

## Inactivation of the Wall-Associated De-*N*-acetylase (PgdA) of *Listeria monocytogenes* Results in Greater Susceptibility of the Cells to Induced Autolysis

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Several species of Gram-positive bacteria have cell wall peptidoglycan (syn. murein) in which not all of the sugar moieties are *N*-acetylated. This has recently been shown to be a secondary effect, caused by the action of a peptidoglycan *N*-acetylglucosamine deacetylase. We have found that the opportunistic pathogen *Listeria monocytogenes* is unusual in having three enzymes with such activity, two of which remain in the cytoplasm. Here, we examine the enzyme (PgdA) that crosses the cytoplasmic membrane and is localized in the cell wall. We purified a hexa-His-tagged form of PgdA to study its activity and constructed a mutant devoid of functional Lmo0415 (PgdA) protein. *L. monocytogenes* PgdA protein exhibited peptidoglycan *N*-acetylglucosamine deacetylase activity with natural substrates (peptidoglycan) from both *L. monocytogenes* and *Escherichia coli* as well as the peptidoglycan sugar chain component *N*-acetylglucosamine, but not with *N*-acetylmuramic acid. As was reported recently [6], inactivation of the structural gene was not lethal for *L. monocytogenes* nor did it affect growth rate or morphology of the cells. However, the *pgdA* mutant was more prone to autolysis induced by such agents as Triton X-100 and EDTA, and is more susceptible to the cationic antimicrobial peptides (CAMP) lysozyme and mutanolysin, using either peptidoglycan muramidases or autolysis-inducing agents. The *pgdA* mutant was also slightly more susceptible than the wild-type strain to the action of certain beta-lactam antibiotics. Our results indicate that protein PgdA plays a protective physiological role for listerial cells.

**Keywords:** *Listeria monocytogenes*, peptidoglycan, autolysis, beta-lactams, *N*-acetylglucosamine deacetylase

*Listeria monocytogenes* is a ubiquitous Gram-positive opportunistic pathogen that causes relatively infrequent but often very serious foodborne infections in humans and animals, such as meningitis, meningoencephalitis, septicemia, and gastroenteritis, as well as abortions [34, 23, 19]. The infections are particularly severe in newborns and immunocompromised individuals and frequently result in death, which makes this bacterium a very dangerous pathogen.

*L. monocytogenes* has received considerable attention, and the impressive amount of data accumulated in recent years has made this bacterium one of the best characterized intracellular pathogens. The accumulated knowledge on the pathogenesis, immunology, and cell biology of *L. monocytogenes* provides an excellent basis for its use in bacto-infection and the expression of plasmid-encoded heterologous proteins in various mammalian cells [27, 36]. *L. monocytogenes* elicits a strong cellular immune response following infection and therefore has potential as a good vaccine vector [14]. However, in view of the possibility of serious infection in certain individuals, a search for highly attenuated strains has been initiated and several candidate strains show promise. One of these is a mutant that requires D-alanine and is unable to grow outside the laboratory, particularly in the cytoplasm of eukaryotic host cells [37].

*L. monocytogenes* induces its own uptake into mammalian cells and then moves within cells and spreads from one cell to another by exploiting actin-based motility [10, 11]. The virulence genes, especially the PrfA-dependent gene cluster and its products, have been well characterized [5]. In *Listeria*, many surface proteins have been predicted, but surface localization has only been experimentally confirmed for a limited number of these [13, 7]. Early studies on the primary composition and molecular ratios of amino acids in the murein of *L. monocytogenes* [15, 20, 35] showed that the structure of this macromolecule is consistent with the A<sub>1</sub> variation, according to the classification of Schleifer

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and Kandler [31]. More recently, modifications in this structure have been described, including de-*N*-acetylation of some of the *N*-acetylglucosamine residues [6], as seen also in other bacteria such as *Streptococcus pneumoniae* and *Bacillus subtilis* [39, 40]. De-*N*-acetylation of murein may be of prime importance for *L. monocytogenes* in avoiding the defense systems of the host [4, 6], since this modification, like *O*-acetylation, has been shown to render murein much less susceptible to lysozyme digestion, even though it appears that for some bacteria neither de-*N*-acetylation nor murein *O*-acetylation are the main mechanisms conferring resistance to the lytic action of this enzyme [9, 18]. It has also been shown that de-*N*-acetylation of murein protects it from the action of the host autolytic enzymes (e.g., in the case of *Lactococcus lactis*).

In 2007, Boneca *et al.* [6] were the first to construct and characterize a peptidoglycan *N*-acetylglucosamine deacetylase mutant and to present the muropeptide composition of *L. monocytogenes* peptidoglycan, which indicated deacetylation of 50% of *N*-acetylglucosamine residues. Their results regarding peptidoglycan composition are in accordance with those obtained in our laboratory (Kloszewska, M. 2003. Structural analysis of the cell wall murein of *Listeria monocytogenes*. Ph.D thesis. University of Warsaw, Poland). Inactivation of *pgdA* revealed the key role of this peptidoglycan modification in bacterial virulence, because the mutant was extremely sensitive to the bacteriolytic activity of lysozyme and was rapidly destroyed within macrophage vacuoles. These findings highlight the central role of peptidoglycan *N*-deacetylation in *Listeria* virulence, through survival in the gastrointestinal tract, in professional phagocytes, evasion of the action of host lysozyme, and in the modulation of the inflammatory response. The observations

of Boneca *et al.* [6] indicate that a major consequence of peptidoglycan *N*-deacetylation is increased survival at early and also later stages of the infection process. Their results conclusively demonstrate that *N*-deacetylation is an important modification of *Listeria* peptidoglycan, which allows this human pathogen to evade the innate immune system.

We have found that the *L. monocytogenes* genome codes for three enzymes with peptidoglycan de-*N*-acetylase activity, two of which are located in the cytoplasm and are essential for the growth and viability of *L. monocytogenes* (submitted). In this study, which complements that of Boneca *et al.* [6], we describe the purification and the characterization of the non-cytoplasmic *N*-acetylglucosamine deacetylase (PgdA) in *L. monocytogenes*.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

*Listeria monocytogenes* strains were grown in Tryptone Soy Yeast Extract Broth (TSYEB; Oxoid) at 37°C with constant shaking (150 rpm) unless otherwise stated, or on plates of Tryptone Soy Yeast Extract Agar (TSYEA; Oxoid). *E. coli* DH5 $\alpha$  and XL-1 Blue MRF' were grown in Luria-Bertani broth [LB or LB agar (1%, w/v)] at 37°C. Ampicillin (100  $\mu$ g/ml) and erythromycin (300  $\mu$ g/ml for *E. coli* and 1–5  $\mu$ g/ml for *L. monocytogenes*) were added to the broth or agar as required. When necessary, IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) (0.1 mM) and X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) (20  $\mu$ g/ml) were spread on agar plates 30 min prior to plating. The bacterial strains and plasmids used in this study are shown in Table 1.

### DNA Isolation and Manipulations

Standard protocols were used for recombinant DNA techniques [29]. DNA fragments used in the cloning procedures and PCR

**Table 1.** Bacterial strains and plasmids used in this study.

Strain	Relevant genotype or phenotype	Reference or source
<i>E. coli</i> TOP 10	F <sup>-</sup> <i>mcrA</i> $\Delta$ ( <i>mrr-hadRMS-mcrBC</i> ) $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74 deoR recA1 araD139 <math>\Delta</math>(<i>araA-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i> (Invitrogen)</i>	Novagen
<i>E. coli</i> DH5 $\alpha$	F <sup>-</sup> $\Phi$ 80d <i>lacZM15</i> ( <i>lacZYA-orgF</i> ) U169 <i>deoR recA1 endA1 hsdR17 phoA supE44</i> $\lambda^-$ <i>thi-1 gyrA96 relA1</i>	Novagen
<i>L. monocytogenes</i> EGD	Wild type	Institut Pasteur (France)
<i>L. monocytogenes</i> MK	<i>pgdA</i> ( <i>lmo0415</i> ) mutant	This study
Plasmids		
pGEM-T Easy	Ap <sup>r</sup> ; 3.0 kb cloning vector with T overhangs	Promega
pAUL-A	Em <sup>r</sup> ; 9.2 kb vector plasmid for insertional mutagenesis; thermosensitive replicon from pE194	[30]
pAUL-A:: <i>lmo0415</i>	Em <sup>r</sup> ; 9.7 kb; 0.516 kb fragment of gene <i>lmo0415</i> , cloned into the EcoRI (MCS) site in pAULA	This study
pBAD/ <i>Myc</i> -HisB	Ap <sup>r</sup> ; 4.1 kb expression vector; <i>araBAD</i> promoter	Invitrogen
pBAD/ <i>Myc</i> -HisB/ <i>lmo0415</i>	Ap <sup>r</sup> ; 1.7 kb PCR product of gene <i>lmo0415</i> <i>L. monocytogenes</i> cloned into the NcoI/EcoRI (MCS) site in pBAD/ <i>Myc</i> -HisB	This study

MCS, multiple cloning site.

products were isolated from agarose gels with the DNA Gel-Out extraction kit (A&A Biotechnology). Plasmid DNA from *E. coli* was isolated and purified with the Plasmid Miniprep Plus kit (A&A Biotechnology). The isolation of plasmid and chromosomal DNA from *L. monocytogenes* was performed as previously described [22], starting with digestion of the bacterial cell wall with 5–10 mg/ml in Glucose–Tris–EDTA (GTE) buffer for 1 h at 37°C.

### Construction of the *pgdA* Mutant

The *pgdA* (*lmo0415*) gene was inactivated by insertion duplication mutagenesis (by single crossing-over plasmid integration). An internal fragment of the gene was amplified from the chromosomal DNA of *L. monocytogenes* EGD by PCR using *Taq* polymerase and the primers *Plmo0415* (5'-GCA TAG AAT GGC CGA GCA AG-3') and *Llmo0415* (5'-AAG TAC GCC TGG TGT TAC CG-3'). *Taq* polymerase was chosen because of the short length of the fragment amplified (516 bp). The PCR was performed in a total volume of 50 µl with 20 ng of template DNA, 50 pmol of each primer, and 1 unit of *Taq* DNA polymerase, using the following thermocycle: 94°C for 5 min, then 30 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 7 min. After purification using a Promega DNA purification kit, the amplified 516 bp fragment was cloned into the pGEM-T Easy vector (Promega)

to produce plasmid pGEM1 and its identity was confirmed by sequencing. Plasmid pGEM1 was digested with *EcoRI*, and the released insert was isolated following agarose gel electrophoresis using the DNA Gel-Out extraction kit (A&A Biotechnology). The fragment was ligated with pAUL-A (*Em*<sup>r</sup>) (pre-digested with *EcoRI*) [28, 30] and the obtained construct, named pAUL-A::*lmo0415*, was verified by restriction analysis. This plasmid was introduced into *L. monocytogenes* cells by electroporation at 30°C. Since *L. monocytogenes* has a thick cell wall containing teichoic acids linked to murein, electrocompetent cells were prepared in the presence of penicillin, as described by Park and Stewart [26]. Following electrotransformation, colonies of *L. monocytogenes* EGD resistant to 1 µg/ml erythromycin (plasmid marker) were selected. Transformants were grown in TSYEB containing 1 µg/ml erythromycin at 30°C for 12 h. The cultures were diluted 1/100 into fresh TSYEB containing erythromycin (2 µg/ml) at 42°C and incubated overnight. These cultures were then spread onto plates containing erythromycin and incubated at 42°C for 12 h. Single colonies lacking the autonomous form of the plasmid were obtained, in which, as a result of homologous recombination (single “crossing-over”), the plasmid had been incorporated into a specific site on the chromosome. The site-specific insertion of pAUL-A::*lmo0415* in the chromosome of *L. monocytogenes* was confirmed by PCR and Southern blot hybridization (data not shown) and the

### A

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1 - MKIRWIRLSL VAILLIAVVF IGVIGFQKYQ FSKSRNKVIM QMDRLMKDQD GGNFRRLDKK
61 - ENGVEIISYI PKTTEKKDNE IIQKEIGKAT DAEVKKLNRD KETQGIIFYT YQKHRMAEQE
121 - ISYKAVQSEY VKEGRTKFVL KDKKDICKNI VTDAETGALL TLGEVLIKSN QTKLNLKTAV
181 - EEELIKTGDF SLKDVGNL GK IKSLVKWNQT DFEITNSEII LPVKIPGAPE PKKVKVKLAD
241 - IASSVNKRYL PSSVKVPEVP KAKTNKRIAL TFDDGPSSSV TPGVLDTLKR HNVKATFFVL
301 - GSSVIQNPGL VKRELEEGHQ VGSHSWDHPQ LTKQSTQEVY NQILKTQKAV FDQTYGFPTT
361 - MRPPYGAVNK QVAEEIGLPI IQWSVDTE DW KYRNAGIVTK KVLGATDGA IVMHDIHKT
421 - TAASLDTTLT KLKSQGYEFV TIDELYGEKL QIGKQYFDKT DSRMVK

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### B

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1. Q8Y9V5 262 KRIALTFDDGPSSVTPGVLDTLKRHNVKATFFVLGSSVIQNPGLVKRELEEGHQVGC 323
2. Q92EM2 262 KRIALTFDDGPSSVTPGVLDTLKRHNVKATFFVLGSSVIQNPGLVKRELEEGHQVGC 323
3. A0AFL4 262 KRIALTFDDGPSSVTPGVLDTLKRHNVKATFFVLGSSVIQNPGLVKRELEEGHQVGC 323
4. Q99Z68 237 KIYALTFDDGPPTTTPQVLDLFAKQAKGTFMIGSKVNNENITKRVSDAGHETA 298
5. O34798 274 KVIALTFDDGPPATTNQILDSLKKYKGATFFVLGSRVQYYEILLRMLKEGNEVG 335
6. Q97PW1 264 KVIALTFDDGPPATTPQVLETLAKNDIKATFFVLGKNVSGNEDLVKRIKSEGHVVG 325
7. Q9CIS3 172 KRIALTFDDGPPNTTPQLLKIESDAQVPATFFALGQAQACQIILKEADRGNEVA 233

1. Q8Y9V5 330 HSWDHPQLTKSTQEVYNQILKQKAVFQTGYFPTTMRPPYGAVNKAAEEIGLPII 381
2. Q92EM2 330 HSWDHPQLTKSTQEVYNQILKQKAVFQTGYFPTTMRPPYGAVNKAAEEIGLPII 381
3. A0AFL4 330 HSWDHPQLTKSTQEVYNQILKQKAVFQTGYFPTTMRPPYGAVNKAAEEIGLPII 381
4. Q99Z68 305 HTWDHFNLLIN SVSEIQHOVNMNQAI EACGKKPRYL RPPYGATNAQQSSGLTQM 356
5. O3479 342 HSWSHPILLTR SVKEALKQINDTQDIIEISGYRPTLV RPPYGCINDRSQMKMDVA 393
6. Q97PW1 332 HSWSHPILSQ SLDEAKKQITDIEDVLT VLGSSSKIMRPPYGATIDRNSLDLSFI 383
7. Q9CIS3 240 HTWDHKDLVT SPDQKQIEESANQLINITGKNVTLFRPPYGSYKNSVLSQTNLS 291

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**Fig. 1. A.** Amino acid sequence of Lmo0415 protein; region of signal sequence is shown in bold; conserved domain polysaccharide deacetylase (pfam01522.13) is bold and shaded in grey. **B.** Primary sequence alignment of representative members of the CE4 family enzymes; catalytic domain characteristic Polysaccharide deacetylase (NCBI Conserved Domain).

The black shades and white letters are identical residues, dark gray shades and white letters are considerable residues (more than 60% of similarity), and gray shades are poorly conserved residues (up to 60% of similarity). 1, *L. monocytogenes* EGD; 2, *L. innocua*, 3, *L. welshimerii* (ATCC 35897); 4, *Streptococcus pyogenes* serotype M1; 5, *Bacillus subtilis*; 6, *Streptococcus pneumoniae*; 7 *Lactococcus lactis*.

strain obtained was designated MK. The nonpolar effect of insertion into *lmo0415* (mutant MK) was confirmed by RT-PCR using primer pairs specific for downstream/upstream genes.

### Expression and Purification of His-Tagged PgdA (Lmo0415) Protein in *E. coli*

The PgdA (Lmo0415) protein with a C-terminal hexa-His-Tag was expressed in *E. coli* TOP10 using expression vector pBAD/*Myc*-HisB (Amp<sup>r</sup>; Invitrogen). A 1,680 bp fragment carrying the entire PgdA ORF was PCR-amplified using primers hisL415 (5'-GGTGCCATGGATAATGTGAAAATTAGGTGG-3') and hisP415 (5'-CCGAATTCCTTTCACCATTCTTGAATCTGTI-3'). The left primer was designed such that the obtained PCR product contained the start codon ATG as well as a sequence cleaved by the restriction enzyme NcoI (underlined). The right primer was designed to introduce a EcoRI restriction site (underlined) and no translation terminator sequence. The PCR was performed in a total volume of 50  $\mu$ l with 20 ng of template DNA, 50 pmol of primers, and 2 units of Pfu DNA polymerase using the following thermocycle: 94°C for 5 min, then 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min, and a final extension step at 72°C for 7 min. The amplified fragment was digested with NcoI/EcoRI as was the expression vector pBAD/*Myc*-HisB. Ligation of the fragment and vector produced a construct named pBAD/*Myc*-HisB/*lmo0415*, subsequently used for the production of PgdA-His6 with a histidine tag at its C terminus. The cloned insert (1,680 bp) was sequenced and found to be identical to the Lmo0415 coding region (GenBank Accession No. AL591975).

LB medium containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 100  $\mu$ g/ml ampicillin was inoculated with *E. coli* TOP10 carrying pBAD/*Myc*-HisB/*lmo0415* that had been grown overnight in medium supplemented with glucose to 0.4% (w/v). The culture was incubated at 37°C to an OD<sub>600</sub> of 0.6 and then expression was induced by the addition of arabinose to 0.1%. The culture was transferred to 30°C for the period of expression (2 h). A culture of *E. coli* TOP10 containing the empty pBAD/*Myc*-HisB vector was treated in the same way as a control. The cultures were harvested by centrifugation and the bacterial pellet was resuspended in 10 ml of a buffer containing 50 mM NaHPO<sub>4</sub>, 500 mM NaCl, 20 mM imidazole, 10 mM  $\beta$ -mercaptoethanol, 0.1% Tween 20, and 100 mM PMSF. The cells were then disrupted by sonication in ice in a VCX-600 ultrasonicator (Sonics and Materials, U.S.A.) at an amplitude of 20%. After sonication, the cellular debris was pelleted by centrifugation at 40,000  $\times$ g for 1 h and the supernatant was loaded onto a 5 ml Ni-NTA Agarose column (Qiagen) that had been equilibrated with 100 ml of the above buffer. The column was washed with 100 ml of buffer containing 50 mM NaHPO<sub>4</sub>, 500 mM NaCl, 40 mM imidazole, and 10% glycerol. The proteins were then eluted with increased (50 mM increments) concentrations of imidazole (50 mM to 1 M in 50 mM increments). Fractions were collected and analyzed by SDS-PAGE using a 12% (w/v) polyacrylamide separating gel. The PgdA-His6 protein eluted at an imidazole concentration of 500 mM. Following the addition of glycerol to a final concentration of 10% (v/v), all fractions were stored at -20°C. The total yield of PgdA-His6 was ~4 mg/l of induced culture.

### Western Blot Detection

Purified PgdA-His6 protein was separated on a 12% SDS-PAGE gel, and electrophoretically transferred (30 mA; overnight) to a polyvinylidene difluoride membrane (BioRad ImmunBlot PVDF Membrane;

0.2  $\mu$ m). The membrane was blocked for 1 h with 3% (v/v) skimmed milk in PBS and then incubated for 1 h in TBS/Tween buffer containing antibody Ni-NTA conjugated with alkaline phosphatase (dilution 1/1,000; Qiagen) at room temperature. After a brief wash with TBS/Tween buffer, the PVDF membrane was treated for 5–15 min in the dark with the chromogen NBT/BCIP (nitroblue tetrazolium/bromochloroindolyphosphate). The reaction was terminated after the appearance of a brown band, by washing the membrane in distilled water.

### Cell Wall Isolation

Cell of *Escherichia coli* DH5 $\alpha$ , and *L. monocytogenes* EGD and MK were harvested by centrifugation as above and the cell pellets resuspended in 1/40 of the original culture volume of ice-cold saline. Glass beads (diameter 150–215  $\mu$ m; Sigma) were added (1 g per 1 ml of cell suspension) and the cells were disrupted by ten 1-min periods of sonication in a VCX-600 ultrasonicator (Sonics and Materials, U.S.A.) at an amplitude of 20%. The crude cell wall preparations were sedimented by centrifugation in a Beckman ultracentrifuge (25 min, 100,000  $\times$ g at 4°C). Depending on the nature of the experiments, the cell walls were either washed in the appropriate buffer and resuspended in the same, or treated with 4% SDS (final concentration).

### Purification of Murein

Each cell pellet was resuspended in ice-cold water and added dropwise to the same volume of boiling 8% (w/v) SDS with vigorous stirring throughout [8, 16]. Boiling was continued for 30 min and the suspension was left at room temperature overnight. Sacculi were collected by centrifugation (30 min, 150,000  $\times$ g at 22°C) and the pellet was washed five times with distilled water at room temperature. After each wash, the pellet was resuspended homogeneously and centrifuged as before. The SDS-free pellet [17] was resuspended in 49% (v/v) hydrofluoric acid and incubated for 40 h with stirring in an ice bath to remove teichoic acid. The murein was recovered by centrifugation and washed repeatedly in water as above to remove all hydrofluoric acid. The murein was resuspended in 10 mM Tris-HCl buffer (pH 7.0) and treated with  $\alpha$ -amylase (100  $\mu$ g/ml) for 2 h at 37°C, and then pre-digested pronase E (200  $\mu$ g/ml) was added and the incubation was continued for 90 min at 60°C [16]. Finally, the sample was mixed with 8% SDS and incubated for 15 min at 100°C. SDS was removed by washing in distilled water and centrifuging as described above. *N*-Acetylation of murein was performed with acetic anhydride in the presence of NaHCO<sub>3</sub> as described by Hayashi *et al.* [17]. Murein and muropeptide concentrations were calculated from their diamino acid content. Samples were hydrolyzed in 6 N HCl (12 h, 105°C), vacuum dried, and resuspended in an appropriate volume of distilled water. *N*-Acetylated murein prepared from wild type and the mutant was analyzed by HPLC-MS/MS after digestion with the muramidase mutanolysin M1 or lysozyme (Sigma-Aldrich). The concentration of murein was 1 mg/ml and that of the muropeptides was in the range of 0.2–0.5 mg/ml.

### Assay for Deacetylase Activity

Deacetylase activity of the recombinant PgdA-His6 protein was determined by its ability to release acetic acid from the substrate in a spectrophotometric assay (Ultraspec 2100pro spectrophotometer; Amersham Biosciences). Mureins derived from *E. coli* DH5 $\alpha$ , and *L. monocytogenes* EGD and MK cell walls (1/mg/ml) were used as substrates. Murein suspensions with an OD<sub>600</sub> of 0.8 were prepared

in a 20 mM Tris-HCl (pH 7.5). Purified PgdA-His6 was added to a final concentration of 10 µg/ml. The PgdA-His6 protein was omitted from no-enzyme control reactions. All reactions were supplemented with albumin and sodium azide to final concentrations of 0.1% and 0.02%, respectively. After incubation at 37°C for 4 h, the reactions were centrifuged and the supernatants collected, and the acetic acid released by deacetylase activity was measured spectrophotometrically with an F-kit assay (Roche).

In a separate experiment, the activity of lysozyme against murein treated with purified PgdA-His6 compared with untreated murein was examined. Murein was treated with the recombinant deacetylase as described above and after centrifugation was resuspended in 50 mM potassium phosphate (pH 6.3). Lysozyme was added to a final concentration of 20 µg/ml and the samples were incubated with shaking for 4 h at 37°C. Changes in absorbance were monitored at 600 nm.

In additional experiments intended to determine the substrate specificity of the deacetylase, *N*-acetylglucosamine, *N*-acetylmuramic acid, and muropeptides (see "Purification of Murein" above) were treated with PgdA-His6. The products of these reactions were analyzed by HPLC with tandem mass spectrometry (HPLC-MS/MS).

#### HPLC-MS/MS Analysis

Stock solutions of *N*-acetylglucosamine, *D*-glucosamine and *N*-acetylmuramic acid (Sigma-Aldrich) were prepared in redistilled water (2 mg/ml). The concentration of the muropeptide preparations was 0.2 mg/ml. All the solutions were stored at -20°C and used within one week. For all analyses, 20 µl of each substrate was taken and PgdA-His6 was added to a final concentration of 10 or 20 µg/ml. The no-enzyme control reaction was the same volume of substrate solution with the addition of water or buffer instead of PgdA-His6. The final volume of each sample was 180 µl. After incubation at 37°C for 4 or 8 h, the reactions were centrifuged, and the supernatants collected and analyzed by HPLC-MS/MS.

The Shimadzu LC system consisted of binary pumps LC20-AD, degasser DGU-20A5, column oven CTO-20AC, autosampler SIL-20AC, and UV detector SPD 20A (205 nm), and this was connected to a 3200 QTRAP Mass spectrometer (Applied Biosystem/MDS SCIEX). The MS system equipped with an electrospray ionization source (ESI) operated in positive-ion mode and with a quadrupole mass analyzer in scan mode from 50 to 500 *m/z*. The same ESI conditions were used throughout the analysis: capillary temperature of 200°C, curtain gas at 0.3 MPa, auxiliary gas at 0.3 MPa, discharge current of 3 µA, and positive ionization mode source voltage of 5.5 kV. Nitrogen was used as both the curtain and auxiliary gas. Compounds for HPLC-MS were separated on a Zorbax SB C-18 column (50×4.6 mm, 1.8 µm; Agilent) with a mobile phase consisting of 2 mM formic acid-methanol. The use of a short column and small particle size allowed the separation of the studied compounds in less than 15 min. Samples were infused into the electrospray source *via* a 50 µm i.d. PEEK capillary using a Harvard Apparatus pump at 10 µl/min. Continuous mass spectra were obtained by scanning *m/z* from 50 to 500. Gradient elution was performed by increasing the concentration of methanol in the mobile phase: time 3 min, 3% MeOH; time 15 min, 15% MeOH and 15% MeOH followed by 9 min for stabilization in the initial conditions. The flow rate was 0.3 ml/min at 55°C. Compounds were identified by comparing retention times and *m/z* values obtained by MS and MS<sup>2</sup> with the mass spectra of standards separated under the same conditions.

#### Induced Cell Lysis

Cultures of *L. monocytogenes* strains were grown to mid-exponential phase (OD<sub>600</sub> of 0.80) in TSYEB. Cells were harvested by centrifugation and resuspended in 25 mM phosphate buffer (pH 6.4) that had been pre-warmed to 37°C. To determine the effect of muramidase-induced lysis, lysozyme (10 µg/ml) or mutanolysin (40 µg/ml) was added. To determine the effect of Triton X-100 or EDTA, the cells were grown to an OD<sub>600</sub> of 0.6, harvested, and resuspended in 50 mM Tris-HCl (pH 7.5) containing 0.1% (v/v) Triton X-100 or 1 mM EDTA. Incubation was continued at 37°C with shaking, and lysis of the cell suspensions was monitored spectrophotometrically (Novaspec II spectrophotometer LKB-13; Pharmacia) at 600 nm.

#### Cell Lysis During Growth

Cultures of mutant and wild type of *L. monocytogenes* were grown to early exponential phase (OD<sub>600</sub> of 0.20) in TSYEB. Lysozyme was then added to 10 µg/ml and the lysis of the cultures was monitored spectrophotometrically as described above.

#### Susceptibility to Antimicrobial Compounds

The susceptibility of the *L. monocytogenes* strains to antimicrobial compounds was tested by the disc diffusion method using a panel of antibiotics: ampicillin - Amp (10 µg per disc), penicillin - P (10 µg), oxacillin - OX (5 µg), tetracycline - TE (5 µg), imipenem (10 µg), cephalotin - KF (30 µg), cefotaxime - CTX (30 µg). Cultures were grown in TSYEB at 37°C and diluted in the same broth to an OD<sub>600</sub> of ~0.1. The diluted *L. monocytogenes* inoculum (100 µl) was spread on TSYEA plates. Antibiotic discs were placed on the inoculated plates and these were then incubated for 24 h at 37°C. The diameters (in millimeters) of the clear zones of growth inhibition around the discs (including the 6-mm disc diameter) were measured using precision calipers [25].

#### Minimal Inhibitory Concentration (MIC) Determinations

MICs of the antibiotics ampicillin, penicillin, oxacillin, tetracycline, cephalotin, and cefotaxime (Sigma) for the *L. monocytogenes* strains were determined by the broth microdilution method of the National Committee for Clinical Laboratory Standards [25]. The final concentrations of the antibiotics were between 0.039 and 5 µg/ml. Each MIC determination was repeated at least two times.

## RESULTS

#### Analysis of the *L. monocytogenes* *pgdA* Sequence

The gene *pgdA* (*lmo0415*) encodes a protein of 466 amino acid residues, with a *pI* of 10.07 and mass of 52,495 Da, carrying a putative N-terminal signal peptide, predicted by the ExpASY Proteomics server (DAS, PSORT, and SignalP); (<http://www.expasy.org>). Putative open reading frames were examined using the Clone program (Sci Ed Software) and deduced amino acid sequences were compared with the NCBI Entrez protein databases by BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequence analysis of *lmo0415* (DAS and TMHMM programs) indicated a potential 22 amino acid signal sequence in the N-terminal

region of this protein between residues 5 and 26: N-WIRLSLVAILIIVVFIGVIGF-C. The presence of this type of sequence suggests that this protein is exported from the cytoplasm and processed by a signal peptidase, so it could be localized in the cell wall. Sequence comparison analysis of Lmo0415 revealed the presence of a conserved C-terminal domain characteristic of polysaccharide deacetylases. This domain is found in polysaccharide deacetylase. This family of enzymes includes *Rhizobium* NodB (nodulation protein homolog B), which is a chitooligosaccharide deacetylase, chitin deacetylase from yeast, and endoxylanases that hydrolyze glucosidic bonds in xylan. The highest identity exhibited by the PgdA protein was to analogous domains detected in homologous proteins (NCBI Conserved Domain). Among these homologs were proteins from other *Listeria* species (Fig. 1B). The construction of a model of the conserved domain of PgdA (modeled residue range: 263–461 from 466 residues), based on template 2c1gA (1.75 Å) using the server Swiss Model (<http://swissmodel.expasy.org>) [1, 21, 33], demonstrates its significant similarity to other proteins of this type belonging to the family CE4 (Fig. 2).

#### Role of De-N-acetylation in Cell Separation and Autolysis

The growth rates of wild-type *L. monocytogenes* EGD and mutant MK in TSYEB were identical at the various temperatures tested, indicating that inactivation of *pgdA* has no impact on bacterial growth rate (Fig. 7A). No differences between the wild-type and mutant were detected with respect to hemolytic activity on blood agar plates, growth in “MICROBAT – *Listeria* Identification System 12L” (Oxoid), or on Oxoid Chromogenic *Listeria* Agar



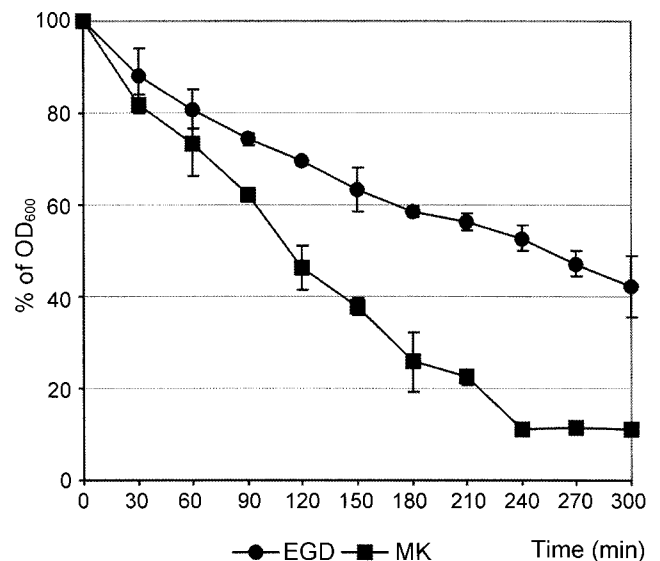
**Fig. 2.** Model of the conserved domain of protein Lmo0415 generated with the use of the Swiss Model server (<http://swissmodel.expasy.org>). Modeled residue range: 263 to 461 from 466 residues, based on template 2c1gA (1.75 Å).

(OCLA). Cells of the EGD and MK strains were also examined by light and electron microscopies when the cultures were in the logarithmic and stationary phases of growth ( $OD_{600}$  of 0.8 and 1.5, respectively). These microscopic observations revealed the similar morphology and size of the bacterial cells (data not shown).

Triton-X-100- or EDTA-induced lysis was examined at log-phase after transferring the bacterial cells ( $OD_{600}$  of 0.80) to pre-warmed ( $37^{\circ}\text{C}$ ) 50 mM Tris-HCl (pH 7.5), containing 0.1% (v/v) Triton X-100 or 1 mM EDTA. The rate of autolysis of MK cells was significantly faster than that of the wild-type cells, with the difference being much clearer in the case of EDTA. At the end of the experiment with EDTA as the inducing agent, the fall in optical density of the cell suspension was 88% for mutant MK, compared with 65% for the wild type (Fig. 3), and in the case of Triton X-100, the respective values were 90% and 82%, respectively (Fig. 4).

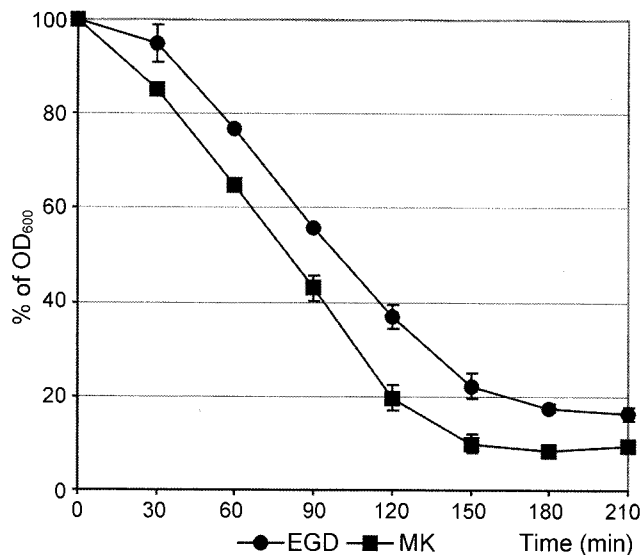
Lysozyme- or mutanolysin-induced cell lysis was examined after transferring the bacterial cells ( $OD_{600}$  of 0.80) to 25 mM phosphate buffer (pH 6.4) pre-warmed to  $37^{\circ}\text{C}$  and supplemented with lysozyme (10  $\mu\text{g}/\text{ml}$ ) or mutanolysin (40  $\mu\text{g}/\text{ml}$ ).

The rate of lysis induced by either lysozyme or mutanolysin was faster in the MK cells from the very start of the experiment. After 15 min in the presence of lysozyme, the optical density of the MK cell suspension had dropped by 40%, compared with 16% for the parental cells. The greatest difference between the optical densities of the mutant and wild-type cell suspensions was observed in the



**Fig. 3.** EDTA-stimulated autolysis of *L. monocytogenes* EGD and mutant MK.

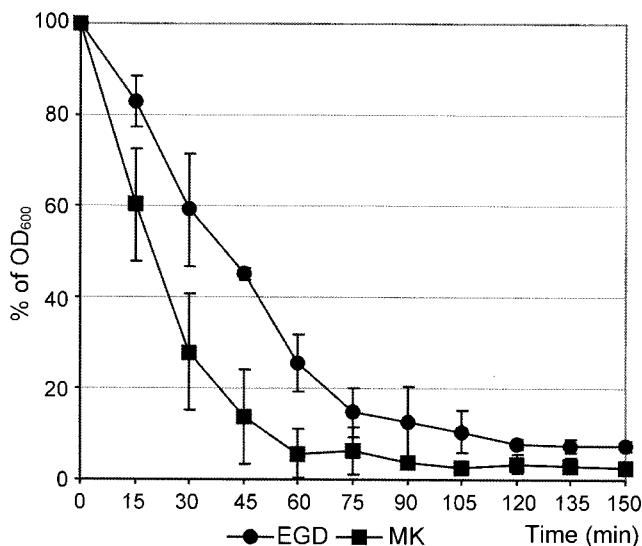
Cells were grown in TSB to  $OD_{600}$  0.80, and then harvested, washed, and resuspended at  $OD_{600}$  0.80 in 50 mM Tris-HCl (pH 7.5) with 1 mM EDTA, pre-warmed to  $37^{\circ}\text{C}$ . Autolysis was measured as a decline in optical density. The results are means from three independent experiments.



**Fig. 4.** Triton-X-100-stimulated autolysis of *L. monocytogenes* EGD and mutant MK.

Cells were grown in TSB to OD<sub>600</sub> 0.60, and then harvested, washed, and resuspended at OD<sub>600</sub> 0.80 in 50 mM Tris-HCL (pH 7.5) with 0.1% (v/v) Triton X-100, pre-warmed to 37 °C. Autolysis was measured as a decline in optical density. The results are means from three independent experiments.

45<sup>th</sup> minute of the experiment, when the decreases in optical density were about 85% and 55%, respectively. The induced lysis of mutant MK terminated at around the 60<sup>th</sup> minute when the same optical density was approximately 10% of the original value, whereas in the case of *L. monocytogenes* EGD, an equivalent optical density was



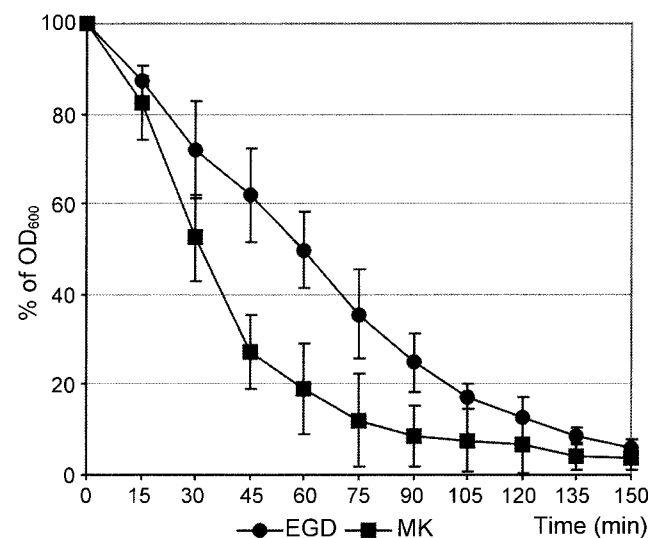
**Fig. 5.** Lysozyme-stimulated autolysis of *L. monocytogenes* EGD and mutant MK.

Cells were grown in TSB to OD<sub>600</sub> 0.60, and then harvested, washed, and resuspended at OD<sub>600</sub> 0.80 in 25 mM phosphate buffer (pH 6.4), pre-warmed to 37 °C. To determine the effect of muramidase-induced lysis, lysozyme (10 µg/ml) was added. Autolysis was measured as a decline in optical density. The results are means from three independent experiments.

observed only after 120 min of treatment (Fig. 5). When lysis of the cells was induced by mutanolysin, the optical density had dropped by 50% after 30 min in the case of mutant MK, compared with 28% for the parent. As for the lysozyme treatment, the greatest difference between the optical densities of the two cell suspensions (around 35%) was observed in the 45<sup>th</sup> minute. In the case of mutant MK, lysis terminated after about 105 min, whereas the same optical density (10% of the original value) was achieved about 30 min later (Fig. 6) for the parent strain.

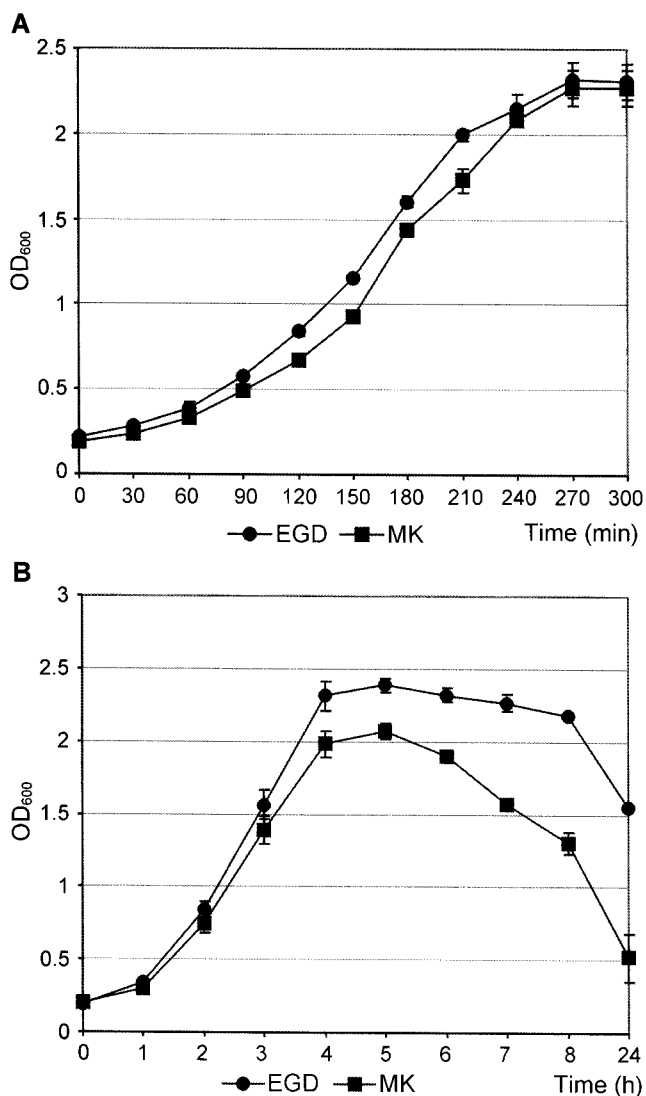
Cell lysis during growth in TSYEB was determined by the addition of lysozyme (10 µg/ml) to cultures of the mutant and wild-type *L. monocytogenes* strains in early exponential phase (OD<sub>600</sub> of 0.20). In general, the cells of strain MK were more susceptible to lysozyme and the difference in optical density was most pronounced when the culture entered the stationary phase of growth, reaching an OD<sub>600</sub> of 0.9 (Fig. 7B).

In addition, samples were taken every hour during the growth of the cultured samples and plated on solid TSYEA. The colonies were counted after 24 h incubation at 37 °C to compare the number of cells of the parent strain EGD and the mutant MK that survived the action of lysozyme (Fig. 8). The results confirmed the earlier observations presented in Fig. 7B, the number of EGD and MK cells in the logarithmic phase of the culture in the presence of lysozyme was the same, but when the cultures reached stationary phase, the number of MK cells showed a slight drop compared with the wild-type cells, confirming the greater susceptibility of the mutant to lysozyme.



**Fig. 6.** Mutanolysin-stimulated autolysis of *L. monocytogenes* EGD and mutant MK.

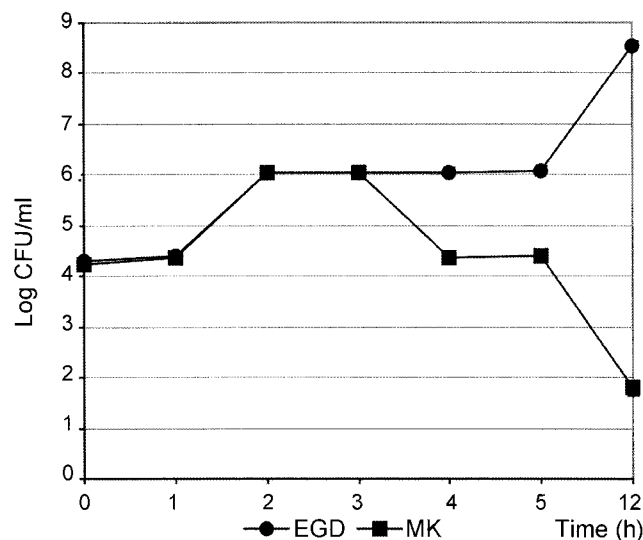
Cells were grown in TSB to OD<sub>600</sub> 0.60, and then harvested, washed, and resuspended at OD<sub>600</sub> 0.80 in 25 mM phosphate buffer (pH 6.4), pre-warmed to 37 °C. To determine the effect of muramidase-induced lysis, mutanolysin (40 µg/ml) was added. Autolysis was measured as a decline in optical density. The results are means from three independent experiments.



**Fig. 7.** A. Growth kinetics in broth at 37°C of *L. monocytogenes* EGD and MK. B. Growth of the *L. monocytogenes* EGD and MK mutant in the presence of lysozyme. Cells were grown to early exponential phase (OD<sub>600</sub> 0.20) in TSB. To determine the effect of lysozyme-induced lysis, 10 µg/ml was added. Growth was measured as a decline in optical density. The results are means from three independent experiments.

**Susceptibility to Antimicrobial Compounds**

The susceptibility of the *L. monocytogenes* strains to antibiotics was tested by the disc diffusion method and



**Fig. 8.** Survivability of the *L. monocytogenes* EGD and MK mutant in the presence of lysozyme. During the course of the culture (Fig. 7B), samples were taken every hour and plated on solid TSB medium. Colonies were counted after 24 h incubation at 37 °C and the results obtained allowed comparing the number of cells of the parental strain EGD and mutant MK that survived the action of lysozyme. The results are means from three independent experiments.

MIC determinations. Differences were observed for only 3 beta-lactam antibiotics and these pointed to the slightly greater susceptibility of the mutant MK compared with the parental strain EGD (Table 2).

**PgdA Purification and Activity Determination**

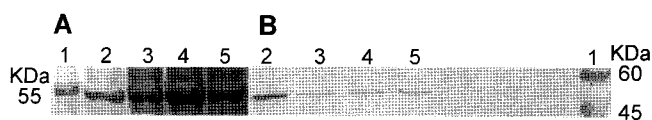
Protein PgdA with a C-terminal hexa-His-tag was expressed in *E. coli* TOP10, purified on a Ni-NTA agarose column (Qiagen), and eluted with 500 mM imidazole. The concentration of the purified protein was 4 mg/ml. Analysis of fractions by SDS-PAGE using 12% (w/v) polyacrylamide separating gels demonstrated a major protein of approximately 52 kDa, which was shown to be PgdA-His6 by Western Blot detection (Figs. 9A and 9B).

To confirm the enzymatic activity of PgdA-His6, reactions were performed in which the substrate was murein isolated from either *L. monocytogenes* EGD or mutant MK. Peptidoglycan de-N-acetylase activity removes the acetyl groups from N-acetylglucosamine, resulting in the release of acetic acid into the reaction mixture, which can be

**Table 2.** Susceptibility of *L. monocytogenes* EGD and mutant MK to select beta-lactam antibiotics.

Antibiotic	Drug code	Disc drug concentration (µg)	Diffusion zone breakpoint (mm)/MIC of antibiotics (µg/ml)	
			<i>L. monocytogenes</i> EGD	<i>L. monocytogenes</i> MK
Ampicillin	AMP	10	10.9/0.156	12.9/0.078
Penicillin	P	10	12.4/0.078	14.8/0.039
Oxacillin	OX	5	8.8/2.5	12.9/1.5

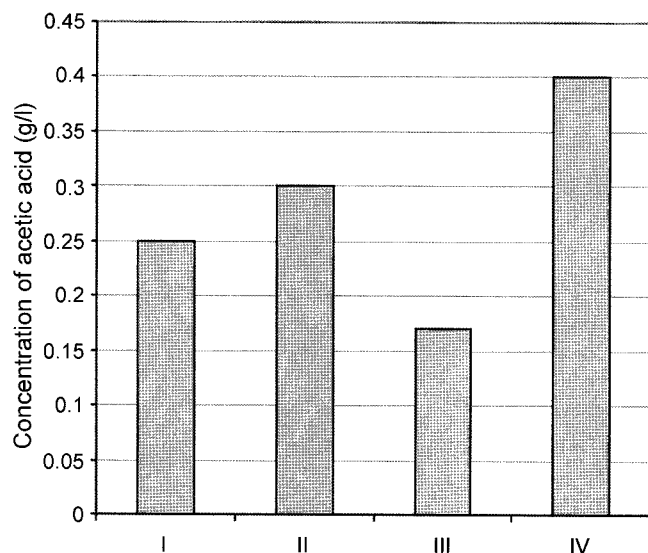




**Fig. 9.** **A.** Pgda-His6 (pBAD/Myc-HisB/lmo0415) separated on 12% SDS-PAGE gel, stained with Coomassie blue. **B.** Western blot of purified Pgda-His6.

Lane 1, molecular weight markers (in kDa) standard; lanes 2–5, fractions after purification on a Ni-NTA agarose column.

detected with an acetic acid determination kit (Roche). The data presented in Fig. 10 confirm the increased concentration of acetic acid after murein preparations had been incubated with Pgda-His6, compared with the control without enzyme. A particularly significant increase in the concentration of acetic acid was observed when murein from mutant MK was the substrate. Compared with the no-enzyme control, an increase from 0.17 g/l to 0.4 g/l was detected; that is, 2.35-fold. This result also confirmed the absence or very low level of de-*N*-acetylated sugar moieties in the murein of mutant MK. Roughly half of the glucosamine moieties in the murein of wild-type *Listeria monocytogenes* are deacetylated, so there would be less substrate for the enzyme on a weight basis compared with the murein of mutant MK. Consequently, the murein of mutant MK treated with purified Pgda-His6 would be expected to release more measurable acetic acid than the wild-type murein, which is exactly what was found.



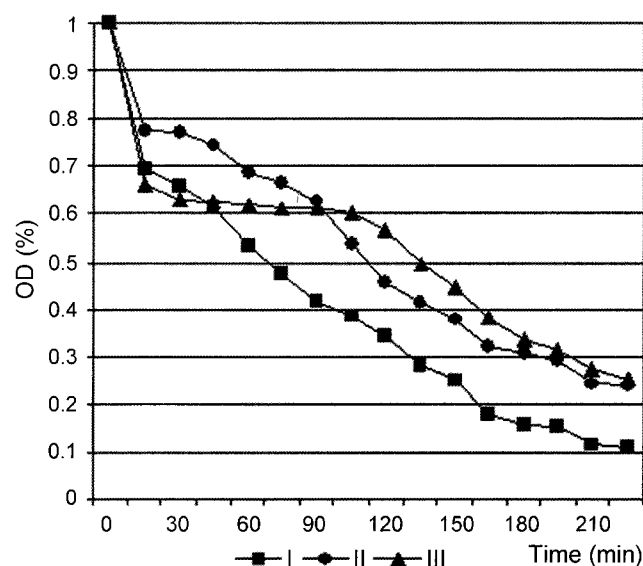
**Fig. 10.** Acetic acid content in different murein samples.

**I,** murein of *L. monocytogenes* EGD; **II,** murein of *L. monocytogenes* EGD after incubation with purified Pgda-His6 enzyme; **III,** murein of *L. monocytogenes* MK (Pgda-); **IV,** murein of *L. monocytogenes* MK (Pgda-) after incubation with purified Pgda-His6 enzyme. Murein derived from *L. monocytogenes* EGD and MK cell walls (1 mg/ml) were used as substrates, and purified Pgda-His6 was added to a final concentration of 10 µg/ml. The control was murein without the addition of Pgda-His6. After incubation at 37°C for 4 h, acetic acid released from the substrates by deacetylase activity (supernatants) was measured with an F-kit (Roche).

An additional experiment was carried out to confirm the deacetylase activity of Pgda-His6. Purified murein (1 mg/ml) from *Escherichia coli* DH5α, which is practically 100% *N*-acetylated, was incubated overnight at 37°C with Pgda-His6, at 10 µg/ml. Three parallel reactions were run: a and b, with addition of protein Pgda-His6 obtained from two independent purifications on Ni-NTA agarose columns; and c, (control), without the acetylase. Lysozyme was then added at 20 µg/ml to each reaction and incubation was continued. At every 15 min for 4.5 h, the absorbance of the samples at 600 nm was measured. As shown in Fig. 11, the absorbance of the preparations of murein digested with lysozyme following treatment with protein Pgda-His6 was about 10% higher than that of the no-enzyme sample. As expected, the enzymatic activity of Pgda-His6, which involves the removal of acetyl groups from *N*-acetylglucosamine, had decreased the degree of *N*-acetylation in the *E. coli* murein and reduced its susceptibility to lysozyme.

The substrate specificity of purified Pgda-His6 was then determined by analyzing its action against *N*-acetylglucosamine, *N*-acetylmuramic acid, or mucopeptides by HPLC-MS/MS. Pgda-His6 (or water/buffer as a control) was added to each substrate solution, and after incubation at 37°C for 4 or 8 h, the reaction products in the supernatants were analyzed by HPLC-MS/MS.

It has been demonstrated that optimization of the ESI parameters plays a key role in the achievement of adequate



**Fig. 11.** Lysozyme digestion of *E. coli* murein.

**I,** *E. coli* murein digested with lysozyme; **II,** *E. coli* murein incubated with purified enzyme Pgda-His6 (1), followed by digestion with lysozyme; **III,** *E. coli* murein incubated with purified enzyme Pgda-His6 (2), followed by digestion with lysozyme. *E. coli* murein was treated with the deacetylase (as described in Fig. 10) and then lysozyme was added to a final concentration of 20 µg/ml and the samples were incubated with shaking for 4 h at 37°C. Changes in absorbance were followed at 600 nm. (1) or (2) mean two different, independent purifications of enzyme Pgda-His6.

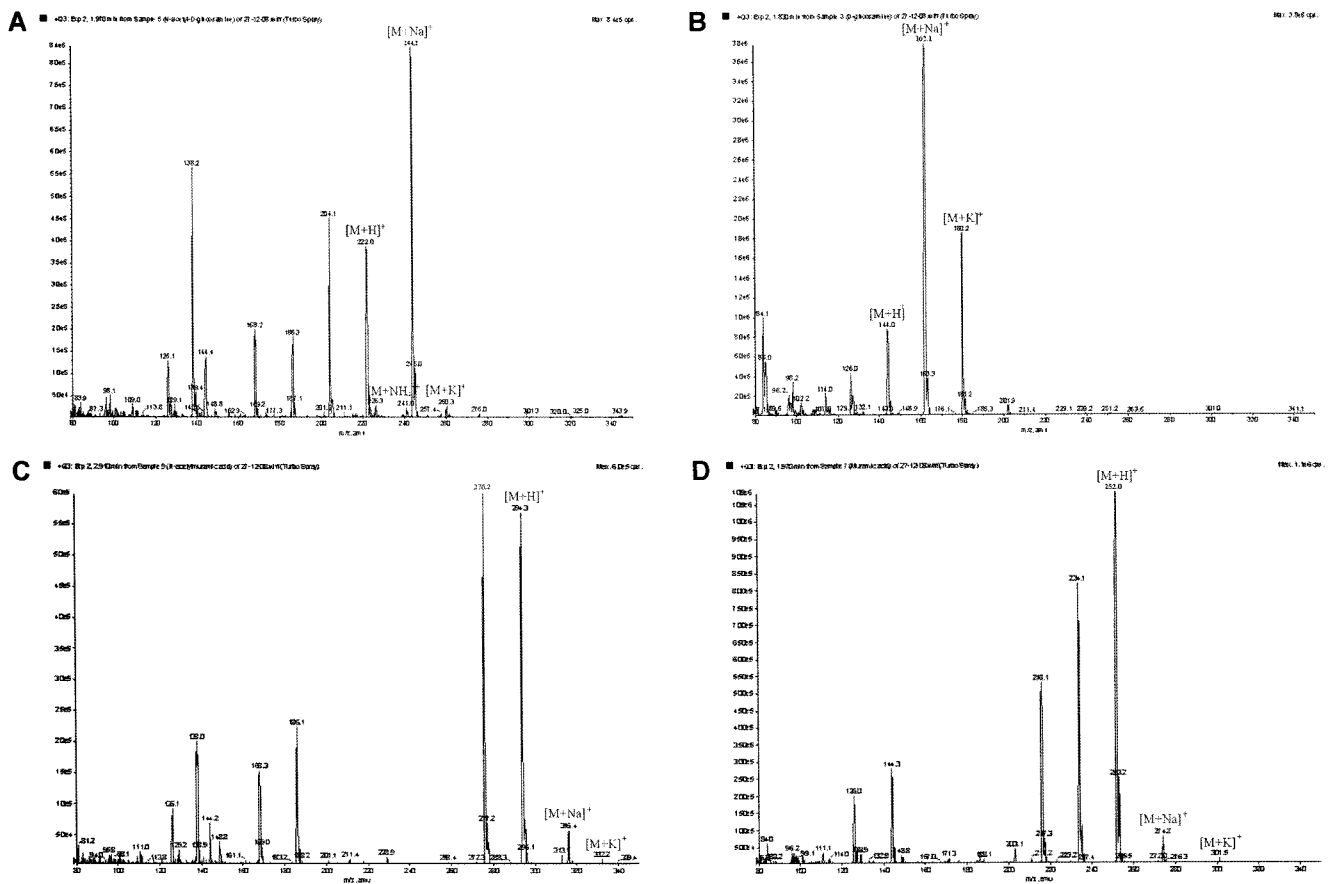
**Table 3.** LC/MS/MS characteristics of investigated compounds in the positive mode.

Compound	Retention time (min)	MRM	DP (V)	CE (V)
D-Glucosamine	1.8	180/162	21	13
<i>N</i> -Acetyl-D-glucosamine	2.0	222/138	41	21
		222/204	41	11
Muramic acid	2.0	252/234	21	15
		252/126	21	25
<i>N</i> -Acetyl-muramic acid	2.9	294/138	26	23
		294/276	26	13

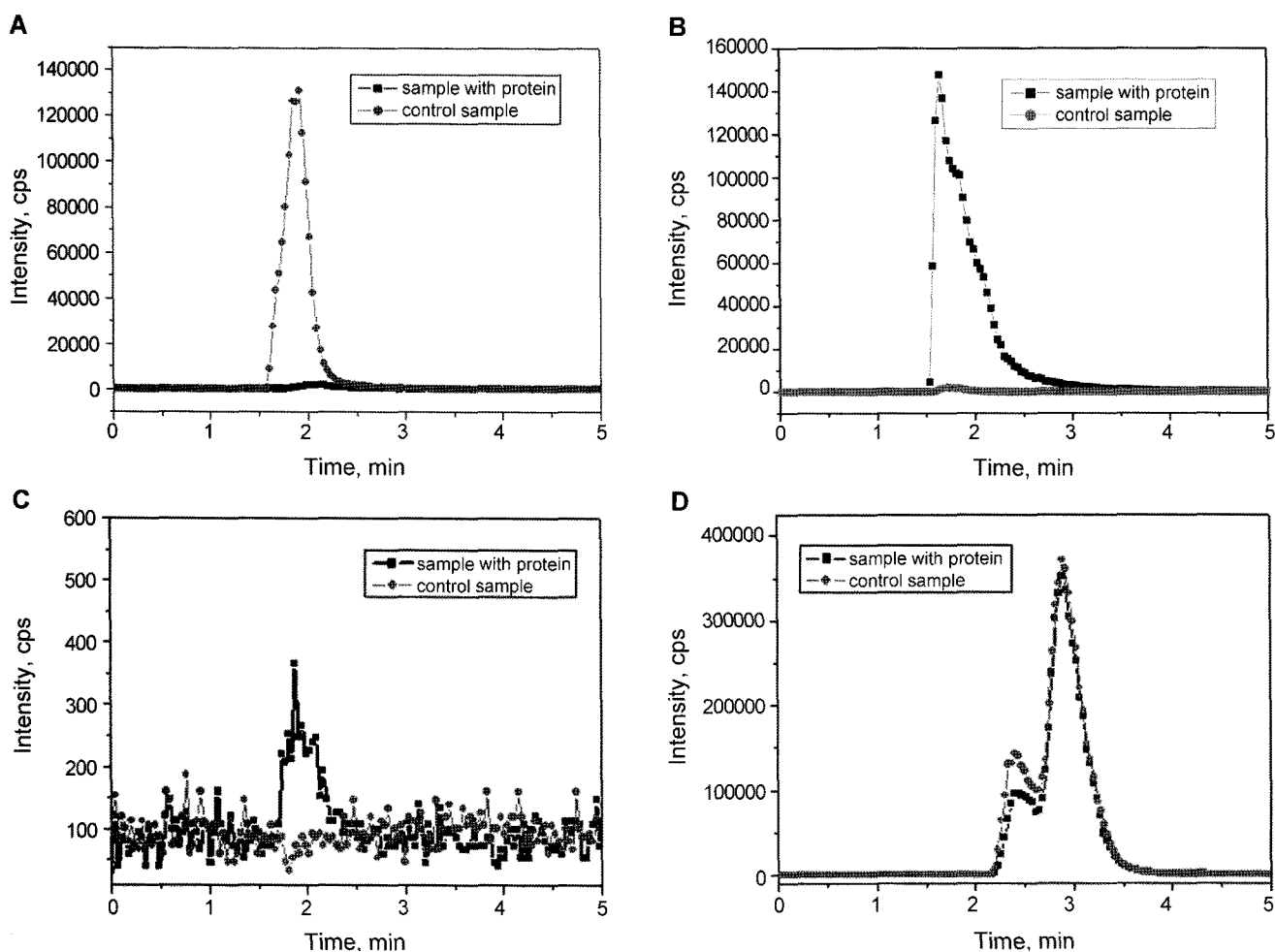
MS signals for any analyte. Before analysis was started, the MS/MS parameters were assessed in both negative and positive modes in infusion mode. The best signal/noise ratio and sensitivity were obtained in the positive mode. Since the positive ionization mode with electrospray source ionization mode was utilized, the relative ionization efficiencies and diagnostic utilities of CAD (collision-activated dissociation) spectra for all protonated compounds were compared. The spectra collected in the positive ion mode contain  $[M+H]^+$  ions and adducts with cations:  $[M+Na]^+$ ,  $[M+NH_4]^+$ , and  $[M+K]^+$ . The declustering potential (DP) and collision energy (CE), measured as fragmentor voltage value, were

optimized in infusion mode for each protonated compound in the range from 10 to 400 V and 5 to 130 V, respectively. The voltage required for significant fragmentation was compound-specific (Table 3). The developed method includes two pairs of ion-transition monitorings (MRM) of the main residue of *N*-acetyl-D-glucosamine, *N*-acetyl-muramic acid, and muramic acid, and one for D-glucosamine.

Three of the investigated compounds (D-glucosamine, *N*-acetyl-D-glucosamine, and muramic acids) were eluted at similar retention times of around 2 min. However, the use of a mass spectrometer as the detector permitted the identification of the unresolved compounds. Fig. 12 presents



**Fig. 12.** ESI-MS spectra of the investigated compounds: **A.** *N*-Acetyl-D-glucosamine; **B.** D-Glucosamine; **C.** *N*-Acetylmuramic acid; **D.** Muramic acid.  $[M+H]^+$  ions and adducts with cations:  $[M+Na]^+$ ,  $[M+NH_4]^+$ ,  $[M+K]^+$  indicated.



**Fig. 13.** HPLC-MS/MS analysis of PgdA-His6 activity.

MRM spectra of sample with purified enzyme PgdA-His6 (sample with protein) and without (control sample): **A**, Comparison of MRM 222138 for *N*-acetyl- $\text{D}$ -glucosamine; **B**, **C**, Comparison of MRM 180162 for  $\text{D}$ -glucosamine; **D**, Comparison of MRM 294276 for *N*-acetylmuramic acid. In **A** and **B**, the substrate was *N*-acetyl- $\text{D}$ -glucosamine. In **C**, the substrate was *N*-acetylated muropeptides. In **D**, the substrate was *N*-acetylmuramic acid.

MS spectra of the investigated compounds obtained under optimized conditions. The obtained transitions were used in MRM mode for confirmation of the tested compounds in samples with the PgdA-His6 protein. Fig. 13 presents a comparison of MRM data obtained for samples incubated with and without this protein. From the results of these experiments, it may be concluded that *N*-acetylglucosamine is a substrate for PgdA-His6, whereas *N*-acetylmuramic acid is not. The action of purified PgdA-His6 resulted in a decrease of *N*-acetylglucosamine (Fig. 13A) with a concomitant increase in  $\text{D}$ -glucosamine (Fig. 13B). The extent of this transformation depended on the amount of active enzyme in the reaction. In contrast, the concentration of *N*-acetylmuramic acid did not change after incubation with PgdA-His6 compared with the no-enzyme control sample (Fig. 13D). The appearance of  $\text{D}$ -glucosamine in the reaction containing muropeptides demonstrated that *N*-acetylated murein after lysozyme digestion also constitutes a substrate for PgdA-His6 (Fig. 13C).

## DISCUSSION

Modifications of the sugar backbone of murein have been recognized for many years, and have recently been elegantly reviewed by Vollmer [40]. *O*-Acetylation of the C-6 hydroxyl group of *N*-acetylmuramyl residues has been studied quite intensively and found in both Gram-negative (*e.g.*, *Neisseria gonorrhoeae*, *Proteus mirabilis*) and Gram-positive (*e.g.*, *Staphylococcus aureus*, *Enterococcus faecalis*) bacteria [18, 40]. This modification has been shown to affect the susceptibility of murein to degradation by mureolytic enzymes. Recently, the activity of the enzyme peptidoglycan *O*-acetyltransferase was found to directly affect both lysozyme resistance and pathogenicity in *S. aureus* [3]. More surprisingly, a peptidoglycan *O*-acetyltransferase mutant of *Streptococcus pneumoniae* showed attenuated penicillin resistance [12].

Another modification of the sugar moieties of murein is their incomplete *N*-acetylation. This observation was made

many years ago and reported for a number of Gram-positive bacterial species [2, 17, 32]. More recently, Vollmer and Tomasz [38, 39] showed that gene *pgdA* of *S. pneumoniae* codes for a peptidoglycan *N*-acetylglucosamine deacetylase, which removes *N*-acetyl groups from sugar moieties [38] and is a putative virulence factor in this bacterium [39]. Subsequently, *N*-acetylglucosamine de-*N*-acetylases were found in several other bacterial species, such as *Enterococcus faecalis* [18] and *Lactococcus lactis*, in which peptidoglycan *N*-acetylglucosamine deacetylation decreases autolysis [24].

Peptidoglycan *N*-deacetylation has recently been very elegantly shown to play a critical role in evasion of the host innate immune system by *Listeria monocytogenes* [6]. In our studies, which complement those of Boneca *et al.* [6], we have focused on the *N*-acetylglucosamine de-*N*-acetylases of *L. monocytogenes* and have found that this bacterium synthesizes three such enzymes, two of which are localized in the cytoplasm and one is associated with the cell. We have submitted the results of our studies on the former elsewhere, and in this report we describe our findings on the latter. Analysis of the nucleotide sequence of the *L. monocytogenes* EGD genome revealed gene *lmo0415* (*pgdA*), coding for a potential polysaccharide deacetylase, with analogs in other *Listeria* species. A mutant, designated MK, was constructed in which gene *pgdA* was inactivated by insertion duplication mutagenesis. This mutant was fully viable and its growth rates were identical with those of the wild-type parent strain at various temperatures and in different growth media. The morphology of the parent and mutant cells was identical. These results demonstrate that the putative deacetylase encoded by gene *pgdA* is not necessary for the viability of the cells. Similar results have been reported for a mutant of *S. pneumoniae* lacking *N*-acetylglucosamine de-*N*-acetylase activity [38]. To examine the physiological role of the *pgdA* gene product in the *L. monocytogenes* cell, a series of experiments involving both the constructed mutant and the purified recombinant enzyme was carried out. Examination of Triton-X-100- or EDTA-induced lysis of *L. monocytogenes* cells showed that the mutant was more prone to induced autolysis of the cell wall murein than the parental strain. This may indicate that fully acetylated murein is a better substrate for the host autolysins than the partially de-*N*-acetylated macromolecule. Similar results were obtained when the cells were spun down, resuspended in buffer, and treated with lysozyme or mutanolysin. An experiment designed to investigate the effect of lysozyme as a cationic antimicrobial peptide (CAMP) under normal growth conditions revealed that the mutant MK cells were slightly more susceptible to the action of this enzyme in general, but that the difference was much more pronounced in the stationary phase of growth, as shown not only by changes in the optical density of cultures but also by the number of viable survivors. Taken together, these findings indicate differences

in the degree of *N*-acetylation of murein between the parent strains and the *pgdA* mutant. To confirm this and the assumption that PgdA has peptidoglycan *N*-acetylglucosamine deacetylase activity, a PgdA-His6 fusion protein was expressed in *E. coli*, purified, and used to treat murein isolated from *L. monocytogenes* EGD and mutant MK. The obtained results indicate that the murein of the mutant has a higher content of acetyl groups compared with that of the wild type, which reflects the activity of PgdA in the latter. The deacetylase activity of PgdA-His6 catalyzed the removal of these acetyl groups from mutant peptidoglycan, releasing them as acetic acid into the reaction mixture, thus complementing the effect of the mutation in gene *lmo0415* *in vitro*. These results are compatible with our earlier unpublished observations pointing to the partial de-*N*-acetylation of glucosamine residues in wild-type *L. monocytogenes* cells. In separate experiments involving HPLC with tandem mass spectrometry, we have proven that the substrate for the purified *L. monocytogenes* enzyme is *N*-acetylglucosamine and not *N*-acetylmuramic acid. To finally confirm the *N*-acetylglucosamine de-*N*-acetylase activity of PgdA-His6, the enzyme was used to treat fully *N*-acetylated murein from *Escherichia coli* DH5 $\alpha$ , followed by incubation of the murein with lysozyme. Following digestion with PgdA-His6, the *E. coli* murein became less susceptible to the action of the muramidase, reflecting the removal of acetyl groups from *N*-acetylglucosamine, thus making the *E. coli* murein a worse substrate for lysozyme.

In light of the results presented by Crisóstomo *et al.* [12], we also tested the susceptibility of mutant MK to antimicrobial compounds and found that, in contrast to the *S. pneumoniae* mutant lacking peptidoglycan *O*-acetyl transferase activity, mutant MK was slightly more susceptible to three beta-lactams among the various antibiotics tested. This finding is compatible with the results of the test of induced cell lysis in the parent strain and mutant, which indicate that fully *N*-acetylated murein is a poorer substrate for host autolysins.

In conclusion, the cell-wall-associated *N*-acetylglucosamine de-*N*-acetylase of *L. monocytogenes*, PgdA, is responsible for altering the primary structure of the cell wall murein [6], making it more susceptible to the action of its own autolytic enzymes, exogenous muramidases, and certain beta-lactam antibiotics. Consequently, protein PgdA plays a protective physiological role for listerial cells.

## Acknowledgments

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