Peptidoglycan *N*-acetylglucosamine deacetylation decreases autolysis in *Lactococcus lactis*

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The gene *xynD* (renamed *pgdA*) of *Lactococcus lactis* IL1403 was shown to encode a peptidoglycan *N*-acetylglucosamine deacetylase. Inactivation of *pgdA* in *L. lactis* led to fully acetylated peptidoglycan, whereas cloning of *pgdA* on a multicopy plasmid vector resulted in an increased degree of peptidoglycan deacetylation, as shown by analysis of peptidoglycan constituent muropeptides. An increased amount of *N*-unsubstituted glucosamine residues in peptidoglycan resulted in a reduction of the rate of autolysis of *L. lactis* cells. The activity of the *L. lactis* major autolysin AcmA was tested on *L. lactis* cells or peptidoglycan with different degrees of de-*N*-acetylation. Deacetylated peptidoglycan exhibited decreased susceptibility to AcmA hydrolysis. This reduced susceptibility to AcmA did not result from reduced AcmA binding to peptidoglycan with an increasing degree of de-*N*-acetylation. In conclusion, enzymic *N*-acetylglucosamine deacetylation protects peptidoglycan from hydrolysis by the major autolysin AcmA in *L. lactis* cells, and this leads to decreased cellular autolysis.

INTRODUCTION

Peptidoglycan is the major cell wall component of Gram-positive bacteria, and ensures the stability and rigidity of the cell wall (Delcour *et al.*, 1999). During growth, bacteria synthesize enzymes capable of hydrolysing their own peptidoglycan. These peptidoglycan hydrolases (PGHs) are required for cell separation after division and are involved in several other cellular processes that require peptidoglycan remodelling, such as peptidoglycan turnover and cell wall expansion (Smith *et al.*, 2000). Under conditions which result in cessation of peptidoglycan synthesis, such as stationary phase or exposure to antibiotics, PGH activity may cause cellular autolysis, leading to the release of intracellular components.

Since PGHs are potentially lethal enzymes and are present in the cells during bacterial growth, it is usually assumed that their activities are regulated at the post-translational level (Shockman & Høltje, 1994; Smith *et al.*, 2000). Different mechanisms of PGH control have been proposed, including proteolytic maturation (Buist *et al.*, 1998; Poquet *et al.*, 2000; Shockman, 1992), interaction with a specific modifier protein (Lazarevic *et al.*, 1992), regulation by the membrane proton motive force (Calamita *et al.*, 2001;

Kemper *et al.*, 1993), and interaction with secondary cell wall polymers such as teichoic acids (Palumbo *et al.*, 2006; Wecke *et al.*, 1997). Finally, structural modifications of the peptidoglycan substrate by *O*-acetylation (Pfeffer *et al.*, 2006) or de-*N*-acetylation (Atrih *et al.*, 1999) may control the action of PGHs.

The peptidoglycan hydrolase complement of *Lactococcus lactis*, the model Gram-positive lactic acid bacterium, comprises five PGHs, including three *N*-acetylglucosaminidases (Buist *et al.*, 1995; Huard *et al.*, 2004, 2003) and one γ -D-glutaminyll-L-lysyl-endopeptidase (Redko *et al.*, 2007). The autolysin AcmA is involved in cell separation and is the major effector of cellular autolysis in stationary phase (Buist *et al.*, 1995). The AcmA protein has two domains: an N-terminal catalytic domain endowed with *N*-acetylglucosaminidase specificity (Steen *et al.*, 2005a), and a C-terminal domain with three LysM modules involved in cell wall binding and which recognize peptidoglycan (Steen *et al.*, 2003).

Our objective was to examine the impact of structural modifications of peptidoglycan on *L. lactis* cellular autolysis and on the activity of AcmA autolysin in the cells. Peptidoglycan consists of glycan strands made of alternating β -1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) which are cross-linked by short peptide chains. Its structure can be analysed by identification of the constituent muropeptides obtained after muramidase digestion. Recently, we performed a detailed analysis of *L. lactis* peptidoglycan structure and observed several deacetylated muropeptides (Courtin *et al.*,

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Abbreviations: PGH, peptidoglycan hydrolase; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; SCO, single crossing-over; PSD, post-source decay; PMF, peptide mass fingerprinting.

2006). A gene (*xynD*) encoding a putative peptidoglycan deacetylase was identified in the *L. lactis* IL1403 genome on the basis of sequence similarity with the peptidoglycan GlcNAc deacetylase PgdA from *Streptococcus pneumoniae* (Vollmer & Tomasz, 2000). These peptidoglycan deacetylases belong to the carbohydrate esterase family 4 (CE4), which also includes chitin deacetylases, acetylxylose esterases and chitooligosaccharide deacetylase [Psylinakis *et al.*, 2005; Carbohydrate Active Enzymes database (<http://www.cazy.org/>); Coutinho & Henrissat, 1999].

In this study, we show that *L. lactis xynD* (renamed *pgdA*) encodes a peptidoglycan GlcNAc deacetylase. In addition, we show that an increase in the degree of peptidoglycan de-N-acetylation results in decreased autolysis of *L. lactis* cells, due to a reduced susceptibility of deacetylated peptidoglycan to the major autolysin AcmA.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37 °C in Luria-Bertani (LB; Difco) medium with shaking. *L. lactis* strains were grown in M17 medium (Difco) supplemented with 0.5% (w/v) glucose at 30 °C. Growth and autolysis were monitored by measuring OD₆₀₀ with a

spectrophotometer (Uvikon XL, Bio-Tek Instruments). Plasmids were selected by addition of antibiotics as follows: for *L. lactis*, erythromycin (5 µg ml⁻¹), chloramphenicol (5 µg ml⁻¹); for *E. coli*, ampicillin (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹), chloramphenicol (10 µg ml⁻¹).

General recombinant DNA techniques. Molecular cloning techniques were performed using standard procedures (Sambrook *et al.*, 1989). Restriction enzymes (New England Biolabs), T4 DNA ligase (Epicenter), Taq DNA polymerase (Qiagen) and the TripleMaster PCR System (Eppendorf) were used as recommended by the manufacturers. Oligonucleotides were purchased from Invitrogen. Plasmids were extracted using the QIAprep Spin Miniprep kit (Qiagen). PCR was performed with a GeneAmp 2400 PCR system (Perkin-Elmer). DNA sequences were determined with an Applied Biosystems 370A automated DNA sequencer, and with ABI PRISM dye terminator cycle sequencing and dye primer cycle sequencing kits (Perkin-Elmer). Preparation of competent cells and electrotransformation of *L. lactis* were carried out as described elsewhere (Holo & Nes, 1989).

Construction of the *pgdA* mutant and complementation of the mutant. The gene *pgdA* (*xynD*) was inactivated in *L. lactis* strain IL6288 by single crossing-over (SCO) plasmid integration. An internal *pgdA* fragment was PCR-amplified with primers 5'-TACCTTGCTTATAGGAGCG-3' and 5'-CAGGCTTGGGCTTGTTTTC-3', using IL1403 DNA as template. The resulting 597 bp fragment was cloned into the pGEM-T Easy vector and sequenced. It was then cloned into the *NcoI* and *SacI* restriction sites of the pJIM2242

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant genotype or phenotype	Source or reference
<i>E. coli</i> strains		
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB⁺ lac^f lacZAM15]</i>	
BL21(DE3)	F ⁻ <i>ompT hsdS_B(r_Bm_B) gal dcm</i> bearing T7 RNA polymerase gene (λDE3 lysogen)	Studier & Moffatt (1986)
BL21(DE3)pLysE	Cm ^r , BL21(DE3) containing pLysE plasmid producing T7 lysozyme, an inhibitor of T7 RNA polymerase	Invitrogen
TIL933	Cm ^r Ap ^r Kan ^r , BL21(DE3)pLysE derivative containing pET11a-Kan	This study
TIL934	Cm ^r Ap ^r Kan ^r , BL21(DE3)pLysE derivative expressing AcmA with hexa-His-tag with pET11a-Kan plasmid	This study
<i>L. lactis</i> strains		
IL1403	Plasmid-free strain	Chopin <i>et al.</i> (1984)
IL6288	IL1403 derivative cured of its six prophages	M.-C. Chopin*
TIL926	Em ^r , IL6288 derivative, <i>pgdA</i> mutant obtained by SCO integration of pJIM2242 plasmid containing a 597 bp <i>pgdA</i> internal fragment	This study
TIL926(pJIM <i>pgdA</i>)	Em ^r Cm ^r , TIL926 derivative containing pJIM <i>pgdA</i> plasmid	This study
TIL926(pJIM)	Em ^r Cm ^r , TIL926 derivative containing pJIM2242	This study
PA1001(pPA3)	Derivative of NZ9000 lacking <i>acmA</i> and <i>htrA</i> and containing pPA3 plasmid	Bosma <i>et al.</i> (2006)
MG1363 <i>acmAΔ1</i>	Derivative of MG1363 carrying an internal deletion in <i>acmA</i>	Buist <i>et al.</i> (1995)
Plasmids		
pGEM-T Easy	Ap ^r , cloning vector with T overhangs	Promega
pET11a	Ap ^r , expression vector with T7 <i>lac</i> promoter	Novagen
pET11a-Kan	Ap ^r Kan ^r , pET11a derivative with kanamycin-resistance cassette cloned into <i>Clal</i> site	Chich <i>et al.</i> (1995)
pJIM2242	Em ^r , derivative of pORI28 non-replicative vector in <i>L. lactis</i>	Guedon <i>et al.</i> (2001)
pJIM2246	Cm ^r , high-copy lactococcal cloning vector	Renault <i>et al.</i> (1996)
pJIM <i>pgdA</i>	Cm ^r , pJIM2246 derivative carrying <i>pgdA</i> gene under control of its own promoter	This study
pPA3	Cm ^r , pNZ8048 derivative containing <i>c-myc</i> , 3' end of <i>acmA</i> (nt 835–1492) under control of nisin-inducible promoter and <i>usp45</i> signal sequence	Bosma <i>et al.</i> (2006)

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plasmid vector, a non-replicative plasmid in *L. lactis*. The resulting plasmid was produced in *E. coli* TG1 and used to transform *L. lactis* IL6288. Integration of this plasmid by SCO into the IL6288 chromosome was performed with helper plasmid pGhost8, as described elsewhere (Guedon *et al.*, 2001). Erythromycin-resistant clone TIL926 was selected. Plasmid integration was verified by PCR and Southern blot hybridization.

Complementation of the IL6288 *pgdA*-negative mutant was obtained by cloning the *pgdA* gene into the pJIM2246 multicopy plasmid vector. A 1351 bp DNA fragment encoding *pgdA* with its putative promoter and terminator was amplified by PCR from IL1403 total DNA with primers 5'-AAACTGCAGGGATATGTTAAGATAGGAG-G-3' (*Pst*I site underlined) and 5'-ATATGCGGCCGCTCAAGTTTCCCATAAGTTGTC-3' (*Not*I site underlined). The fragment was digested with *Pst*I and *Not*I and cloned into a *Pst*I/*Not*I-linearized pJIM2246 vector. The resulting plasmid was produced in *E. coli* TG1 and used to transform TIL926. The resulting strain was named TIL926(pJIM*pgdA*). A negative control strain was obtained by transformation of TIL926 with pJIM2246 empty plasmid.

Cell wall peptidoglycan preparation. Peptidoglycan from *L. lactis* strains was prepared as described previously (Courtin *et al.*, 2006). Cells from a 500 ml exponentially growing culture (OD₆₀₀ 0.3) were chilled on ice and harvested by centrifugation (4200 g, 10 min, 4 °C). Cells were suspended in 20 ml deionized H₂O and boiled for 10 min. They were then resuspended in 1 ml 5 % (w/v) SDS and boiled for 25 min. The pellet obtained by centrifugation at 20 000 g for 10 min was resuspended in 1 ml 4 % (w/v) SDS and boiled again for 15 min. Cell walls were recovered by centrifugation at 20 000 g for 10 min and washed six times with deionized H₂O to remove SDS. To eliminate proteins, the cell wall pellet was treated with Pronase (2 mg ml⁻¹) for 90 min at 60 °C, then by trypsin (200 µg ml⁻¹) for 16 h at 37 °C. The pellet containing peptidoglycan was treated with 48 % hydrofluoric acid overnight at 4 °C to eliminate teichoic acids, washed twice with 0.25 M Tris/HCl, pH 8.0, and then four times with deionized H₂O. The final pellet was freeze-dried and stored at -20 °C.

Structural analysis of *L. lactis* peptidoglycan. Purified peptidoglycan (4 mg dry weight in 500 µl) was digested with mutanolysin (Sigma; 2500 U ml⁻¹) in 25 mM sodium phosphate buffer, pH 5.5, for 19 h at 37 °C under rotational shaking. The enzyme was inactivated by boiling the sample for 3 min and insoluble material was removed by centrifugation. The soluble muropeptides were reduced with sodium borohydride and then separated by reversed-phase HPLC using a Hypersil ODS column (C18; 250 × 4.6 mm internal diameter; 5 µm particle size; Thermo Hypersil-Keystone) at 50 °C using ammonium phosphate buffer and a methanol linear gradient, as described previously (Courtin *et al.*, 2006). Peaks were analysed without desalting by MALDI-TOF MS with a Voyager DE STR mass spectrometer (Applied Biosystems), as described previously (Courtin *et al.*, 2006).

For MALDI-post-source decay (PSD) analysis (Chaurand *et al.*, 1999), the purified muropeptides were desalted by HPLC on a Betasil C18 column (250 × 4.6 mm internal diameter; 5 µm particle size; Thermo Hypersil-Keystone) equilibrated with solvent A [H₂O with 0.115 % trifluoroacetic acid (TFA)] at 50 °C. The muropeptides were eluted with a linear gradient from 0 to 33 % of solvent B (60 % acetonitrile with 0.1 % TFA) in 40 min. The desalted muropeptides were then dried with a SpeedVac concentrator, and resuspended in 10 µl 50 % acetonitrile for MALDI-PSD analysis.

Expression and purification of His-tagged AcmA protein in *E. coli*. The major autolysin AcmA from *L. lactis* IL1403 was expressed in *E. coli* BL21(DE3)pLysE without its putative signal sequence and with a C-terminal hexa-His-tag. With the primers AUT1 (5'-GGAA-TTCCATATGGCAACCAATTCCACAGAA-3'; *Nde*I site underlined)

and AUT4 (5'-CGGGATCCTTGTGATGGTGTGATGGTGTGATGTTAA-TACGAAGATATTGACCAAT-3'; *Bam*HI site underlined; encoding a hexa-His-tag) selected from the IL1403 genome sequence, a 1145 kb DNA fragment was PCR-amplified from *L. lactis* IL1403 DNA. This fragment was cloned into the expression vector pET-11a-Kan (Chich *et al.*, 1995) under the control of the T7 promoter with the *lac* operator. The resulting plasmid was produced in *E. coli* TG1 and used to transform *E. coli* BL21(DE3)pLysE. The resulting strain was named TIL934. To induce expression of recombinant hexa-His-tagged AcmA, IPTG was added at a final concentration of 1 mM to the culture at an OD₆₀₀ of 0.6. The control strain BL21(DE3)pLysE containing the empty pET11a-Kan vector (TIL933) was treated in a similar way. Bacteria were grown at 37 °C until IPTG addition and were then transferred to 30 °C during the expression time (3 h) to avoid inclusion body formation. Cells were harvested by centrifugation and disrupted by one passage at a pressure of 1600 bar with a Basic Z Cell Disruption System (Constant Systems). The soluble fraction containing the recombinant protein was recovered after centrifugation at 15 000 g for 15 min at 4 °C. The hexa-His-tagged protein was purified on a His-Trap FF column (1 ml, Amersham Biosciences) according to the manufacturer's instructions using a fast protein liquid chromatography system (Amersham Biosciences). Elution was carried out with an imidazole concentration gradient (20–500 mM in 15 min). Fractions were collected and analysed by SDS-PAGE and zymogram. Fractions containing AcmA eluted at an imidazole concentration between 300 and 400 mM.

SDS-PAGE and renaturing SDS-PAGE (zymogram). SDS-PAGE was performed with 10 % (w/v) polyacrylamide separating gels. Renaturing SDS-PAGE was performed as previously described (Huard *et al.*, 2003). The polyacrylamide gels contained 0.2 % (w/v) *Micrococcus luteus* ATCC 4698 (Sigma) or 0.4 % (w/v) *L. lactis* autoclaved cells, or 0.08 % (w/v) *L. lactis* peptidoglycan as enzyme substrates. After sample migration in the gels, the gels were washed for 30 min in deionized H₂O at room temperature and then incubated in 50 mM Tris/HCl, pH 7.0, containing 0.1 % (v/v) Triton X-100, overnight at 37 °C. The gels were subsequently washed for 30 min in deionized H₂O, then stained with 0.1 % Methylene Blue in 0.01 % (w/v) KOH for 2 h at room temperature and destained in deionized H₂O. Gel images were generated with a DuoScan T1200 scanner (Agfa-Gevaert).

Protein identification by peptide mass fingerprinting (PMF). Proteins were digested with trypsin and the masses of tryptic peptides were analysed by MALDI-TOF MS, as described previously (Guillot *et al.*, 2003), with a Voyager DE STR instrument (Applied Biosystems) by the Plateau d'Analyses Protéomiques par Séquençage et Spectrométrie de Masse (PAPSS) at INRA, Jouy-en-Josas. Database searches were conducted with the MS-Fit software (<http://prospec-tor.ucsf.edu>) either on an *L. lactis*-specific database containing protein sequences deduced from the genome sequence of *L. lactis* IL1403 or on the SWISS-PROT database.

Triton X-100-induced autolysis in buffer solution. *L. lactis* strains were grown in M17 medium to mid-exponential phase (OD₆₀₀ 0.8). Cells were harvested by centrifugation at 5000 g for 10 min at 4 °C, washed once with 50 mM potassium phosphate buffer, pH 7.0, and resuspended at an OD₆₀₀ of 1 in 50 mM potassium phosphate buffer, pH 7.0, supplemented with 0.05 % Triton X-100 (Cornett & Shockman, 1978). Cell suspensions were then transferred into 100-well sterile microplates and incubated at 30 °C. Autolysis was monitored by measuring the OD₆₀₀ of the cell suspensions with an automated incubator/optical density reader (Microbiology Workstation Bioscreen C, Labsystems).

Assay of AcmA activity in *L. lactis* autoclaved cells. *L. lactis* autoclaved cells were used as a substrate for measuring AcmA activity

and were prepared as follows. *L. lactis* strains were grown in M17 medium up to the end of exponential phase. Cells were harvested by centrifugation at 5000 *g* for 15 min at 4 °C, washed once with 10 mM Tris/HCl, pH 7.5, at 4 °C, washed once with deionized H₂O, and freeze-dried. The dried samples were resuspended in deionized H₂O at 10 % (w/v), autoclaved and stored at 4 °C.

Autoclaved cells were diluted in 25 mM Tris/HCl, pH 7, to OD₆₀₀ 0.5. Purified hexa-His-tagged AcmA (~1.5 µg ml⁻¹ final concentration) was added in a final volume of 1 ml, and the OD₆₀₀ of the cell suspension was monitored with a spectrophotometer (Uvikon XL, Bio-Tek Instruments). The extent of autolysis was expressed as the percentage decrease in OD₆₀₀.

Binding of c-Myc-PA fusion protein to peptidoglycan. The c-Myc-PA (PA3) fusion protein corresponds to the cell wall binding domain of AcmA (PA; C-terminal 218 aa of AcmA) fused to a c-Myc epitope at its N terminus (Bosma *et al.*, 2006). It was produced in the supernatant of strain *L. lactis* PA1001(pPA3) (a kind gift of K. Leenhouts, BioMade, Groningen, The Netherlands). The strain was grown at 30 °C in M17 glucose medium containing 10 ng nisin ml⁻¹ to induce the production of c-Myc-PA fusion protein. The supernatant was recovered at the end of the exponential growth phase and filtered through a 0.22 µm pore-size filter. Peptidoglycan (150 µg) extracted from the different *L. lactis* strains was resuspended in 1 ml of culture supernatant containing c-Myc-PA diluted 10, 20 or 50 times in culture supernatant of *L. lactis* MG1363acmΔΔ1. The suspensions were incubated for 1 h at room temperature under rotational shaking and centrifuged for 30 min at 35 000 *g*, and the pellet was washed twice with PBS. Then, the pellet was resuspended in 40 µl denaturing buffer (50 mM Tris/HCl, pH 6.8, 2 % SDS, 10 %, v/v, glycerol, 0.1 % Bromophenol Blue and 100 mM DTT), boiled for 3 min and centrifuged for 10 min at 35 000 *g* before SDS-PAGE. Supernatant (10 µl) was analysed by SDS-PAGE with a 12.5 % polyacrylamide gel. c-Myc-PA protein bound to peptidoglycan was detected by Western blot hybridization after transfer of the proteins onto a nitrocellulose membrane (Bio-Rad), as described elsewhere (Towbin *et al.*, 1979). Membranes were incubated successively with monoclonal anti-c-Myc antibody (Clontech) and rabbit anti-mouse antibodies coupled to horseradish peroxidase (Amersham), and revealed by chemiluminescence with a Western Lightning kit (Perkin Elmer) according to the manufacturer's instructions. The resulting light was detected on Hyperfilm ECL (Amersham). The film was scanned with a Duoscan T1200 scanner (Agfa-Gevaert) and quantitative analysis of revealed bands was performed with ImageJ software (<http://rsb.info.nih.gov/ij/>). The relative amount of c-Myc-PA protein in the different samples was standardized against the amount of c-Myc-PA contained in 5 µl *L. lactis* PA1001(pPA3) supernatant, which was run on each gel.

RESULTS

Analysis of *L. lactis* *pgdA* sequence

The gene *pgdA* (*xynD*) encodes a 372-residue protein with a putative N-terminal signal peptide, predicted by Psort software (<http://psort.hgc.jp>) to be uncleavable. The PgdA amino acid sequence exhibits 31 % identity to the *S. pneumoniae* peptidoglycan GlcNAc deacetylase sequence (Vollmer & Tomasz, 2000). In addition, *L. lactis* PgdA contains a conserved zinc-binding triad (Asp-184, His-234, His-238) identified in the active site of its *S. pneumoniae* homologue (Blair *et al.*, 2005). Northern blot analysis

indicated that the *pgdA* gene is transcribed during the exponential growth phase in *L. lactis* IL1403 (data not shown). The transcript size (1.3 kb) indicated a monocistronic organization of the gene, in agreement with the detection of two putative rho-independent transcription terminators upstream and downstream of *pgdA*.

L. lactis *pgdA* encodes a peptidoglycan GlcNAc deacetylase

To investigate the role of *pgdA*, the gene was inactivated in *L. lactis* subsp. *lactis* strain IL6288. This strain is a derivative of IL1403 obtained after curing the six identified prophages (Chopin *et al.*, 2001) from the chromosome (A. Aucouturier and M.-C. Chopin, personal communication). Inactivation of *pgdA* was obtained by SCO integration of the non-replicative vector pJIM2242 containing a 597 bp internal *pgdA* fragment.

The peptidoglycan structure of the IL6288 *pgdA* mutant (TIL926) was analysed and compared with that of the parental strain IL6288. After peptidoglycan digestion with mutanolysin, a muramidase, the resulting muropeptides were separated by HPLC (Fig. 1). Four peaks (indicated by arrows) present in the IL6288 muropeptide profile (Fig. 1a) were absent from the TIL926 muropeptide profile (Fig. 1b). These peaks (A, B, E, F) (Fig. 1c) were restored in the muropeptide profile of the complemented strain TIL926(pJIM*pgdA*), obtained by cloning *pgdA* into the multicopy plasmid vector pJIM2246. Also, three other minor peaks (C, D, G) appeared in the complemented mutant (Fig. 1c). Peaks A–G were analysed by MALDI-TOF MS. Their measured *m/z* values exhibited a 42 Da mass defect compared to the masses expected for the molecular sodiated ions corresponding to *L. lactis* fully acetylated muropeptides (Table 2). This 42 Da mass defect corresponds to the loss of one acetyl group. Thus, all the peaks A–G contained deacetylated muropeptides. From these results, we conclude that *pgdA* encodes a peptidoglycan deacetylase. The percentage of deacetylated muropeptides was calculated to be 9.8 % in the parental strain IL6288 at the beginning of exponential growth, whereas it was 15.8 % in the complemented strain TIL926(pJIM*pgdA*). These data indicate that *pgdA* is overexpressed in TIL926(pJIM*pgdA*), most probably because *pgdA* was cloned on a high-copy-number plasmid.

Peak A, containing the deacetylated monomer disaccharide tripeptide with Asn side chain (*m/z* 920.45), was subjected to MALDI-PSD analysis, which allowed the analysis of muropeptide fragment ions (Fig. 2). A fragment with *m/z* of 760.6 corresponded to the loss of one glucosamine. Several other fragments resulted from the loss of one glucosamine (*m/z* 161), whereas no fragment corresponding to the loss of one GlcNAc (*m/z* 203) was observed. These results indicate that muropeptide A contains glucosamine instead of GlcNAc, and that deacetylation occurs on GlcNAc. Thus, the *L. lactis* *pgdA* gene encodes a peptidoglycan GlcNAc deacetylase.

Fig. 1. Reversed-phase HPLC separation of mucopeptides from *L. lactis* IL6288 (a), IL6288 *pgdA* mutant (TIL926) (b) and complemented mutant TIL926(pJIM*pgdA*) (c). Arrows indicate peaks which contain deacetylated mucopeptides.

Influence of peptidoglycan de-*N*-acetylation on lysozyme sensitivity

We tested the lysozyme sensitivity of IL6288 and its derivative strains by plating cultures of each strain on M17

agar plates containing lysozyme concentrations from 0 to 3 mg ml⁻¹. The parental strain IL6288 was resistant to 1 mg lysozyme ml⁻¹, whereas the *pgdA*-negative mutant TIL926 was sensitive to 1 mg ml⁻¹. In contrast, the complemented strain TIL926(pJIM*pgdA*) with a higher

Table 2. Structures, molecular masses and quantities of deacetylated mucopeptides

Peak*	Observed <i>m/z</i>	Calculated [M + Na] ⁺ †	Δ <i>m</i>	Identification‡	Percentage§	
					IL6288	TIL926(pJIM <i>pgdA</i>)
A	920.45	962.43	-41.98	Tri-N (deAc)	1.3	2.5
B	921.35	963.41	-42.06	Tri-D (deAc)	0.7	1.4
C	991.49	1033.47	-41.98	Tetra-N (deAc)	0.4	1.0
D	1062.42	1104.50	-42.08	Penta-N (deAc)	0.4	0.7
E	1912.92	1954.90	-41.98	Tri-N-Tetra-N (deAc)	2.5	3.7
F	1912.90	1954.90	-42.00	Tri-N-Tetra-N (deAc)	3.1	4.7
G	2905.22	2947.36	-42.14	Tri-N-Tetra-N-Tetra-N (deAc)	1.4	1.8
				Total	9.8	15.8

*Peak identification refers to the chromatograms in Fig. 1.

†Calculated [M + Na]⁺ corresponds to the non-deacetylated mucopeptides from *L. lactis*. Sodiated molecular ions were the most abundant ones in MALDI-TOF mass spectra for all mucopeptides.

‡Tri, disaccharide tripeptide (L-Ala-D-iGln-L-Lys); Tetra, disaccharide tetrapeptide (L-Ala-D-iGln-L-Lys-D-Ala); Penta, disaccharide pentapeptide (L-Ala-D-iGln-L-Lys-D-Ala-D-Ala). Disaccharide=GlcNAc-MurNAc; D, Asp; N, Asn; deAc, deacetylation; iGln, isoGln.

§The percentage for each peak was calculated as the ratio of its area over the sum of all the peak areas.

Fig. 2. MALDI-PSD analysis of reduced mucopeptide A purified by reversed-phase HPLC (see Fig. 1). The $[M+Na]^+$ parental ion (m/z 920.9) was selected for PSD analysis. (a) MALDI-PSD TOF mass spectrum of sodiated ions corresponding to fragments that issued from mucopeptide A; (b) chemical structure determined for mucopeptide A and fragmentation pattern. The dashed arrows indicate N-terminal fragment ions (a and b ions) and a C-terminal fragment ion (ion y) identified on the spectrum. Several fragments result from the loss of one glucosamine (m/z 161).

degree of peptidoglycan deacetylation showed increased resistance to lysozyme and grew in the presence of 2 mg lysozyme ml^{-1} , while the parent strain did not.

Influence of peptidoglycan de-*N*-acetylation on cell separation and autolysis

The growth rates of the *pgdA*-negative mutant and the wild-type strain were identical in M17 glucose medium, and the growth rates of the complemented strain TIL926(pJIM*pgdA*) and the control TIL926(pJIM) strain were identical in M17 glucose medium containing chloramphenicol. These results indicate that inactivation or overexpression of *pgdA* has no impact on bacterial growth rate. Microscopic observation of the different strains revealed that strain TIL926(pJIM*pgdA*) forms long chains of cells (Fig. 3c), unlike the parental strain IL6288 (Fig. 3a) and the *pgdA*-negative mutant TIL926 (Fig. 3b). The chain length for TIL926(pJIM*pgdA*) (15–20 cells) was

shorter than that obtained with the MG1363 *acmA* mutant (50–100 cells per chain). The defect of cell separation in TIL926(pJIM*pgdA*) may result from the resistance of deacetylated peptidoglycan to endogenous autolysins.

Autolysis of the different strains after they had reached stationary phase was followed over 6 days. During this period, TIL926(pJIM*pgdA*) culture did not exhibit autolysis, as measured by the decrease in OD_{600} , and behaved like the MG1363*acmA* mutant (data not shown). No difference in the rate and extent of autolysis was found among the wild-type IL6288 strain, the *pgdA*-negative mutant and the control strain TIL926(pJIM). For the three strains, the decrease in OD_{600} reached 10 % after 4 days, and then remained stable.

Autolysis was also compared after transfer of bacterial cells to 50 mM potassium phosphate buffer, pH 7.0, containing 0.05 % Triton X-100. As shown in Fig. 4, the *pgdA* mutant TIL926 exhibited a similar initial autolysis rate to that of

Fig. 3. Phase-contrast microscopy of *L. lactis* IL6288 (a), the TIL926 mutant (b) and the complemented TIL926(pJIMpgdA) mutant (c).

the parental IL6288 strain, but a higher final autolysis level. In contrast, strain TIL926(pJIMpgdA), with a higher degree of peptidoglycan de-N-acetylation, showed a reduced rate of autolysis compared to IL6288. The control strain TIL926(pJIM) exhibited an autolysis rate and a final extent of autolysis identical to those of TIL926 without plasmid (data not shown).

Influence of GlcNAc deacetylation on peptidoglycan susceptibility to AcmA autolysin

Since AcmA autolysin is the major enzyme involved in *L. lactis* autolysis and is involved in cell separation after cell division (Buist *et al.*, 1995), we tested the activity of AcmA *in vitro* on peptidoglycan substrates with different degrees of de-N-acetylation.

AcmA without its putative signal sequence and with a C-terminal hexa-His-tag was produced in *E. coli*

BL21(DE3)pLysE under control of the T7 promoter. After induction with IPTG, a low amount of recombinant AcmA-His was obtained (data not shown). The protein was purified by affinity chromatography on a His-Trap column. The major protein detected at 45 kDa in the purified fraction analysed by SDS-PAGE corresponded to AcmA-His (Fig. 5a, lane 1), as confirmed by PMF analysis. In addition, its activity could be detected by zymogram assay with *M. luteus* cells as substrate (Fig. 5b, lane 1). Three minor contaminant proteins were co-purified, and were identified by PMF as *E. coli* proteins not related to PGHs (Fig. 5a, lane 1). These contaminant proteins did not exhibit peptidoglycan-hydrolysing activity as checked by zymogram assay (Fig. 5b, lane 1). Thus, we used the partially purified AcmA-His for activity tests.

AcmA activity was first tested on autoclaved cells obtained from the three strains which exhibited different degrees of peptidoglycan de-N-acetylation: IL6288 (9.8 %), *pgdA*-negative mutant TIL926 (0 %) and complemented strain TIL926(pJIMpgdA) (15.8 %). Activity was measured by the optical density decrease of the cell suspensions. As shown

Fig. 4. Autolysis of *L. lactis* IL6288 and its derivative mutants in buffer containing Triton X-100. Cells were grown in M17 glucose medium to OD₆₀₀ 0.8, and washed and resuspended at OD₆₀₀ 1 in 50 mM potassium phosphate, pH 7.0, containing 0.05 % Triton X-100 at 30 °C. Lysis of IL6288 (■), *pgdA* mutant TIL926 (□) and TIL926(pJIMpgdA) (▲) was monitored by measuring the decrease in OD₆₀₀ of the cell suspensions. Optical density values are the mean of three experiments made with one culture, and sd values were close to zero. The results are representative of three independent experiments.

Fig. 5. SDS-PAGE (a) and zymogram (b) analysis of purified AcmA-His. Proteins were separated on 10 % polyacrylamide gels. (a) Protein bands were stained with Coomassie Blue; (b) the gel contained 0.2 % (w/v) *M. luteus* cells. Lane 1 contained His-Trap column eluate (300 ng protein). P1, P2 and P3 designate proteins which were co-purified with AcmA-His. They were identified by PMF analysis as *E. coli* proteins: P1, UDP-D-glucuronate dehydrogenase (YfbG); P2, 50S ribosomal protein L2; P3, FKBP-type peptidyl-prolyl *cis-trans* isomerase (SlyD). M, molecular mass markers.

in Fig. 6a, the highest activity was found towards cells originating from the negative mutant TIL926 with fully acetylated peptidoglycan. AcmA activity towards cells from TIL926(pJIM*pgdA*) with the highest degree of de-*N*-acetylation was lower than towards parental strain IL6288 cells. These results indicate that de-*N*-acetylation of peptidoglycan reduces its susceptibility to the autolysin AcmA.

We further confirmed this result by testing AcmA-His activity in a zymogram assay with purified peptidoglycan as substrate included into polyacrylamide gels. As shown in Fig. 6b, when the same amounts of AcmA-His were loaded on the gels, the activity band detected on peptidoglycan from the *pgdA*-negative mutant was more intense than that on peptidoglycan from TIL926(pJIM*pgdA*), confirming the lower activity of AcmA towards de-*N*-acetylated peptidoglycan compared to fully acetylated peptidoglycan.

Influence of GlcNAc deacetylation on AcmA binding to *L. lactis* peptidoglycan

AcmA produced by *L. lactis* cells is found both attached to the cells and in the culture supernatant (Buist *et al.*, 1995). It has to bind the peptidoglycan of the cell wall through its C-terminal LysM-containing domain to be able to lyse the cells (Steen *et al.*, 2003). We investigated whether peptidoglycan deacetylation influences substrate binding of AcmA.

First, using the zymogram technique, we compared the amount of AcmA bound to the cells and the amount present in culture supernatant for IL6288 and its derivative mutants. No difference was found in the intensity of the AcmA activity band on micrococci used as substrate between the *pgdA* mutant and wild-type IL6288 or the overexpressing strain (data not shown). Breakdown products of AcmA, which retained activity in the zymogram (Poquet *et al.*, 2000; Steen *et al.*, 2003), were barely detected in IL6288 and were present in similar

amounts in its derivative mutants, indicating no difference in AcmA degradation among the tested strains.

Second, we examined whether peptidoglycan de-*N*-acetylation affects AcmA binding to purified peptidoglycan. AcmA consists of two domains: an active site domain and a C-terminal region containing three highly homologous repeats of 45 aa, named LysM domains, which bind peptidoglycan (Steen *et al.*, 2003). To study the binding of AcmA to peptidoglycan with different degrees of de-*N*-acetylation, we used a fusion protein consisting of the C-terminal peptidoglycan-binding domain of AcmA fused to a c-Myc epitope (c-Myc-PA) at its N terminus (Bosma *et al.*, 2006). Peptidoglycan extracted from IL6288, *pgdA* mutant TIL926 and *pgdA*-overexpressing strain TIL926(pJIM*pgdA*) was incubated with different concentrations of c-Myc-PA. The amount of fusion protein bound to each peptidoglycan was analysed by Western blotting with specific anti-c-Myc antibody (Fig. 7). A band with the expected molecular mass (28 kDa) was detected. Quantitative analysis of the immunodetected band in each sample (data not shown) indicated that equal amounts of c-Myc-PA bound to peptidoglycan from the wild-type, *pgdA* mutant and *pgdA*-overexpressing strains, with different degrees of deacetylation. These results indicate that GlcNAc deacetylation does not modify AcmA binding to peptidoglycan in strain TIL926(pJIM*pgdA*), but most probably affects the efficiency of peptidoglycan chain cleavage by AcmA.

DISCUSSION

The aim of the study was to evaluate the impact of peptidoglycan structural modification on *L. lactis* autolysis. We have identified a peptidoglycan deacetylase (Pgda) in *L. lactis* which deacetylates GlcNAc residues. We have shown that the increase of *N*-unsubstituted glucosamine residues in peptidoglycan results in decreased autolysis of

Fig. 6. (a) Activity of AcmA-His towards autoclaved cells with different degrees of de-*N*-acetylation. Purified AcmA-His was incubated with autoclaved cells prepared from *L. lactis* IL6288 (■), *pgdA* mutant TIL926 (□) and TIL926(pJIM*pgdA*) (▲), and the decrease in OD₆₀₀ of the cell suspensions was monitored. The results shown are representative of three independent experiments in which similar results were obtained. (b) Zymogram analysis of AcmA-His activity towards peptidoglycan substrates included in the gels. Lanes: 1, peptidoglycan from *pgdA* mutant TIL926; 2, peptidoglycan from TIL926(pJIM*pgdA*). Purified AcmA-His (~450 ng) was loaded on each gel.

Fig. 7. Western blot analysis of the binding of c-Myc-PA fusion protein to purified peptidoglycan with different degrees of de-*N*-acetylation. Peptidoglycan (150 µg) extracted from IL6288, *pgdA* mutant TIL926 and TIL926(pJIM*pgdA*) was incubated with culture supernatant of PA1001(pPA3) containing c-Myc-PA protein diluted 10, 20 or 50 times in MG1363*acmAΔ1* culture supernatant. Bound c-Myc-PA protein was detected with specific anti-c-Myc antibody. A control sample [5 µl PA1001(pPA3) supernatant] was included in each experiment and was used as a standard for the quantitative analysis of the immunodetected bands.

L. lactis cells due to reduced susceptibility of the modified peptidoglycan to the hydrolytic activity of the *L. lactis* major autolysin AcmA.

GlcNAc deacetylation of peptidoglycan negatively influences the hydrolytic activity of AcmA, which is a glucosaminidase, able to hydrolyse β -1,4-glycosidic bonds between GlcNAc and MurNAc. Deacetylation of peptidoglycan aminosugars has previously been shown to affect the hydrolytic activity of PGHs with different specificities, either positively or negatively. For example, de-*N*-acetylation of peptidoglycan GlcNAc confers resistance to lysozyme, an exogenous muramidase, upon several bacterial species, such as *S. pneumoniae* (Vollmer & Tomasz, 2000), *Bacillus cereus* (Hayashi *et al.*, 1973), *Listeria monocytogenes* (Boneca *et al.*, 2007) and *L. lactis* (this study). In contrast, a muramidase purified from *Clostridium acetobutylicum* has been found to act on non-acetylated peptidoglycan alone (Croux *et al.*, 1992). Also, the *S. pneumoniae* LytA amidase has higher activity towards non-modified *S. pneumoniae* peptidoglycan with a high degree of deacetylation than towards chemically acetylated peptidoglycan (Vollmer & Tomasz, 2000). In *B. cereus*, glycosidases have been identified with a preference for either peptidoglycan with *N*-unsubstituted glucosamine or peptidoglycan with acetylated aminosugars (Kawagishi *et al.*, 1980).

AcmA consists of two domains: an active site domain and a C-terminal region containing three highly homologous repeats of 45 aa, named LysM domains, which bind peptidoglycan. We found that equal amounts of AcmA C-terminal domain bound to peptidoglycan with the different degrees of de-*N*-acetylation tested (15.8 % in the *pgdA*-overexpressing strain vs 0 % in the *pgdA*-negative mutant). Thus, the reduced autolysis of *L. lactis* cells with an increased degree of peptidoglycan deacetylation does

not seem to result from reduced AcmA binding to the cell wall peptidoglycan substrate. The decreased autolysis therefore probably results from reduced efficiency of cleavage by AcmA of GlcNH₂-MurNAc bonds compared to GlcNAc-MurNAc bonds.

The degree of peptidoglycan GlcNAc deacetylation in *L. lactis* strain IL6288 (9.8 %) was low compared to that observed in *S. pneumoniae* (over 80 % in strain R36A; Vollmer & Tomasz, 2000), *B. cereus* (40–100 %; Hayashi *et al.*, 1973) and *L. monocytogenes* (50 %; Boneca *et al.*, 2007). However, this appears to be sufficient to modulate the sensitivity of *L. lactis* cells to lysozyme and to AcmA autolysis.

The autolysis of *L. lactis* is of special interest with respect to its use as a starter in dairy fermentations. It has been shown that bacterial autolysis during cheese ripening enhances the contribution of intracellular enzymes to cheese flavour formation (Lortal & Chapot-Chartier, 2005). For another proposed application of *L. lactis*, i.e. its use as a delivery vehicle for antigens and therapeutic molecules in the digestive tract of humans and animals (Nouaille *et al.*, 2003), autolysis is also a critical parameter to consider for optimal delivery of molecules (Grangette *et al.*, 2004). The autolytic properties of *L. lactis* have been observed to be strain-dependent (Lortal & Chapot-Chartier, 2005). In this study, we observed that the degree of peptidoglycan deacetylation influences autolytic properties. Variation in the degree of peptidoglycan deacetylation could be one of the factors that contributes to the variability of autolytic properties among natural strains.

Since PGHs are potentially lethal enzymes for the cells that produce them, their activities are thought to be regulated at the post-translational level (Shockman & Höltje, 1994; Smith *et al.*, 2000). This regulation could involve different factors. With respect to AcmA, previous studies have already revealed two mechanisms involved in the control of its activity in *L. lactis* cells. First, AcmA is found to bind to peptidoglycan through its LysM domains at specific loci in the cell wall, around the poles and septum of the cells, whereas AcmA binding on the whole cell surface is hindered by some cell wall constituents, most probably by lipoteichoic acids (Steen *et al.*, 2003). Second, it has been shown that a decrease of the level of D-Ala substitution on lipoteichoic acids leads to increased autolysis through an indirect effect that results from the decreased degradation of AcmA by the extracellular housekeeping protease HtrA (Steen *et al.*, 2005b). The degree of peptidoglycan de-*N*-acetylation could constitute a third mechanism of control of AcmA in cells by decreasing the susceptibility of peptidoglycan to AcmA.

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