Characterization of AtlL, a bifunctional autolysin of *Staphylococcus lugdunensis* with *N*-acetylglucosaminidase and *N*-acetylmuramoyl-L-alanine amidase activities

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Abstract

The nucleotide sequence of *atlL*, a gene encoding a putative *Staphylococcus lugdunensis* peptidoglycan hydrolase, was determined using degenerate consensus PCR and genome walking. This 3837-bp gene encodes a protein, AtlL, that appears as a putative bifunctional autolysin with a 29-amino acid putative signal peptide and two enzymatic putative centres (N-acetylmuramoyl-L-alanine amidase and N-acetylglucosaminidase) interconnected with three imperfect repeated sequences displaying glycine–tryptophan motifs. In order to determine whether both lytic domains were functional, and verify their exact enzymatic activities, gene fragments harbouring both putative domains, AM (*N*-acetylmuramoyl-L-alanine amidase enzymatic centre plus two repeated sequences) and GL (*N*-acetylglucosaminidase enzymatic centre plus one repeated sequence), were isolated, subcloned, and expressed in *Escherichia coli*. Purified recombinant AM and GL protein truncations exhibited cell wall lytic activity in zymograms performed with cell walls of *Micrococcus lysodeikticus*, *Bacillus subtilis*, and *S. lugdunensis*. AtlL is expressed during the whole growth, with an overexpression in the early-exponential stage. Liquid chromatography-mass spectrometry analysis of muropeptides generated by digestion of *B. subtilis* cell walls demonstrated the hydrolytic bond specificities and confirmed both of the acetyl domains’ activities as predicted by sequence homology data. AtlL is the first autolysin described in *S. lugdunensis*, with a bifunctional enzymatic activity involved in peptidoglycan hydrolysis.

Introduction

*Staphylococcus lugdunensis* is a coagulase-negative staphylococcus that has emerged as a recognized important human pathogen (Herchline & Ayers, 1991), and behaves much like *Staphylococcus aureus* in terms of virulence, tissue destruction, and clinical course (Vandenesch et al., 1993; Hellbacher et al., 2006; Frank et al., 2008). Bacterial peptidoglycan hydrolases (PGH) of several staphylococcal species (Oshida et al., 1995; Heilmann et al., 1997; Hell et al., 1998; Allignet et al., 2001; Biswas et al., 2006; Yokoi et al., 2008) and other Gram-positive low G+C % bacteria (*Bacillus subtilis* and *Clostridium difficile*) (Smith et al., 2000; Dhalluin et al., 2005) have been described. Atl-like staphylococcal autolysins, Atl (*S. aureus*), AtLE (*Staphylococcus epidermidis*), AtlC (*Staphylococcus caprae*), Aas (*Staphylococcus saprophyticus*), and AtlWM (*Staphylococcus warneri* M) are bifunctional enzymes with *N*-acetylmuramoyl-L-alanine amidase and *N*-acetylglucosaminidase cleavage activity on the peptidoglycan. Together with other PGHs, these enzymes are thought to play a crucial role in bacterial physiology by maintaining a balance between peptidoglycan synthesis and degradation (Perkins, 1980; Shockman & Holtje, 1994; Blackman et al., 1998; Smith et al., 2000) as well as through their
involvement in cell separation (Foster, 1995; Sugai et al., 1995; Baba & Schneewind, 1998). This PGH involvement in bacterial physiology, and probably in antibiotic-induced autolysis (Tomasz & Waks, 1975; Perkins, 1980; Moreillon et al., 1990; Yamada et al., 1996; Sugai, 1997), further reinforces the importance of understanding bacterial autolysis, especially for a species such as *S. lugdunensis*, in which glycopeptide tolerance has been reported (Bourgeois et al., 2007; Frank et al., 2007). In the present study, we report the first characterization in *S. lugdunensis* of a bifunctional autolysin involved in peptidoglycan hydrolysis.

**Materials and methods**

**Bacterial strains and culture conditions**

*Staphylococcus lugdunensis* ATCC 43809 strain, stored frozen in glycerol broth at –80 °C, was subcultured on Columbia blood agar (BioRad) or trypticase soy broth (Difco) before use in experiments. *Escherichia coli* TOP10 strain (Invitrogen) was used as the cloning and expression strain. Luria–Bertani (LB) and 2XYT broths (Q.BioGene) were used for the culture of transformed *E. coli* cells at 37 °C with shaking (225 r.p.m.). When required, ampicillin (100 μg mL⁻¹) or l-arabinose (0.2% w/v) was added. *Bacillus subtilis* 168 HR (Foster, 1991) was cultivated in LB broth (Q.BioGene) at 37 °C with shaking (200 r.p.m.) and was used (1) in zymograms and (2) to prepare cell wall extracts for the study of hydrolytic bond specificity.

**General DNA techniques**

Chromosomal DNA from *S. lugdunensis* used as a template for PCR was extracted from bacterial cells with a MagnaPure LC DNA system (DNA isolation kit III; Roche) according to the manufacturer’s instructions, except that 10 ng mL⁻¹ lysozyme (Sigma), 200 μg mL⁻¹ lysozyme (Sigma), and 200 μg mL⁻¹ RNase (Sigma) were added during a supplementary cell lysis step. DNA fragments used in the cloning procedures and PCR products were isolated from agarose gels with the Qiagel quick gel extraction kit (Qiagen). Plasmid DNA from *E. coli* was isolated and purified with the Plasmid midi kit (Qiagen).

**PCR amplification and genome walking for *atll* characterization**

In order to determine the *atll* sequence, two conserved regions, AM-cat (for the *N*-acetylmuramoyl-1-alanine amidase catalytic domain) and GL-cat (for the *N*-acetylglycosaminidase catalytic domain), were highlighted after the alignment of six bifunctional staphylococcal autolysin sequences plus the sequence of the autolysin Acd of *C. difficile*. Two partial sequences of *atll* were then amplified using two pairs of degenerated primers designed on the conserved regions, ligated into the pCR2 vector using the TOPO-TA cloning technology (Invitrogen), and sequenced. After determination of these partial sequences, the unknown flanking sequences were amplified using the GenomeWalker Universal Kit (Clontech): adapter-ligated genomic DNA libraries from *S. lugdunensis* ATCC 43809 were obtained by complete digestion of its genomic DNA with four different restriction enzymes (DraI, EcoRV, PvuII, and StuI); each batch of digested products was then ligated to the GenomeWalker Adaptor and used as a template for amplifications with an adaptor primer and successive gene-specific primers. Finally, after genome walking up- and downstream of both partial sequences, a last amplification was carried out to determine the sequence of the central missing region, using specific primers. All primers used are listed in Supporting Information, Table S1. All DNA sequences were determined with an Applied Biosystems 310 automated DNA sequencer using an ABI-PRISM Big Dye terminator v3.1 cycle sequencing kit (Perkin Elmer). Nucleotide sequences were analysed, assembled, and edited using the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The nucleotide sequence accession no. of the *atll* gene is EU518933.

**Cloning, expression, and purification of AM- and GL-His-tagged fusion proteins in *E. coli***

The pBAD/His B expression system (Invitrogen) was used to subclone and express the truncated *AtlL* gene fragments harbouring the AM and GL domains in *E. coli* TOP10 (Invitrogen) as N-terminal hexa-His-tagged. After amplification with the primer pairs AM-exp-F/R and GL-exp-F/R using the High Fidelity PCR enzyme mix (Fermentas), PCR fragments (1782 and 1434 bp) were digested by BglII plus PvuII, and StuI); each batch of digested products was ligated into the pCR2 vector using the TOPO-TA cloning technology (Invitrogen). Overnight cultures of *E. coli* recombinant strains were used to inoculate a 1600-mL volume of 2XYT broth containing ampicillin, which was incubated until an OD600 nm of 0.6. At this point, expression was induced by adding l-arabinose to a final concentration of 0.2% (w/v) for a further 4 h. Cells were then harvested by centrifugation (9500 g, 15 min, 4 °C) and stored at −20 °C until required.

The proteins were purified by affinity chromatography on Ni²⁺-nitrilotriacetic acid agarose (Qiagen) using PolyPrep columns (BioRad) according to the manufacturers’ recommendations.
For the AM-His-tagged fusion protein, purification was achieved under denaturing conditions with 8 M urea; the eluted protein was electrophoresed through sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), and pooled fractions were refolded by sequential dialysis steps at 4°C against 100 mM sodium phosphate (pH 7.0), 1 mM EDTA, 4 M urea, 5% (w/v) sucrose, 100 mM NaCl, 100 mM KCl, 100 mM L-arginine, and 4 mM MgCl₂ with successive dilutions in 100 mM phosphate buffer (pH 7.0). Soluble protein fractions were collected after centrifugation, concentrated in Amicon 10 membrane centrifugal filters devices (Millipore), and stored at −20°C.

For the GL-His-tagged fusion protein, purification was achieved under native conditions. The eluted protein was electrophoresed by SDS-PAGE, and pooled fractions were dialysed overnight at 4°C twice with very slow stirring against the lysis buffer without imidazole, concentrated in Amicon 10 membrane centrifugal filters devices (Millipore), and stored at −20°C.

Zymograms

Surface-associated cell wall lytic enzymes of Staphylococcus lugdunensis were extracted by LiCl and SDS extraction procedures, respectively, as described (Groicher et al., 2000; Leclerc & Asselin, 1989). SDS-PAGE was performed with 15% polyacrylamide separating gels. Lytic activity was detected using SDS polyacrylamide gels containing 0.2% (w/v) Micrococcus lysodeikticus ATCC 4698 (Sigma) (Leclerc & Asselin, 1989) and B. subtilis 168 HR (Foster, 1991). Following electrophoresis, the gels were gently shaken at 37°C for 16 h in 250 mL of 25 mM Tris-HCl (pH 8.0) containing 1% (w/v) Triton X-100 to allow protein renaturation. Gels were stained with 1% (w/v) methylene blue (Sigma) in 0.01% (w/v) KOH and subsequently destained with distilled water to visualize bands of lytic activity.

Determination of peptidoglycan hydrolytic bond specificities

Peptidoglycan (2 mg) from B. subtilis 168 HR vegetative cells, prepared as described previously for Lactococcus lactis (Meyrand et al., 2007), was incubated overnight at 37°C with purified AM- or GL-His recombinant protein (75 μg) in a final volume of 250-μL sodium phosphate buffer, pH 8.0. Mutanolysin (Sigma), a PGH with muramidase activity, was used as a digestion control. Samples were boiled for 3 min to stop the reaction. The insoluble material was removed by centrifugation at 14,000 g for 15 min. Half of the soluble mucopeptide fraction obtained by the GL-His recombinant protein was further redigested with mutanolysin (2500 U mL⁻¹). The soluble mucopeptides obtained after digestion were reduced with sodium borohydride to a final concentration of 8 mg mL⁻¹ (Atrih et al., 1999). The reduced mucopeptides were then separated by reverse-phase HPLC (RP-HPLC) using a Hypersil ODS column (C18, 250 × 4.6 mm; 5 μm; ThermoHypersil-Keystone) at 50°C using ammonium phosphate buffer and a methanol linear gradient (Courtin et al., 2006). Peaks were analysed without desalting by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS with a Voyager DE STR mass spectrometer (Applied Biosystems) (Courtin et al., 2006).

Results and discussion

Characterization of a putative bifunctional PGH gene in the S. lugdunensis ATCC 43809 genome

Because the S. lugdunensis genome had not yet been sequenced, we first aligned autolysins from six staphylococcal species and C. difficile, which are all Gram-positive low G+C % bacteria. This alignment highlighted two conserved regions, which allowed us to design degenerated consensus primers and perform PCR amplification and sequencing steps. These partial sequences were completed by walking PCR, and finally by specific PCR to identify the whole atlL ORF. This approach, previously used for elucidation of partial genome sequences for phylogenetic purposes and identification of emerging pathogens (Renesto et al., 2000; Drancourt et al., 2001; Inokuma et al., 2001), was effective for isolation of the atlL S. lugdunensis gene.

The atlL gene would encode a protein of 1279 amino acids with a deduced molecular mass of 140.69 kDa. The hydrophobicity blot analysis (Kyte & Doolittle, 1982) indicates that AtlL mainly consists of hydrophilic amino acids. The N-terminal part of the protein is a strong hydrophobic region with a putative signal peptide of 29 amino acids. The N-terminal part of the protein is a strong hydrophobic region with a putative signal peptide of 29 amino acids as assessed by SIGNALP (http://www.cbs.dtu.dk/services/SignalP/). The overall sequence and the domain architecture (Fig. 1) exhibit significant similarities to other staphylococcal autolysins such as Atl (Oshida et al., 1995) and AtLE (Heilmann et al., 1997). The AtLE protein harbours a structural organization with two putative catalytic domains: an N-acetylmuramoyl-l-alanine amidase domain and an N-acetylgalactosaminidase domain. Three repeated sequences are present between these two catalytic domains, with a glycine–tryptophan (GW) dipeptide motif in the central part and at the end of each repeat. These imperfect repeats are thought to interact with cell wall polymers such as teichoic or lipoteichoic acids and to participate in targeting these enzymes towards their substrate, i.e. the peptidoglycan (Biené & Cossart, 2007). The amino acid sequences of the deduced AM and GL domains (the enzymatic centre plus two or one GW repeats, respectively) revealed significant identity percentages with the corresponding domains of S. aureus Atl (Oshida et al., 1995).
1995) and *S. epidermidis* AtlE (Heilmann *et al.*, 1997) (62% for the AM domain and 55–59% for the GL domain) (Fig. 1), suggesting that AtIL may represent the major autolysin of *S. lugdunensis* involved in cell division as reported for AtI in *S. aureus* (Sugai *et al.*, 1995). Quantitative PCR of *atlL* gene expression indicates that *atlL* is transcribed throughout all phases of growth (early-exponential, mid-exponential, and stationary growth phases), with an increased level (5.5-fold) at the early-exponential stage (data not shown), which is consistent with the hypothesis of an involvement in cell division. Further studies are required to reinforce this hypothesis, especially because *S. lugdunensis* may have a unique evolution path, different from other pathogenic staphylococci suggested by a relative lack of genomic diversity, and a prevalent susceptibility to numerous antimicrobial agents (Frank *et al.*, 2008).

**AM and GL AtIL domains, proteins with bacteriolytic activity**

In order to verify the lytic functionality of two domains in the AtIL protein, it was decided to express and purify each domain as a truncated protein. Because involvement of the central repeats in the lytic activity has been demonstrated (Hell *et al.*, 1998), alignment of AtIL with AtI and AtlIE was performed to determine which sequence repeat(s) should be subcloned with each catalytic domain in order to obtain optimal hydrolytic activity. The two putative domains (the enzymatic centre plus GW repeats), AM (Asn195-Val789) and GL (Thr804-Pro1275), were expressed in *E. coli* as His-tagged proteins. Purified AM and GL were, respectively, characterized as 65 and 55 kDa proteins (Fig. 2).

Both AM and GL domains yielded a clear hydrolysis band in renaturing SDS-PAGE experiments with *M. lysodeikticus* SP, PP, AM, GL, and R.

**Fig. 1.** General organization of *Staphylococcus lugdunensis* AtlIL, *Staphylococcus aureus* AtI (Oshida *et al.*, 1995), and *Staphylococcus epidermidis* AtlE (Heilmann *et al.*, 1997). AtlL sequence data were deduced from homologies to the former proteins. The definitions of the boxed domains output of the SMART web interface (http://smart.embl.de) are as follows: Ami-2, N-acetylmuramoyl-L-alanine amidase; LYZ2, mannosylglycoprotein endo-β-N-acetylglucosaminidase. The identity percentages between AtlL domains and Atl and AtlE domains are also given on the schematic autolysins. SP, signal peptide; PP, propeptide; AM, N-acetylmuramoyl-β-alanine amidase; R, repeated sequence; GL, N-acetylglucosaminidase; Glycine–tryptophane (GW) dipeptide.

**Fig. 2.** SDS-PAGE and renaturing SDS-PAGE of *Staphylococcus lugdunensis* ATCC 43809 proteins extracted with LiCl (lane 1) and with SDS (lane 2), and of *purified GL-His* (lane 3) and *purified AM-His* (lane 4). (a) Coomassie blue staining; (b) renaturing SDS-PAGE in gel containing 0.2% (w/v) *Micrococcus lysodeikticus* cells. The molecular masses (in kDa) of the standard proteins (SeeBlue Plus2 Pre-Stained Standard, Invitrogen) are indicated on the right of gel (a).
cells (Fig. 3b, lanes 3 and 4) as well as with B. subtilis and S. lugdunensis cells (data not shown).

The cell surface-associated proteins released from LiCl and SDS extracts of S. lugdunensis (Fig. 2a, lanes 1 and 2) also revealed, among others, two bands of clearing in the zymogram with M. lysodeikticus cells (Fig. 2b, lanes 1 and 2), whose molecular size was concordant with that of the recombinant proteins, indicating that they most probably correspond to AtlL domains generated by proteolytic processing. Such processing has been proposed for Atl of S. aureus (Komatsuzawa et al., 1997). Several other clearing bands were observed in gels with B. subtilis and S. lugdunensis cells (data not shown) in accordance with the recent in silico prediction of the PGH number (from 15 to 20 in Staphylococcus genomes studied) within Firmicute genomes (Layec et al., 2008).

**Fig. 3.** RP-HPLC analysis of the soluble muropeptides released from Bacillus subtilis vegetative cell walls after incubation with mutanolysin (a) GL (b) or GL and mutanolysin (c) or AM (d). The numbers and letters indicate the peaks analysed by MALDI-TOF MS.

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**Determination of AM and GL hydrolytic bond specificities**

To determine the hydrolytic specificity of each AtlL domain, the recombinant His-tagged domains were used to digest cell walls from B. subtilis 168 HR, whose muropeptides released by mutanolysin digestion have been described previously (Atrih et al., 1999). The soluble muropeptides released from digestion by each recombinant His-tagged domain were completely different from those released from mutanolysin digestion, as revealed by RP-HPLC analysis (Fig. 3a, b and d). These results suggest that neither of the two AtlL domains possessed muramidase activity.

The soluble muropeptides obtained by GL digestion of B. subtilis peptidoglycan were analysed by MALDI-TOF MS.
Four peaks (labelled 1–4 on Fig. 3b) gave molecular ions with m/z values of 893.32, 892.31, 1815.73, and 1814.71, respectively (Table 1). The mixture of soluble muropeptides released from *B. subtilis* peptidoglycan by GL was redigested by mutanolysin. The resulting RP-HPLC profile (Fig. 3c) contained peaks different from the RP-HPLC profile of GL-digestion products; MALDI-TOF MS of these new peaks revealed molecular ions with m/z values in agreement with the loss of one or two N-acetyl-glucosamines (GlcNAc) residues (mass decrease of 203 or 406 Da, respectively) from the muropeptides present in peaks 1, 2, 3, or 4 (Table 1 and Fig. 4). These results indicate that the muropeptides generated by GL hydrolysis can be further cleaved by mutanolysin, and that GlcNAc is present at the reducing end of these muropeptides. Thus, GL hydrolyses peptidoglycan bonds between GlcNAc and N-acetyl-muramic acid (MurNAc) and has N-acetylmuraminidase specificity.

The chromatogram of soluble material obtained from *B. subtilis* peptidoglycan digestion by AM recombinant protein revealed a few peaks with short retention times. MALDI-TOF MS analysis of the five peaks (labelled a–e in Fig. 3d) generated ions with m/z values expected for peptidic moieties of *B. subtilis* muropeptides (dimers, trimers, and tetramers) (Table 1 and Fig. 4). These peptidic structures result from cleavage of amide bonds between MurNAc residues of the peptidoglycan glycan chains and the first L-Ala residues of the peptidic chains. Thus, our results demonstrate that AM has N-acetylmuramoyl-L-alanine amidase activity.

Thus, the recombinant proteins AM and GL were demonstrated to function as N-acetylmuramoyl-L-alanine amidase and N-acetylmuraminidase by combining previously used RP-HPLC and MALDI-TOF MS (Huard et al., 2003; Dhalluin et al., 2005; Biswas et al., 2006). Of note, it is the first time that this strategy has allowed the characterization of both enzymatic activities using a unique cell wall substrate and a unique chromatography system, which allows separation of muropeptides composed of glycans and amino acids or only composed of amino acids.

*Staphylococcus lugdunensis* is recognized as a successful pathogen resembling *S. aureus* in many aspects of clinical manifestations (elevated degree of virulence, destructive clinical course). In addition, tolerance to glycopeptides may be widespread among *S. lugdunensis* strains (Bourgeois et al., 2007; Frank et al., 2007, 2008), in contrast to other coagulase-negative staphylococci. This first description of an autolysin in *S. lugdunensis* will allow further study of peptidoglycan dynamics in this species to delineate whether and how autolysins, most probably via autolysis regulatory systems (Antignac et al., 2007), are involved in lysis induced by antibiotics targeting the bacterial cell wall and, as a consequence, in the drug resistance phenomenon.

### Table 1.

Calculated and observed m/z values for sodiated molecular ions of muropeptides obtained after hydrolysis of *Bacillus subtilis* peptidoglycan by GL or by GL, followed by mutanolysin or by AM and purification by RP-HPLC.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Ion</th>
<th>m/z</th>
<th>Δm (Da)</th>
<th>Muropeptide identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(M+Na)^+</td>
<td>893.32</td>
<td>0.04</td>
<td>ds tri</td>
</tr>
<tr>
<td>2</td>
<td>(M+Na)^+</td>
<td>892.31</td>
<td>0.07</td>
<td>ds tri (NH₂) – tetra</td>
</tr>
<tr>
<td>3</td>
<td>(M+Na)^+</td>
<td>1815.73</td>
<td>0.04</td>
<td>ds tri (NH₂) – ds tetra – tetra</td>
</tr>
<tr>
<td>4</td>
<td>(M+Na)^+</td>
<td>1409.62</td>
<td>0.08</td>
<td>ds tri (NH₂) – ds tetra – tetra (NH₂)</td>
</tr>
<tr>
<td>5</td>
<td>(M+Na)^+</td>
<td>1611.75</td>
<td>0.13</td>
<td>tri (NH₂) – tetra (NH₂)</td>
</tr>
<tr>
<td>6</td>
<td>(M+Na)^+</td>
<td>1408.64</td>
<td>0.13</td>
<td>tri (NH₂) – tetra (NH₂) – tetra</td>
</tr>
<tr>
<td>7</td>
<td>(M+Na)^+</td>
<td>1814.79</td>
<td>0.10</td>
<td>tri (NH₂) – tetra (NH₂) – tetra (NH₂)</td>
</tr>
</tbody>
</table>

Δm, difference between calculated and observed m/z values; ds, disaccharide (MurNAc–GlcNAc); tri, tripeptide; tetra, tetrapeptide; NH₂ indicates the presence of an amidation on the peptidic chain, most probably on mDAP (Atrih et al., 1999). When only one amidation is present, the assignment of the amidation position in dimers is arbitrary.

Peak numbers refer to the peaks on the chromatograms presented in Fig. 3.
Fig. 4. Structure of the muropeptides from Bacillus subtilis peptidoglycan obtained after GL digestion (peaks 1, 2, 3, and 4) or after GL and mutanolysin digestion (peaks $1', 2', 3'', 4''$, and $4'''$) or after AM digestion (peaks a, b, c, d, and e). Numbers and letters refer to the peaks on the chromatograms presented in Fig. 3.

### References


Atrih A, Bacher G, Allmaier G, Williamson MP & Foster SJ (1999) Analysis of peptidoglycan structure from vegetative cells of...


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligonucleotide primers used in this study.

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