washed once with blocking buffer and twice with PBS, and subsequently incubated in blocking buffer containing goat anti-mouse IgG (1: 100 dilution) conjugated to FITC (Sigma-Aldrich) for 2 h. The cells were washed once with blocking buffer and twice with PBS to remove unbound antibodies. One drop of 50 % (v/v) glycerol in PBS was added onto the cells and a coverslip was mounted. Confocal laser scanning microscopy analyses were performed using a Leica TCS SP5 II system. Images were taken with a 63.0× 1.40 oil-immersion objective. Immunofluorescence-stained bacterial cells were excited at 488 nm using an argon laser and detected at an emission bandwidth of 500–595 nm. Images were acquired and processed with Leica LAS AF software.

RESULTS

In silico search for predicted PGHs of *L. buchneri*

Putative PGHs of *L. buchneri* CD034 were identified *in silico* in the genome (GenBank Assembly ID: GCA_000298115.2) (Heinl *et al.*, 2012) by searching for catalytic domains specific for PGHs (Layec *et al.*, 2008). Twenty-three putative enzymes could be affiliated to the known classes of PGHs (Layec *et al.*, 2008) according to their predicted hydrolytic specificities (Table 1). PGHs of bacteria of the phylum *Firmicutes* typically show a modular structure consisting of a catalytic and a cell wall-binding domain (Layec *et al.*, 2008). Only three of the putative *L. buchneri* PGHs were predicted to contain a cell wall-binding domain. Among these is an amidase and an endopeptidase containing the non-catalytic SH3 cell wall-binding domain (Whisstock & Lesk, 1999), and a carboxypeptidase containing the cell wall association domain PBP5_C (Layec *et al.*, 2008). The amino acid sequences of all putative *L. buchneri* CD034 PGHs were aligned with the respective *L. buchneri* NRRL

B-30929 homologues (Liu *et al.*, 2011); the calculated sequence identity was between 97 and 100 % (Table 1).

LbGH25B and *LbGH25N* are 380-aa proteins with a calculated molecular mass of 40.8 kDa. A difference of 23 nt translates to a difference of only 2 aa, with Val182 and Ala260 in *LbGH25B* being replaced by Ile182 and Glu260, respectively, in *LbGH25N*. Either enzyme exhibits a conserved GH25 (Pfam01183) domain following a 29-aa signal peptide including a type I signal peptidase cleavage site at the N terminus, and a C-terminal part with unknown function (Fig. 1).

Evaluation of secondary structure composition and thermal stability of *LbGH25B* and *LbGH25N* by ECD spectroscopy

ECD spectroscopy was used to investigate the secondary structure composition and compare the thermal stability of *LbGH25B* and *LbGH25N*. Fig. 2(a) depicts the ECD spectra in the far-UV region of both enzymes. The ellipticity of α -helices typically shows two minima at 208 and 222 nm, whereas the contribution of β -sheets gives rise to negative ellipticity around 212–214 nm, but with $[\theta]$ values that are five times lower than those for α -helices (Kelly *et al.*, 2005). The spectra of *LbGH25B* and *LbGH25N* show a similar overall fold of both proteins and the typical feature of proteins with a high β -sheet content, which correlates with computed secondary structure predictions. According to the PSIPRED secondary structure prediction server, *LbGH25B* and *LbGH25N* contain 28 % β -sheets, 16 % α -helices and 56 % random coils, and 28 % β -sheets, 17 % α -helices and 56 % random coils, respectively.

To gain more information about the thermal stability of the *N*-acetylmuramidases, temperature-mediated unfolding was followed by ECD spectroscopy in the far-UV region at 222 nm. The melting curves of both enzymes suggest a simple two-state transition with T_m values of 49.0 ± 0.1 and 47.9 ± 0.2 °C for *LbGH25B* and *LbGH25N*, respectively (Fig. 2b). A clear linear relationship between the equilibrium constants and the reciprocal temperature allowed us to calculate the van't Hoff enthalpy for this transition to 413.4 ± 9.2 and 364.4 ± 4.2 kJ mol⁻¹ for *LbGH25B* and *LbGH25N*, respectively.

Hydrolysing activity in zymograms

His-tagged *LbGH25B* and *LbGH25N* (Anzengruber *et al.*, 2014) were purified from *E. coli* by nickel-affinity chromatography and the hydrolytic activity was investigated by zymogram assays. Additionally, enzyme activity of native *LbGH25B* and *LbGH25N* was assayed by applying a *L. buchneri* CD034 and *L. buchneri* NRRL B-30929 cell extract, respectively, to zymogram gels. To unravel a sequence-function relationship, *LbGH25B* was dissected into an N-terminal protein portion containing the GH25 domain and the C-terminal rest of the protein. The protein portions were produced in *E. coli* and assayed for their functionality. All protein variants used for zymography are schematically depicted in Fig. 1.

LbGH25B and *LbGH25N* were isolated at a final yield of 8 and 5 mg per gram of protein of *E. coli* cell pellet, respectively (Fig. 3a, lanes 1 and 2). Purity of the recombinant enzymes was analysed by SDS-PAGE following CBB staining (Fig. 3a), revealing both proteins

migrating as a single band with a molecular mass of ~40 kDa (Fig. 3a, lanes 3 and 4). The N-terminal, GH25 domain-containing region and the C-terminal region of *LbGH25B* as present in extracts of *E. coli* cells carrying the respective expression plasmid (Fig. 3a, lanes 6 and 7) and purified (Fig. 3a, lanes 10 and 11) migrated at ~22 and ~18 kDa, respectively, which corresponds to the calculated masses based on the amino acid sequence of the truncated proteins. As a negative control, *E. coli* was transformed with empty pET28a vector (Fig. 3a, lane 5). Both *L. buchneri* CD034 and *L. buchneri* NRRL B-30929 cell extracts showed the characteristic protein pattern of *L. buchneri* cells, with the abundant protein band at ~60 kDa corresponding to the S-layer glycoproteins SlpB and SlpN, respectively (Anzengruber *et al.*, 2014) (Fig. 3a, lanes 8 and 9).

Aliquots of the protein samples were applied to renaturing SDS-PAGE gels containing co-polymerized *L. buchneri* CD034 cell walls as a substrate (Fig. 3b). Translucent zones were observed for purified recombinant *LbGH25B* and *LbGH25N* as well as for *LbGH25B* and *LbGH25N* from *E. coli* cell extracts (Fig. 3b, lanes 1–4). Presumptive hydrolytic activity of an *E. coli* protein of ~25 kDa was detected in all of the *E. coli* cell extracts (Fig. 3b, lanes 3–7), including the negative control containing the empty plasmid (Fig. 3b, lane 5). Whereas the C-terminal region of *LbGH25B* did not show clearance in the zymogram gel, the N-terminal GH25 domain-containing region showed clearance (indicated by a star in Fig. 3b, lanes 6 and 10), confirming that the GH25 domain of the enzyme is crucial for its lytic activity. In regard to the PGH activity of *L. buchneri* CD034 and *L. buchneri* NRRL B-30929 cell extracts, a clearance zone was observed at ~40 kDa, which can probably be attributed to enzyme activity of native *LbGH25B* and *LbGH25N*, respectively (indicated by arrows in Fig. 3b, lanes 8 and 9). Cell wall hydrolysis by recombinant *LbGH25B* and *LbGH25N* was further confirmed on zymograms with *L. buchneri* NRRL B-30929 cell walls as a substrate (Fig. 3c). *L. rhamnosus* GG cells (Fig. 3d), which exhibit, like *L. buchneri*, an A4α PG chemotype with an interpeptide bridge (Schleifer & Kandler, 1972; Claes *et al.*, 2012), were also shown to be a substrate for full-length *LbGH25B* and its N-terminal GH25 domain-containing region (indicated by the star in Fig. 3d), while the *LbGH25B* C-terminal region did not yield cleared zones. This is in agreement with the results obtained with *L. buchneri* CD034 cell walls. Interestingly, recombinant and native *LbGH25B* and *LbGH25N* were also able to hydrolyse the Gram-negative cell walls of *E. coli*, which are of the A1γ PG chemotype with direct cross-linking between D-Ala⁴ and meso-diaminopimelic acid³ (Schleifer & Kandler, 1972) (cleared band in Fig. 3e).

Hydrolytic specificity of *LbGH25B* and *LbGH25N* on PG

The hydrolytic specificity of *LbGH25B* and *LbGH25N* was determined by assaying the activity of the purified recombinant, His-tagged enzymes on *L. buchneri* CD034 and *L. buchneri* NRRL B-30929 PG, as well as on *L. rhamnosus* GG PG for which reference data were available (Claes *et al.*, 2012). Isolated PGs were incubated with either *LbGH25B*, *LbGH25N* or the *N*-acetylmuramidase mutanolysin, a mixture of two major lytic enzymes from *Streptomyces globisporus* (Kawata *et al.*, 1983). Soluble muropeptides were then separated by reversed-phase HPLC (Fig. 4), with mutanolysin-treated PG serving as a reference. Comparison of the muropeptide profiles obtained from *L. buchneri* CD034 PG digested with either mutanolysin, *LbGH25B* or *LbGH25N* revealed overall identity (Fig.

4a). An identical series of profiles was obtained for *L. buchneri* NRRL B-30929 PG digested with each of the three enzymes (Fig. 4b) as well as for *L. rhamnosus* GG PG (Fig. 4c). Furthermore, reversed-phase HPLC separation profiles of muropeptides obtained from PGs digested with a combination of *LbGH25B* or *LbGH25N* and mutanolysin did not differ from those treated with *LbGH25B* or *LbGH25N* alone (data not shown). This indicates that the muropeptides released by the two *L. buchneri* PGHs and by mutanolysin are identical, suggesting that *LbGH25B* and *LbGH25N* exhibit *N*-acetylmuramidase specificity like mutanolysin, cleaving the β -*N*-acetylmuramyl-(1 $\rightarrow$ 4)-*N*-acetylglucosamine bonds of bacterial PG. *N*-Acetylmuramidase specificity was further confirmed by MALDI-TOF MS analysis of main muropeptides (Table 2). Muropeptide structures were deduced from their determined *m/z* values and revealed acetylation of the GlcNAc–MurNAc disaccharide in both *L. buchneri* and *L. rhamnosus* PGs (Table 2). From this it can be concluded that *L. buchneri* muramidases can digest *O*-acetylated PG.

To exclude the possibility that the observed *N*-acetylmuramidase activity was caused by *E. coli* proteins that could not be completely removed after purification of *LbGH25B* and *LbGH25N*, or from lysozyme used for cell lysis, PGs were digested with recombinant SlpB as a negative control. SlpB was treated in the same way as *LbGH25B* and *LbGH25N* during expression and purification, but no PG-hydrolysing activity was observed (Fig. 4).

Of note, nearly identical muropeptide profiles were obtained from *L. buchneri* CD034 PG and *L. buchneri* NRRL B-30929 PG (Fig. 4a, b), supporting highly similar if not identical PG structures in these organisms.

Although *L. buchneri* and *L. rhamnosus* GG have a similar A4 α PG chemotype with a D-Ala⁴→(D-Asp/D-Asn)→L-Lys³ inter-peptide bridge (Schleifer & Kandler, 1972; Claes *et al.*, 2012), the muropeptide profiles differ due to PG modifications (acetylation of glycan chains, amidation of C-terminal D-Ala, cleavage of peptide chains) present to varying extents.

Cellular localization of *LbGH25B* and *LbGH25N*

To determine the cellular localization of the *L. buchneri* muramidases, cell fractionation followed by Western immunoblotting, an *in vitro* cell wall-binding assay with purified recombinant *LbGH25B* and *LbGH25N*, as well as indirect immunofluorescence microscopy of whole cells, was performed.

Proteins from the cell wall and TCA-precipitated proteins from the cytosol as well as the supernatant of *L. buchneri* CD034 and NRRL B-30929 cells, respectively, were subjected to Western immunoblotting for *LbGH25B* and *LbGH25N* detection. Distinct protein bands were observed in the cell wall fraction, while muramidase-specific antiserum could not detect the enzymes in either the cytosolic fraction or the supernatant (Fig. 5a).

Furthermore, the ability of recombinant *LbGH25B* and *LbGH25N* to attach to isolated cell walls of *L. buchneri* was investigated in an *in vitro* cell wall-binding assay. Due to extensive treatment with denaturing agents, the cell wall preparation used for the assay was free of either native *LbGH25B* or native *LbGH25N* as confirmed by Western immunoblotting (data not shown). The results of the *in vitro* binding assay, as evidenced by densitometric

quantification of the immunoblots, indicated that both recombinant proteins, despite the lack of a known cell wall recognition domain, are able to bind to the cell wall (Fig. 5b). Under the applied assay conditions, 77 ± 4 % of *LbGH25B* and 77 ± 2 % of *LbGH25N* used for the analysis were attached to *L. buchneri* cell walls as determined from three independent experimental repeats.

For localization of the PGHs in *L. buchneri* CD034 and *L. buchneri* NRRL B-30929 cells, muramidase-specific mouse antiserum in combination with FITC-conjugated goat anti-mouse, followed by immunofluorescence microscopy, was used (Fig. 6a, b), revealing a prevalence of the enzymes at the periphery of the bacterial cells. Closer inspection of the fluorescently labelled cells indicated accumulation of the enzymes at the cell poles and near emerging septa of dividing cells. Staining with pre-immune serum as a negative control revealed neither non-specific reactions nor fluorescence of *L. buchneri* cells (Fig. 6c, d). To exclude non-specific reactions of the muramidase-specific anti-serum with muramidases other than *LbGH25B* and *LbGH25N*, or with components of the Gram-positive cell surface, immunofluorescence staining was performed with *L. plantarum* cells. As shown in Fig. 6(e), no non-specific staining was detected.

DISCUSSION

Our aims were to investigate the PGH complement of *L. buchneri* and to biochemically characterize in detail the homologous cell-wall hydrolases *LbGH25B* and *LbGH25N* from *L. buchneri* CD034 and *L. buchneri* NRRL B-30929, respectively.

We provide clear evidence for cell wall-hydrolysing activity of the above enzymes using a combination of zymogram analysis (Leclerc & Asselin, 1989; Lepeuple *et al.*, 1998) and determination of the PG cleavage site. Both *LbGH25B* and *LbGH25N* performed inter-species cell wall-hydrolysing activity by degrading *L. buchneri* CD034, *L. buchneri* NRRL B-30929 and *L. rhamnosus* GG cell walls. Relaxed substrate specificity was corroborated by hydrolysing activity on Gram-negative *E. coli* cell walls. Via mucopeptide analysis with reversed-phase HPLC and MALDI-TOF MS, these enzymes were shown to possess *N*-acetylmuramidase specificity with cleavage of the $\beta(1\rightarrow4)$ glycosidic bond between MurNAc and GlcNAc in the bacterial PG.

There are relatively few reports on bacterial muramidases with experimentally proven specificity in the literature, with none of these originating from lactobacilli (Vollmer *et al.*, 2008b). Two autolytic muramidases, SF muramidase and pesticin, have been described in *Enterococcus faecium* (Kawamura & Shockman, 1983; Barrett *et al.*, 1984) and in *Yersinia pestis* (Vollmer *et al.*, 1997), respectively. Cellosyl produced by *Streptomyces coelicolor* degrades *O*-acetylated PG as present in *Staphylococcus aureus* and other pathogens (Rau *et al.*, 2001). LytC and GHIP were identified in *Streptococcus pneumoniae* (García *et al.*, 1999; Niu *et al.*, 2013). Mutanolysin, consisting of two kinds of *N*-acetylmuramidases, M-1 and M-2, was isolated from culture broth of *Streptomyces globisporus*. While the hydrolysing activity of the M-2 enzyme was suppressed by the presence of *O*-acetylated MurNAc, M-1 was independent of the modification with *O*-acetyl groups (Kawata *et al.*, 1983). In this study, *LbGH25B* and *LbGH25N* were shown to degrade the PG of various

bacterial strains, including *L. buchneri* and *L. rhamnosus* PG, for which we could demonstrate MurNAc acetylation in this study, as well as *E. coli* PG, which does not contain *O*-acetylated MurNAc (Dezlee & Bricas, 1970). This indicates that the hydrolytic activity of the *L. buchneri* *N*-acetylmuramidases is not affected by *O*-acetylation of the PG glycan backbone.

Immunofluorescence microscopy clearly identified the *N*-acetylmuramidases as cell surface enzymes, indicating their possible role in cell wall growth and daughter cell separation, properties which are counted among the numerous functions of PGHs (Vollmer *et al.*, 2008b). During bacterial cell wall growth, PG hydrolytic and synthetic reactions are coordinated temporarily and spatially to avoid cell lysis and guarantee a safe enlargement of the sacculus (Typas *et al.*, 2012; Vollmer, 2012). A direct interaction between peptidoglycan synthases and hydrolases in multi-enzyme complexes has been observed in *E. coli* (Hltje, 1996a, b). In Gram-positive bacteria, synthases are involved in the synthesis of the inner, new PG layers, whereas hydrolases act, distant from the synthases, on the outer layers (Vollmer *et al.*, 2008b). Further studies imply that bacteria have specific inhibitors to control their hydrolases, as was shown in *Pseudomonas aeruginosa* (Clarke *et al.*, 2010). Interestingly, *O*-glycosylation was shown to control the activity of the glucosaminidase Acm2 from *L. plantarum*. Lack of *O*-glycosylation significantly increased enzyme activity, whereas removal of the PG-binding domains of Acm2 reduced this activity. A model was proposed in which access by Acm2 to its substrate may be hindered by *O*-glycosylation (Rolain *et al.*, 2013). A similar control mechanism can be hypothesized for putatively *O*-glycosylated LbGH25B and LbGH25N.

Cell surface localization of LbGH25B and LbGH25N was further confirmed by subcellular fractionation, with no detectable enzyme in the culture supernatant. In contrast, lactobacillar endopeptidases Msp1 and Lc-p75 from *L. rhamnosus* GG and *L. casei* BL23, respectively, were found both attached to the cell wall and secreted in the culture supernatant (Claes *et al.*, 2012; Regulski *et al.*, 2012). Attachment of the *N*-acetylmuramidases to *L. buchneri* cell walls was additionally shown by *in vitro* cell wall-binding assays. Typically, PGHs exhibit a multi-domain structure including a catalytic domain and several copies of a cell wall-binding domain. Frequently found binding domains in GH25-containing proteins are LysM, SH3, PG_binding, CW_binding or Cpl_7 (Layec *et al.*, 2008). Surprisingly, LbGH25B and LbGH25N do not contain any of these known domains, despite being, according to our experiments, cell wall-associated proteins. One might speculate that their basic pI value, which results in an overall basic net charge at neutral and low pH, may enhance their binding to negatively charged cell wall components (Vollmer *et al.*, 2008b). The *N*-acetylmuramidase cellosyl, which also does not exhibit distinct cell-wall binding domains, possesses a basic pI of 9.3 and was shown to contain at its C terminus a prominent, long groove, very likely to be the PG-binding site, as predicted from its crystal structure (Rau *et al.*, 2001).

Bacterial cell wall hydrolases, especially those originating from GRAS organisms, are interesting candidates as a new class of antimicrobials (Parisien *et al.*, 2008). Structural stability is a relevant feature for the pharmaceutical and biotechnological use of proteins. In this regard, thermal denaturation studies can provide an evaluation of conditions that

minimize unfolding and maintain biological activity. ECD demonstrated that *LbGH25B* and *LbGH25N* have similar overall protein folds, mainly composed of β -sheets. To date, the 3D structures of a few GH25 enzymes have been determined, including cellosyl [Protein Data Bank (PDB) code 1JFX] (Rau *et al.*, 2001), BaGH25c from *Bacillus anthracis* (PDB code 2WAG) (Martinez-Fleites *et al.*, 2009) and LytC and GHIP (PDB code 2WW5 and 4FF5, respectively) (Pérez-Dorado *et al.*, 2010; Niu *et al.*, 2013). Cellosyl, which shows, from all crystallized bacterial muramidases, the highest primary structure similarity to *LbGH25B* and *LbGH25N*, mainly at the N-terminal GH25 domain, comprises a single domain composed of an eight-stranded β -barrel flanked by seven α -helices (Rau *et al.*, 2001).

To gain insight into protein stability, temperature-mediated unfolding was performed. The determined T_m values of around 49 °C for *LbGH25B* and *LbGH25N* reflect thermal stabilities of the enzymes as required for biotechnological and pharmacological exploitation. The values obtained are in accordance with that of the muramidase Cpl-7 of *S. pneumoniae* recently studied as an antibacterial agent (Bustamante *et al.*, 2012). Identical thermal stability was observed for *LbGH25B* and *LbGH25N* with a simple two-state transition during thermal denaturation, suggesting that these enzymes are organized as one folding unit, which is in agreement with the single domain architecture of these enzymes based on their amino acid sequence. This has been observed for other bacterial muramidases, including cellosyl and GHIP (Rau *et al.*, 2001; Pérez-Dorado *et al.*, 2010; Niu *et al.*, 2013).

To summarize, the data presented in this study characterize in detail, for the first time, the major *N*-acetylmuramidases from the genus *Lactobacillus* and contribute to the pool of bacterial cell wall hydrolases whose development as novel therapeutics pinpoints advantages over conventional antibiotics, such as low probability of developing resistance (Parisien *et al.*, 2008). Food applications of bacterial cell wall hydrolases are being intensively studied (Callewaert *et al.*, 2011), for instance, in the control of wine-spoiling lactic and acetic acid bacteria, which are due to specific PG structures mostly resistant to lysozyme (Blättel *et al.*, 2009).

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Abbreviations

CBB	Coomassie brilliant blue R-250
ECD	electronic circular dichroism
GdHCl	guanidinium hydrochloride
GH25	glycosyl hydrolase family 25
GlcNAc	<i>N</i> -acetylglucosamine

GRAS	generally regarded as safe
MurNAc	<i>N</i> -acetylmuramic acid
PG	peptidoglycan
PGH	peptidoglycan hydrolase
S-layer	surface layer

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Fig. 1.

Schematic drawing of *LbGH25B* and *LbGH25N* variants. Native (n) *LbGH25B* and *LbGH25N* are both 380-aa proteins with a calculated molecular mass of 40.8 kDa. The amino acids Val182 and Ala260 in *LbGH25B* are replaced by Ile182 and Glu260, respectively, in *LbGH25N*. The putatively glycosylated sequence motif Ser–Ala–Ser–Ser–Ala–Ser is marked in dark grey. Recombinant (r) *LbGH25B* and *LbGH25N* are devoid of their 29-aa signal peptide (SP) but contain a C-terminal hexahistidine tag (black) for purification purposes. *LbGH25B* was dissected into an N-terminal protein portion containing the glycosyl hydrolase family 25 domain (GH25, Pf01183) and the C-terminal remainder of the protein.

Fig. 2. Comparison of secondary structure and thermal stability of *LbGH25B* and *LbGH25N* by ECD spectroscopy. Far-UV ECD spectra at 25 °C (a) and temperature-mediated unfolding followed at 222 nm (b) of *LbGH25B* (solid line) and *LbGH25N* (dotted line). Measurements were performed in 12.5 mM (a) and 50 mM (b) sodium citrate buffer, pH 5.5. The inset in (b) shows the van't Hoff plots.

Fig. 3. SDS-PAGE and zymogram analysis. Purified recombinant *LbGH25B* (lane 1) and *LbGH25N* (lane 2); cell extracts of *E. coli* expressing *LbGH25B* (lane 3) and *LbGH25N* (lane 4); *E. coli* harbouring empty pET28a (lane 5); *E. coli* expressing the GH25 domain (lane 6) or the C-terminal domain (lane 7) of *LbGH25B*; cell extracts of *L. buchneri* CD034 (lane 8) and *L. buchneri* NRRL B-30929 (lane 9) as well as the purified recombinant GH25 domain (lane 10) and C-terminal domain (lane 11) of *LbGH25B* were run on SDS-PAGE (20 % polyacrylamide gel) and stained with CBB (a) or loaded on the zymogram gels (b–e) containing *L. buchneri* CD034 cell walls (20 % PA gel) (b), *L. buchneri* NRRL B-30929 cell walls (12 % PA gel) (c), *L. rhamnosus* GG cells (12 % polyacrylamide gel) (d) or *E. coli* BL21 Star BL21(DE3) cell walls (12 % PA gel) (e) as substrate. Zymogram gels were incubated overnight at 37 °C in potassium phosphate buffer containing 1 mM DTT and visualized by methylene blue staining. Arrows indicate hydrolytic bands of native *LbGH25B* and *LbGH25N*. Asterisks indicate hydrolytic bands of the *LbGH25B* N-terminal GH25 domain-containing region. Note the presence of a hydrolytic protein (~25 kDa) in the *E. coli* cell extracts (b). Mm₁, Precision Plus protein kaleidoscope standard (Bio-Rad); Mm₂, PageRuler Plus pre-stained protein ladder (Thermo Scientific).

Fig. 4.

Reversed-phase HPLC separation profiles of muropeptides obtained from *L. buchneri* CD034 (a), *L. buchneri* NRRL B-30929 (b) and *L. rhamnosus* GG PG (c). PGs were digested by mutanolysin, *LbGH25B*, *LbGH25N* or SlpB (negative control). Main peaks (1–4, A–D) were analysed by MALDI-TOF MS (Table 2).

Fig. 5.

Cellular localization of *LbGH25B* and *LbGH25N* by (a) subcellular fractionation and (b) *in vitro* cell-wall binding. (a) *L. buchneri* CD034 and NRRL B-30929 proteins were isolated from cell walls (lanes 1 and 4), the cytosol (lanes 2 and 5), and the culture supernatant (lanes 3 and 6), run on SDS-PAGE (10 % gel) using equivalent sample amounts, Western blotted and detected with muramidase-specific antiserum. (b) Binding of recombinant *LbGH25B* and *LbGH25N* to cell walls of *L. buchneri* was tested. Proteins were incubated with (+) and without (-) cell walls. After incubation, the reactions were centrifuged to separate cell walls (with bound protein) from unbound protein. Analysis was done by SDS-PAGE (20 % gels) followed by Western immunoblotting using muramidase-specific antiserum. The figure represents one of three independent repeats of the experiment. S, supernatant; P, pellet; Mm, PageRuler prestained protein ladder (Thermo Scientific).

Fig. 6.

Cellular localization of *LbGH25B* and *LbGH25N* by immunofluorescence microscopy. Muramidase-specific mouse antiserum in combination with FITC-conjugated goat anti-mouse IgG was used to visualize *LbGH25B* and *LbGH25N* on *L. buchneri* CD034 (a) and *L. buchneri* NRRL B-30929 (b) cells, respectively. Staining with pre-immune serum as a negative control revealed no fluorescence in *L. buchneri* CD034 (c) and *L. buchneri* NRRL B-30929 (d) cells. As an additional negative control, immunofluorescence staining with muramidase-specific mouse antiserum did not show a non-specific reaction in the Gram-positive bacterium *L. plantarum* CD033 (e).

Table 1

PGHs of *L. buchneri* CD034 and their homologues in *L. buchneri* NRRL B-30929 predicted *in silico* on whole-genome sequences

Locus tag	MM* (kDa)	SP [†]	Putative hydrolytic specificity	Catalytic domain [‡]	CW-binding domain [§]	NRRL B-30929 homologue	Identity (%) [∫]
LBUCD034_0240	40.8	Yes	Muramidase	GH25		Lbuc_0200	99
LBUCD034_0976	30.8	No	Muramidase	GH25		Lbuc_1390	53
LBUCD034_1564	30.5	No	Muramidase	GH25		Lbuc_1506	99
LBUCD034_1723	29.5	No	Muramidase	GH25		Lbuc_1566	100
LBUCD034_1884	34.4	Yes	Muramidase	GH25		Lbuc_1800	99
LBUCD034_2218	33.5	Yes	Muramidase	GH25		Lbuc_2119	99
LBUCD034_0537	23.5	No	Glucosaminidase	Glucosaminidase		Lbuc_0501	99
LBUCD034_1423	28.2	Yes	Glucosaminidase	Glucosaminidase		Lbuc_1294	99
LBUCD034_1424	54.2	Yes	Glucosaminidase	Glucosaminidase		Lbuc_1295	99
LBUCD034_1762	24.0	Yes	Glucosaminidase	Glucosaminidase		Lbuc_1692	100
LBUCD034_0238	25.2	Yes	Amidase	Amidase_2		Lbuc_0198	99
LBUCD034_0862	67.0	No	Amidase	Amidase_2		Lbuc_0803	98
LBUCD034_1137	34.2	No	Amidase	Amidase_3	SH3_3	Lbuc_1003	100
LBUCD034_2087	36.2	Yes	Amidase	Amidase_2		Lbuc_1998	99
LBUCD034_0481	47.7	Yes	D-Alanyl-D-alanine carboxypeptidase	Peptidase_S11		Lbuc_0439	98
LBUCD034_0620	45.1	Yes	D-Alanyl-D-alanine carboxypeptidase	Peptidase_S11	PBP5_C	Lbuc_0602	97
LBUCD034_0662	43.3	Yes	D-Alanyl-D-alanine carboxypeptidase	Peptidase_S11		Lbuc_642	99
LBUCD034_0534	17.7	Yes	Endopeptidase	NLPC_P60		Lbuc_0498	99
LBUCD034_0586	19.2	Yes	Endopeptidase	NLPC_P60		Lbuc_0549	100
LBUCD034_1373	32.9	No	Endopeptidase	NLPC_P60	SH3_3	Lbuc_1248	100
LBUCD034_1574	202.2	No	Endopeptidase	NLPC_P60		Lbuc_1516	99
LBUCD034_1996	30.9	No	Endopeptidase	NLPC_P60		Lbuc_1908	96
LBUCD034_2219	17.8	Yes	Endopeptidase	NLPC_P60		Lbuc_2120	100

* Calculated molecular mass.

[†] SP, signal peptide predicted with SignalP 4.1 server.

[‡] Catalytic domains predicted with Pfam domain prediction. Muramidase (glyco_hydro_25; PF01183), glucosaminidase (PF01832), Amidase_2 (PF01510), Amidase_3 (PF01520), Peptidase_S11 (PF00768), endopeptidase (NLPC_P60; PF00877).

[§] CW (cell wall)-binding domain; SH3_3, (PF08239); PBP5_C (PF07943).

^{||} Locus tag of *L. buchneri* NRRL B-30929 protein, homology search by BLAST algorithm.

[∫] Amino acid sequence identity by BLAST alignment.

Table 2Main mucopeptides from *L. buchneri* NRRL B-30929 and *L. rhamnosus* PG digested with *LbGH25N*

Peak [*]	Proposed structure [†]	Observed <i>m/z</i>	Calculated [‡] [M+Na] ⁺
1	Tri-N	962.18	962.43
2	Tetra-D	1034.37	1034.45
3	Tetra-N	1033.35	1033.47
4	Tetra-N(Ac)	1075.52	1075.48
A	Tri-N	962.33	962.43
B	Tetra-N	1033.43	1033.47
C	Tri-N(Ac)	1004.37	1004.44
D	Tetra-N(Ac)	1075.38	1075.48

* Peak numbers refer to Fig. 4.

[†] Tri, disaccharide tripeptide (L-Ala-D-iGln-L-Lys); Tetra, disaccharide tetrapeptide (L-Ala-D-iGln-L-Lys-D-Ala); Disaccharide, GlcNAc-MurNAc; Ac, acetylation; iGln, isoglutamine; N, D-Asn; D, D-Asp.

[‡] Sodiated molecular ions were the most abundant on MALDI-TOF mass spectra for all mucopeptides.