Activity of the major staphylococcal autolysin Atl

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Abstract

The major autolysin of *Staphylococcus aureus* (AtlA) and of *Staphylococcus epidermidis* (AtlE) are well-studied enzymes. Here we created an atlA deletion mutant in *S. aureus* that formed large cell clusters and was biofilm-negative. In electron micrographs, the mutant cells were distinguished by rough outer cell surface. The mutant could be complemented using the *atlE* gene from *S. epidermidis*. To study the role of the repetitive sequences of *atlE*, we expressed in *Escherichia coli* the amidase domain encoded by the gene, carrying no repeat regions (*amiE*) or two repeat regions (*amiE*-R1,2), or the three repeat regions alone (R1,2,3) as N-terminal His-tag fusion proteins. Only slight differences in the cell wall lytic activity between AmiE and AmiE-R1,2 were observed. The repetitive sequences exhibit a good binding affinity to isolated peptidoglycan and might contribute to the targeting of the amidase to the substrate. AmiE and AmiE-R1,2 have a broad substrate specificity as shown by similar activities with peptidoglycan lacking wall teichoic acid, O-acetylation, or both. As the amidase activity of AtlA and AtlE has not been proved biochemically, we used purified AmiE-R1,2 to determine the exact peptidoglycan cleavage site. We provide the first evidence that the amidase indeed cleaves the amide bond between N-acetyl muramic acid and L-alanine.

Introduction

Pioneering results on the structure and biosynthesis of peptidoglycan (PG) were obtained with *Staphylococcus aureus* (Schleifer & Kandler, 1972; Rogers et al., 1980; Ghuysen & Hakenbeck, 1994). Peptidoglycan of *S. aureus* contains relatively short glycan chains and is highly cross-linked via its peptidic chains (Snowden & Perkins, 1990). In *S. aureus* cells more than 95% of the peptidoglycan subunits are cross-linked. This extremely high degree of cross-linking is possible only because the long and flexible pentaglycine interpeptide bridges are able to span distances between peptides which otherwise are too far apart to be cross-linked (Ghuysen & Hakenbeck, 1994).

Peptidoglycan of *S. aureus* is highly modified. It has almost no free carboxyl groups as the α-carboxyl group of d-glutamic acid is amidated (Tipper et al., 1967; Pucci et al., 1995) and about 50% of the muramic acid residues are 4-N, 6-O-diacetyl-derivatized (Tipper et al., 1971). This latter substitution renders peptidoglycan resistant to human and egg-white lysozyme. As we have shown recently, the major determinant for this high lysozyme resistance is the peptidoglycan-specific O-acetyltransferase, OatA (Bera et al., 2005).

Peptidoglycan must be continuously synthesized to maintain cell integrity and viability and to facilitate cell growth. Newly synthesized peptidoglycan components are incorporated into the intact cell wall layer after cleavage by peptidoglycan hydrolases. The maintenance of this network requires a proper balance between peptidoglycan synthesis and degradation during bacterial growth. In general, peptidoglycan hydrolases are thought to play an important role in cell wall turnover, cell division, and cell separation, and in the lysis of bacteria induced by the β-lactam antibiotics.

The Atl is the most predominant peptidoglycan hydrolase in staphylococci. AtlA of *S. aureus* (Oshida et al., 1995) and AtlE of *Staphylococcus epidermidis* (Heilmann et al., 1997) are quite similar in both sequence and domain organization. The enzymes are bifunctional, composed of an amidase domain and a glucosaminidase domain.
Cells of atlA and atlE mutants form large clusters (Sugai et al., 1995; Heilmann et al., 1997). The wild-type phenotype of *S. epidermidis* AtlE, i.e. moderate cell clustering, primary adhesion to polystyrene, and binding activity to vitronectin and fibronectin, is restored in *atlE* mutants carrying the DNA encoding the 60 kDa amidase domain and the two repeat sequences R1 and R2 (Heilmann et al., 1997). These results identified a new function of AtlE as an adhesin involved in the primary attachment of the cells to a polystyrene surface.

Recently, two very similar, novel autolysin/adhesins in *S. epidermidis* (Aae) (Heilmann et al., 2003) and *S. aureus* (Aaa) (Heilmann et al., 2005) have been described. Both are 35 kDa surface-associated protein and their N-terminal portion contains three repetitive sequences that might be putative peptidoglycan-binding domains (LysM domain) found in a number of enzymes involved in cell-wall metabolism and also in some adhesins. In addition to its bacteriolytic activity, Aaa and Aae also binds to fibrinogen, fibronectin, and vitronectin.

Here, we report the construction and phenotypic characterization of an *S. aureus* *atlA* deletion mutant, demonstrate that the repetitive sequences located between the amidase domain and the glucosaminidase domain bind to peptidoglycan, and identify the exact cleavage site of the amidase.

**Materials and methods**

**Bacterial strains, media, and growth conditions**

The following *Staphylococcus aureus* strains were used: RN4220 (Kreiswirth et al., 1983); SA113, an NCTC 8325 derivative (Iordanescu & Surdeanu, 1976); and SA113ΔtagO, a mutant lacking teichoic acid in the cell wall mutant (Weidenmaier et al., 2004). *Staphylococcus carnosus* TM300 (Götz, 1990), *Escherichia coli* DH5α (Hanahan, 1983), and *E. coli* M15 (Qiagen) were used as cloning and expression strains. All strains were cultivated aerobically at 37 °C unless otherwise indicated, in LB medium supplemented with 0.1% K2HPO4 and 0.1% glucose. For biofilm studies, cells were grown in TSB medium (Difco) containing filter-sterilized glucose (0.5% final concentration). Antibiotics were added when appropriate: ampicillin (100 μg mL⁻¹) and kanamycin (30 μg mL⁻¹) for *E. coli*, and spectinomycin (150 μg mL⁻¹) and chloramphenicol (20 μg mL⁻¹) for *Staphylococcus* species.

**Construction of an *atlA* deletion mutant and in *S. aureus* SA113**

An *atlA* deletion mutant was constructed by homologous recombination using plasmid pBT2 as described earlier (Brückner, 1997), by cloning a spectinomycin selection marker (*spc*) flanked by upstream and downstream regions of the *atlA* gene in the multiple cloning site of pBT2. The entire genomic *atlA* gene was PCR amplified using the primer pair *atlF* *NheI* (5'-ataaatgatccctgaactttgattactccagc-3'); *atlR* *EcoRI* (5'-ttatgatcctgaactttgattactccagc-3'); introduced *NheI* restriction site underlined) and *atlR* *EcoRI* (5'-ttatgatcctgaactttgattactccagc-3'); introduced *EcoRI* restriction site underlined). The PCR product was digested with *EcoRI* and *SphI* (internal site), and the 1.6 kb downstream fragment (d) was cloned in predigested pEC2 (Brückner, 1997), generating plasmid pEC2d. The upstream flanking region (u) was PCR amplified using primers *atlF* *NheI* and *atlR* *BamHI* (5'-ataaatgatccctgaactttgattactccagc-3'; *BamHI* restriction site underlined) and cloned in pBT2, generating plasmid pBT2u. The downstream flanking region was excised from pEC2u with *EcoRI* and HindIII, and the spectinomycin cassette was excised from pIC156 (Steinmetz & Richter, 1994) with *BamHI* and HindIII. The two fragments were ligated to each other and with *BamHI/EcoRI*-digested pBT2d, generating plasmid pBT2ud. This knockout plasmid was introduced into *S. aureus* RN4220 by electroporation, and subsequently into *S. aureus* SA113 (Augustin & Götz, 1990). The SA113 *atl* deletion mutant Δ*atlA::spc* obtained after homologous recombination was identified by its chloramphenicol-sensitive and spectinomycin-resistant phenotype and was verified by DNA sequencing.

**Complementation of mutants**

The *S. aureus* SA113 Δ*atlA::spc* mutant was complemented by the *S. epidermidis* *atlE* gene either in vector pRC14 (Heilmann et al., 1997), in which the *atlE* gene is under the control of its native promoter, or in pTXatlE, in which *atlE* is under control of the inducible xylose promoter of plasmid pTX15 (Peschel et al., 1996). For cloning in pTX15, the primer pair *atlE*-BglII (5'-attgatccctgaactttgattactccagc-3'; *BglII* site is underlined) and *atlE*-PstI (5'-aacttgaattctttgattactccagc-3'; *PstI* site is underlined) was used.

**Construction of the green fluorescent protein (GFP) reporter plasmid**

The *gfp* gene was excised from the vector pgfp-UV (Clontech, Mountain View, CA) and cloned in pCX19, a derivative of pCX15 (Wieland et al., 1995) in which the *cat* gene has been inverted (Dr B. Krismer, Genmedics GMBH, Germany). To optimize *gfp* expression, the *tufA* promoter was PCR amplified from SA113 genomic DNA using the primers 5'-tacatatgctgctggagctggagaagcaatcatcgtctctctcc-3' and 5'-atatgtccagcccattgattactccagc-3' (nucleotide sequence accession number AF274740) and placed in front of the *gfp* gene. Furthermore, the *gfp* ribosomal binding site was altered to AGGAGG for optimal expression in
Gram-positive bacteria. The resulting reporter plasmid pCtufgp was used to monitor biofilm formation.

**Expression of the AtlE amidase domain with and without repeat sequences and expression of repeat sequences alone**

The amidase domain of the *atlE* gene with no repeat regions (*amiE*) and with two repeat regions (*amiE-R1,2*), and the three repeat regions alone (*R1,2,3*) were cloned and expressed as N-terminal His-tag fusion proteins in pQE30 (Qiagen). *amiE* was constructed using the primer pair AM-BglII (5′-caagatgtatctgtaaaaaact-3′) and AM-PstI (5′- aactgca gatattgagccccaaggtgctacttgcttcg-3′). *amiE-R1,2* was PCR amplified from *S. epidermidis* O-47 genomic DNA using the primer pair AM-BglII and AR2-PstI (5′-aactgcaattctagataaaaaacat-3′) digested with BglII and PstI, and inserted into BamHI/PstI-cleaved pQE30 to generate plasmid pQEamiE. The repeat regions *R1,2,3* were PCR amplified with the primer pair R1-BglII (5′-caagatgtataaaacctaccaaccc-3′) and AR3-PstI (5′-aactgcaattctagataaaaaacat-3′) and AR3-PstI (5′-aactgcaattctagataaaaaacat-3′). All constructs were expressed in *E. coli* M15, and the proteins were located in the cytoplasm.

**Purification of the His-tagged amidase**

*Escherichia coli* M15 cells carrying the constructs encoding the His-tagged proteins were grown in B medium to an OD578 nm of 0.5; expression was then induced with 1 mM isopropyl β-D-thiogalactoside. Cells were harvested by centrifugation, and proteins were purified under denaturing conditions with 8 M urea according to the recommendations of the manufacturer (Qiagen). Proteins were renatured by sequential dialysis steps at 4 °C: (1) against 100 mM sodium phosphate (pH 7.0), 1 mM EDTA, 4 M urea, 5% sucrose, 100 mM NaCl, 100 mM KCl, 100 mM L-arginine, 4 mM MgCl2, for 48 h without stirring; (2) against the buffer in step 1 diluted 1 : 1 with 100 mM phosphate buffer (pH 7.0) for 24 h without stirring; (3) against the buffer in step 2 diluted 1 : 1 with 100 mM phosphate buffer (pH 7.0) for 12 h without stirring; and (4) against 100 mM phosphate buffer (pH 7.0) for 48 h with very slow stirring. Insoluble protein aggregates were removed by centrifugation. Soluble protein fractions were collected and stored at −20 °C.

**Microscopy**

For electron microscopy, bacterial cells in the stationary phase were harvested, washed twice with 100 mM phosphate buffer (pH 7.0), and fixed using the glutaraldehyde/OsO4 method. After washing, agar blocks were dehydrated in an ethanol series beginning with 50% ethanol and finally placed in water-free acetone. Samples were then embedded in Spurr’s resin and polymerized at 60 °C for 2 days. Ultrathin sections were cut with an ultramicrotome with a diamond knife. Samples were poststained with 1% uranyl acetate for 1 h and Reynold’s lead citrate for 20 min and examined with a Zeiss EM 109 transmission electron microscope at 80 kV.

**Biofilm assay**

Bacterial cells carrying pCtufgp were grown in TSB medium supplemented with 0.5% glucose. Cultures were diluted 1 : 200 and incubated for 24 h on top of glass slides soaked with medium in a Petri dish. Biofilms on glass slides were viewed using a confocal microscope.

**Zymogram**

Proteins were analyzed by Tricine-10% SDS-PAGE (Schagger & von Jagow, 1987) and zymograms. Protein concentration was measured using the Biorad protein detection kit with bovine serum albumin as standard. Proteins were visualized by staining with Coomassie brilliant blue R-250. Bacteriolytic enzyme profiles were obtained with zymograms of polyacrylamide gels containing heat-inactivated *Micrococcus luteus* or *S. aureus* cells as described earlier (Heilmann et al., 1997).

**Peptidoglycan preparation and binding assay**

Peptidoglycan was isolated from stationary phase cultures of *S. aureus* SA113 and SA113 ΔtagO as described previously (de Jonge et al., 1992; Bera et al., 2005). Peptidoglycan binding was assayed at 4 °C with 1–2 μg purified (His6)-Ami or (His6)-Ami-R1,2, or (His6)-R1,2,3. Ni2+-NTA-puriﬁed proteins were mixed with 50 μg of *S. aureus* peptidoglycan in 500 μL of 100 mM phosphate buffer (pH 7.0) for 10 min at 4 °C. Unbound proteins in the supernatant were precipitated with 10% trichloroacetic acid. Bound protein retained in the peptidoglycan pellet after centrifuging the incubation mixture at 13 000 g for 5 min was washed with 100 mM phosphate buffer (pH 7.0) and then dissolved in SDS sample buffer. Samples were analyzed by electrophoresis in SDS-10% polyacrylamide gels.

**High-performance liquid chromatography (HPLC) separation of muropeptides**

Approximately 2 mg purified peptidoglycan was digested with either 100 μg (His6)-Ami-R1,2 or 5 μg lysostaphin or with both enzymes together for 16 h in 100 mM phosphate buffer (pH 7.0) in a total volume of 1 mL. Digestions were terminated by heating the samples at 90 °C for 5 min. Insoluble muropeptides were removed by centrifugation, and soluble fractions were dried in a rotary evaporator. The dried muropeptides were resuspended in water and reduced with sodium borohydride. Excess borohydride was destroyed by adding 20% phosphoric acid. Digested samples...
were applied to a Nucleosil 100 C18 column (Grom; 5 μm; 150 x 4 mm) at room temperature with a water/0.05% TFA : 25% acetonitrile/0.05% TFA gradient for 25 min at flow rate of 0.5 mL min⁻¹. Peaks were detected at 206 nm.

**Results**

**Construction of the ΔatlA::spc mutant and complementation**

In the genome of *Staphylococcus aureus* SA113, atlA is flanked upstream by genes encoding a proposed autolysin transcription attenuator and a putative acetyl transferase and downstream by a gene encoding a putative transcription regulator. The atlA mutant was generated in SA113 by homologous recombination using the temperature-sensitive plasmid pBT2ud, which contains the spectinomycin resistance gene (spc cassette) flanked by upstream (u) and downstream regions (d) of atlA (Fig. 1a). The isolated SA113 ΔatlA::spc mutant could be complemented by the *Staphylococcus epidermidis* atlE gene encoded on plasmid pRC14 (Heilmann et al., 1997) and by pTXatlE, in which atlE is transcribed by a xylose-inducible promoter (Fig. 1b).

**Microscopy studies**

The growth rate of the cells was not affected by the ΔatlA::spc mutation (not shown). However, cells of the SA113 ΔatlA::spc mutant formed huge clusters visualized by phase-contrast microscopy (Fig. 2a). When grown overnight without shaking, the mutant cells sedimented in the flask, whereas wild-type cells remained suspended. This result corroborates earlier observations that AtlA is involved in cell separation (Sugai et al., 1995) and suggests that the cluster formation in the mutant arises from hampered cell separation. We also compared the cell wall architecture of the wild-type and the ΔatlA::spc mutant using transmission electron microscopy (Fig. 2b). In ultra-thin sections, the ΔatlA::spc mutant is distinguished by a rough outer cell surface, particularly near the cell division site. A high proportion of the mutant cells were abnormally tetrameric, which indicates that cell separation was hindered.

**Role of AtlA in initial attachment**

The ability to form a biofilm is one of the virulence properties of *S. aureus*. Two distinct processes are involved: an initial attachment to the surface and the formation of

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**Fig. 1.** Allelic replacement of atlA in *Staphylococcus aureus* SA113 (a) and of the atlE expression vectors (b). The SA113 genomic atlA gene was replaced by the spectinomycin (spc) resistant cassette of pBT2ud by homologous recombination. spc in the vector is flanked by 1.3- and 1.6-kb regions found upstream and downstream of the atlA gene in the genome. pBT2ud was introduced into SA113 by transformation, and clones were screened for allelic replacement (chloramphenicol sensitivity and spectinomycin resistance). (b) Linear map of atlE expression vectors. atlE is transcribed from native promoter in pRC14 and from the xylose-inducible promoter in pTXatlE.
multilayer cell clustering facilitated by a slimy substance called the polysaccharide intercellular adhesin, PIA (Götz, 2002). The biofilm-forming capacity of the wild-type, the ΔatlA::spc mutant, and the complemented mutant was compared by cultivating cells on polystyrene or glass surfaces in a biofilm assay using the GFP encoded on plasmid pCtuf-gfp (Cramton et al., 2001) (Fig. 3a). All strains synthesized the GFP. Cells were grown for 24 h, and cells loosely bound to the surfaces were then washed away with phosphate-buffered saline. Cells adhering to the surfaces were monitored by fluorescence microscopy (Fig. 3b). The ΔatlA::spc mutant was deficient in primary attachment, whereas the wild-type and the complemented mutant were not. This result further supports the role of autolysin in biofilm formation.

The C-terminal repetitive sequences of the amidase are required for peptidoglycan binding

As the ΔatlA::spc mutant of S. aureus could be complemented by DNA encoding the AtlE amidase domain, the atlA and atlE genes and their corresponding enzymes are exchangeable and apparently have similar functions. We used the atlE-derived amidase constructs (Heilmann et al., 1997) for the remaining studies on the function of the repetitive sequences and biochemical analysis of the cleavage site.

We expressed the amidase domain of the atlE gene with no repeat regions (amiE) or with two repeat regions (amiE-R1,2), or the three repeat regions alone (R1,2,3) as N-terminal His-tag fusion proteins in Escherichia coli (Fig. 4a). The corresponding Ni2+-NTA purified proteins – the 62 kDa AmiE-R1,2, the 25 kDa AmiE, and the 53 kDa three repeat region R1,2,3 – formed homogenous bands in SDS-polyacrylamide gels (Fig. 4b). In the corresponding zymogram with heat-killed S. aureus cells (Fig. 4b) and in the lysis assay with isolated peptidoglycan (Fig. 4c), only AmiE and AmiE-R1,2 revealed cell lytic activity; the repeat sequences showed no activity as expected.

To study the interaction of these proteins with peptidoglycan of S. aureus, the proteins were incubated with purified peptidoglycan in phosphate buffer for 10 min at 4 °C. The mixtures were then centrifuged, and content of the three proteins in the supernatants and peptidoglycan pellets were analyzed by SDS-PAGE (Fig. 5a). AmiE-R1,2 and the R1,2,3 proteins were recovered in the peptidoglycan pellet, i.e. they bound to purified peptidoglycan. AmiE was only found in the supernatant, which indicated that the amidase domain alone has no, or only very low, peptidoglycan binding ability.

To determine the binding capacity of S. aureus peptidoglycan to the repeat domains, a dose-dependent binding assay was carried out with 50 μg purified peptidoglycan and increasing amounts (1–5 μg) of R1,2,3 (Fig. 5b). The binding capacity of 50 μg of peptidoglycan to the repeat domains was saturated by approximately 2 μg R1,2,3; higher concentrations remained more and more unbound and were recovered in the supernatant.
We also investigated whether the presence of cell wall teichoic acid (WTA) plays a role in peptidoglycan binding. We isolated peptidoglycan from the *S. aureus* SA113 ΔtagO mutant (Weidenmaier et al., 2004), and used it in the same type of dose-dependent binding assay. Essentially the same pattern as for native peptidoglycan was obtained (not shown), ruling out WTA as a binding anchor for the repeat sequences R1,2,3.

**Peptidoglycan hydrolysis activity of amidase**

*Staphylococcus aureus* peptidoglycan was solubilized with amidase or lysostaphin or both together. The soluble fractions were recovered and analyzed by reversed-phase HPLC (Fig. 6). Peaks 1 and 2 in Fig. 6a are products of amidase and lysostaphin double digestion as they were absent in amidase- or lysostaphin-digested samples. These two peaks were analyzed by liquid chromatography-mass spectrometry (LC-MS) and generated ions with m/z of 702.3 and 759.2 (Fig. 6a). The masses are in close agreement with the peptide subunit structure 1-Ala-d-iGln-l-Lys-(Gly)\(X\)-d-Ala-(Gly)\(Y\), where \(X+Y=5\) and 6, respectively. As lysostaphin cleaves within the glycine pentapeptide bridge (between Gly 2 and Gly 3, and Gly 3 and Gly 4), it is evident that amidase cleaves the amide bond between N-acetylmuramic acid and L-alanine.

**Discussion**

The major staphylococcal amidase is curious in so far, as the *atl* null mutant is affected in many respects and still its growth is hardly decreased in complex medium. The mutant cells form huge cell clusters, are defective in cell separation, form tetrad-shaped multicells that were septated but not separated from each other, are unable to proper adhere to various surfaces that is causative for the biofilm-negative phenotype, and they produce much lower amounts of secreted and cell-wall bound proteins. Apparently, whenever the major amidase gets mutated (Takahashi et al., 2002) or attacked, abnormal cluster formation occurs. For example, the addition of protease to exponentially growing cultures that degrades the major amidase led to the formation tetrad-shaped staphylococcal multicells (Yabu et al., 1997).

The question is why does such a multiple disadvantage of an *atl* null mutant has ultimately so little impact on growth. We think that the recently described cell wall hydrolase Aaa (autolysin/adhesin from *S. aureus*) (Heilmann et al., 2005) counterbalances to some extent the deleterious effect in the *DatlA::spc* mutant. This is corroborated by the zymogram (data not shown) that shows that the lytic activity of Aaa is increased in the *atl* mutant. This illustrates that staphylococci are able to cope with the loss of Atl by up regulation of the *aaa* expression. How this regulation occurs is unknown,
but it appears to be a general phenomenon as it was also observed in the \textit{atlE} mutant \textit{S. epidermidis} (Heilmann et al., 1997). However, Aaa up regulation is not sufficient to prevent cell aggregation, tetrad formation or to bring back the ability of biofilm formation. It appears that up regulation of \textit{aaa} is a regime for the extreme emergency; it allows survival but does not cure the severe consequences of an \textit{atl} mutation.

We also attempted to determine the role of the three repetitive sequences located between the amidase and the glucosaminidase domains of the autolysins (Fig. 4a). As \textit{AtlA} and \textit{AtlE} show essentially the same genetic organization, we chose \textit{AtlE} for further studies. We have previously shown that an extracellular protease cleaves the two catalytic domains at valine-303 and leucine-845 in such a way that the amidase contains two repeat sequences (R1 and R2) at its C-terminus, and the glucosaminidase contains the R3 repeat at its N-terminus (Heilmann et al., 1997). It has been suggested that the repeat domains direct the 62 kDa amidase and 51 kDa glucosaminidase to a specific receptor at the equatorial surface ring of staphylococci, thereby allowing localized peptidoglycan hydrolysis and separation of the dividing cells (Sugai et al., 1997; Baba & Schneewind, 1998).

Here, we investigated peptidoglycan binding and lytic activity of the amidase with and without the repeats (Fig. 4a). Slight difference in lytic activity was observed in zymogram and in liquid assays (Figs 4b and c). Therefore, the repeat domains contribute little to the lytic activity. However, the repeat sequences play a role in peptidoglycan binding (Fig. 5a). As both amidase and glucosaminidase are

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig5}
\caption{The role of the repeat sequences in binding to peptidoglycan. (a) Peptidoglycan binding assay. Approximately 1 \( \mu \)g of AmiE-R\(_{1,2}\), R\(_{1,2,3}\), or AmiE was incubated with 50 \( \mu \)g purified peptidoglycan for 10 min at 4 \( ^\circ \)C in 100 mM phosphate buffer (pH 7.0). After centrifugation, peptidoglycan-bound (pellet) and unbound proteins (supernatant) were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. The control contained no peptidoglycan. (b) Dose-dependent binding of R\(_{1,2,3}\) to peptidoglycan lacking cell wall teichoic acid (WTA). Peptidoglycan was isolated from \textit{Staphylococcus aureus} SA113 \textit{ΔtagO}, a mutant deficient in WTA. Peptidoglycan (50 \( \mu \)g) was mixed with 1, 2, 3, 4, and 5 \( \mu \)g of R\(_{1,2,3}\) and analyzed as described above.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig6}
\caption{Reversed-phase HPLC (a) and LC-MS (b) analysis of the soluble muropeptides released from \textit{Staphylococcus aureus} peptidoglycan after incubation with AmiE-R\(_{1,2}\) and lysostaphin. Digested muropeptides were reduced with sodium borohydride and applied to a C18 nucleosil column. Peptides were eluted with a water/0.05\% TFA to 25\% acetonitrile/0.05\% TFA gradient in 25 min. Peaks were detected by absorbance at 206 nm. Peaks 1 and 2 are the two major peaks generated from amidase-lysostaphin double digestion. HPLC fractions containing peaks 1 and 2 were analyzed by LC-MS. The observed \( m/z \) of the ion signals are 702.3 and 759.2, which indicates the structures \( \text{L-Ala-D-iGln-L-Lys-(Gly)\(_2\)-D-Ala-(Gly)\(_3\)} \) and \( \text{L-Ala-D-iGln-L-Lys-(Gly)\(_3\)-D-Ala-(Gly)\(_3\)} \), respectively.}
\end{figure}
preferentially located at the equatorial surface ring, we speculate that the repeat sequences R\textsubscript{1,2,3} probably have a higher affinity to premature peptidoglycan structures. The cell wall targeting function of repeat sequences from autolysin/adhesin protein Aas of \textit{Staphylococcus saprophyticus} has been previously studied and similar result obtained (Hell \textit{et al.}, 2003).

We also addressed the question whether the Ami or Ami-R\textsubscript{1,2} show different activities with peptidoglycan that lacks either WTA, O-acetylation, or both. \textit{S. aureus} peptidoglycan is modified during the course of its biosynthesis. One modification comprises the linkage of WTA (Weidenmaier \textit{et al.}, 2004) the other modification is the O-acetylation of the muramic acid residues at the C\textsubscript{6} position (Bera \textit{et al.}, 2005). Both modification steps are thought to be carried out extracellular. Here we used isolated peptidoglycan lacking O-acetylation or WTA for binding studies with R\textsubscript{1,2,3} and found no evidence for preferential binding of either of these unmodified peptidoglycan structures. Also no difference in lysis activity was observed. Therefore, WTA apparently does not act as a binding anchor for the repeat sequences as we speculated earlier. The question by what particular cell wall structure or component Atl proteins are attached to the equatorial surface ring is still unanswered.

The Atl-derived amidase has been referred to as a N-acetylmuramyl-1-alanine amidase. However, no clear biochemical proof has been carried out. The designation was based on sequence similarity of its N-terminus with the N-terminus of the \textit{Bacillus subtilis} cell wall hydrolase CwlA and the pneumococcal Ejl bacteriophage amidase (Kuroda & Sekiguchi, 1990; Diaz \textit{et al.}, 1992). We biochemically determined the exact cleavage site of amidase. The obtained masses of the two cleavage products obtained (Fig. 6) matched precisely with the sizes expected when peptidoglycan is cleaved between N-acetylmuramic acid and -alanine.

This study shows that the Atl-derived amidase is necessary to cleave the amide bond that links the peptide subunit to the muramic acid residues in the glycan strands of the murein netting. As this is one of the crucial stress-bearing bonds in the murein sacculus, amidases are potentially autolytic enzymes. The specific function of Atl is the splitting of the septum during cell division to allow the separation of the daughter cells. Consequently, mutants deficient in \textit{atl} are growing in large cell clusters. If one considers the multiple phenotypic alterations associated with the \textit{atl} mutants, particularly the difficulties in the separation of daughter cells after cell division, it is surprising that growth and virulence are not more affected in the mutants.

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