

# Targeting of muralytic enzymes to the cell division site of Gram-positive bacteria: repeat domains direct autolysin to the equatorial surface ring of *Staphylococcus aureus*

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***Staphylococcus aureus* secretes autolysin (Atl) to complete cell division by hydrolyzing its thick cell wall layer at a designated site, known as the equatorial surface ring. Secreted pro-Atl (1256 amino acids) is cleaved at residues 198 and 775 to generate a propeptide, amidase and glucosaminidase, respectively. Here we examined the mechanism that directs amidase and glucosaminidase to the cell division site on the staphylococcal surface. Targeting of pro-Atl to the cell surface occurred prior to its proteolytic processing. Three repeat domains (R1, R2 and R3) located at the center of pro-Atl are necessary and sufficient for the targeting of reporter proteins to the equatorial surface ring. Pro-Atl cleavage at residue 775 separates the polypeptide such that R1 and R2 are linked to the C-terminus of amidase, whereas R3 is located at the N-terminus of glucosaminidase. Thus, it appears that the repeat domains direct pro-Atl, amidase and glucosaminidase to a specific receptor at the equatorial surface ring of staphylococci, thereby allowing localized peptidoglycan hydrolysis and separation of the dividing cells.**

**Keywords:** amidase/autolysin/equatorial surface ring/  
glucosaminidase/*Staphylococcus aureus*

## Introduction

The cell wall envelope of Gram-positive bacteria can be viewed as a macromolecular organelle that requires the proper functioning of several enzymatic machines for its concerted assembly and turnover (Strominger *et al.*, 1967; Labischinski and Maidhof, 1994). Cell wall assembly is thought to proceed as the polymerization of peptidoglycan precursor molecules at designated sites of the envelope (Cole, 1965; Matsushashi, 1994; van Heijenoort, 1994) and is catalyzed by a group of penicillin-binding proteins (Ghuysen, 1991). Wall turnover is also localized and occurs at the sites of future cell division (Díaz *et al.*, 1989; Hobot and Rogers, 1991). Staphylococci are spherical cells which divide perpendicular to the previous division plane, requiring cell wall hydrolysis at an equatorial surface ring (Giesbrecht *et al.*, 1976; Yamada *et al.*, 1996). This localized hydrolysis of the rigid peptidoglycan sacculus has been visualized by electron microscopy and is necessary to complete cell division and separation (Giesbrecht *et al.*, 1976, 1985, 1992). Nevertheless, the mechanism by which staphylococci hydrolyze peptidoglycan at the equatorial

ring structure hitherto has not been resolved (Shockman and Höltje, 1994).

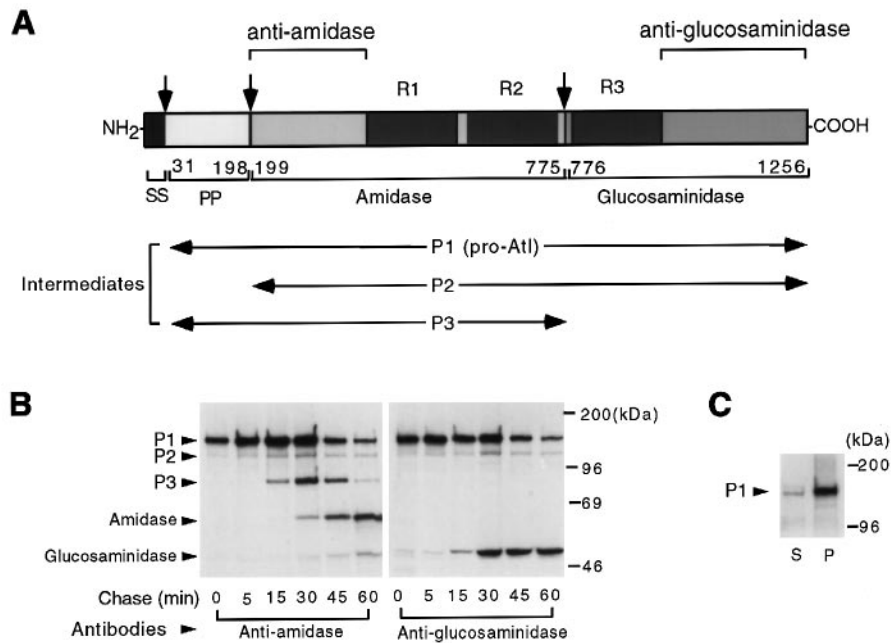
Mutant staphylococci defective in peptidoglycan hydrolysis at the equatorial surface ring grow as large clusters of non-separated cells (Sugai *et al.*, 1995). These mutant cells harbor a mutation in the *atl* gene (Sugai *et al.*, 1995), which has been cloned and sequenced from *Staphylococcus aureus* (Oshida *et al.*, 1995) as well as from the related organism *S.epidermidis* (Heilmann *et al.*, 1997). The large Atl precursor protein (1256 amino acids) is exported from the staphylococcal cytoplasm by an N-terminal signal sequence (Oshida *et al.*, 1995). Two cleavage events, one at residue 198 and another at position 775 of pro-Atl, generate mature amidase (62 kDa) and glucosaminidase (51 kDa) as well as an N-terminal propeptide of unknown function (Oshida *et al.*, 1995). When separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and assayed for enzymatic activity by overlaying bacterial cells as a substrate, pro-Atl, amidase and glucosaminidase all displayed murein hydrolase activity (Oshida *et al.*, 1995). Amidase and glucosaminidase have been purified from either the culture medium or after salt extraction of staphylococci (Sugai *et al.*, 1995; Komatsuzawa *et al.*, 1997). When examined by immunoelectron microscopy with specifically raised antisera, amidase and glucosaminidase were found localized to the equatorial ring on the staphylococcal cell surface that marks the future cell division site (Yamada *et al.*, 1996). Thus, addressing amidase and glucosaminidase to a designated site within the bacterial envelope presumably permits localized murein hydrolysis without destroying the entire cell wall sacculus (Giesbrecht *et al.*, 1985).

Here we investigated the elements necessary and sufficient for pro-Atl targeting to the equatorial ring on the staphylococcal surface. Pro-Atl, amidase and glucosaminidase all bound to the staphylococci. Cytoplasmic export and surface targeting occurred rapidly (<5 min), whereas processing of pro-Atl required >40 min. Three repeat domains, R1, R2 and R3, are positioned at the center of pro-Atl such that mature amidase and glucosaminidase retain either two C-terminal (R1, R2) or one N-terminal (R3) repeat domain, respectively. The repeat domains were each sufficient to direct reporter proteins to the equatorial surface ring, either when secreted by staphylococci or when added externally to the cells. We measured 10<sup>8</sup> repeat domain-binding sites per colony-forming unit (c.f.u.) and propose the existence of a specific receptor that is positioned at the equatorial ring on the staphylococcal surface.

## Results

### **Autolysin processing and targeting to the staphylococcal surface**

Previous work left unresolved which of the Atl species, pro-Atl, amidase or glucosaminidase, is targeted to the



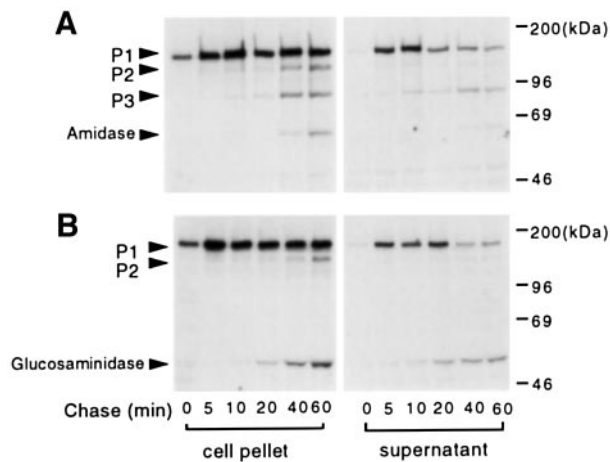
**Fig. 1.** Processing of staphylococcal autolysin (Atl). (A) The drawing depicts the primary structure of the Atl precursor which is exported by an N-terminal signal sequence (SS). Two proteolytic cleavage events at residues 198 and 775 generate the P2 or P3 intermediates as well as the mature pro-peptide (PP), amidase and glucosaminidase. Amino acid positions are relative to the first methionine of Atl. Hatched segments indicate the three repeat domains (R1, R2 and R3). (B) Pulse-chase analysis of autolysin processing. Cultures of *S.aureus* strain OS2 were labeled with [<sup>35</sup>S]methionine for 2 min followed by a chase with non-radioactive amino acids. At timed intervals, processing was stopped by precipitation of protein with TCA. Proteins were solubilized in hot SDS, immunoprecipitated with anti-amidase or anti-glucosaminidase [epitopes used to raise these antibodies are shown in (A)] and analyzed by 8% SDS-PAGE and fluorography. (C) Pro-Atl targeting to the staphylococcal surface. Pulse-labeled staphylococcal cultures were centrifuged and the sedimented cells (P, pellet) were separated from the culture medium in the supernatant (S). Proteins in both samples were precipitated with TCA, solubilized in hot SDS, immunoprecipitated with anti-amidase and analyzed by SDS-PAGE and fluorography. The migration of protein size markers during SDS-PAGE is indicated in kDa.

cell surface and where pro-Atl processing occurs (Sugai *et al.*, 1995; Komatsuzawa *et al.*, 1997). To address these questions, we first measured the secretion and processing of Atl in a pulse-chase experiment. Strain OS2 staphylococci were grown in minimal medium and newly synthesized polypeptide was labeled with [<sup>35</sup>S]methionine for 2 min, after which all further labeling was quenched by the addition of excess non-radioactive methionine (chase). At timed intervals during the chase, Atl processing was stopped by precipitation of proteins with ice-cold trichloroacetic acid (TCA), followed by an acetone wash. Precipitated proteins were solubilized by boiling in a buffer containing 4% SDS prior to immunoprecipitation, SDS-PAGE and fluorography (Figure 1). In *S.aureus* cells, this procedure solubilizes proteins that are either secreted into the medium or non-covalently associated with the cell surface, whereas cytoplasmic, membrane or cell wall-linked proteins remain insoluble after TCA precipitation and boiling in SDS (Schneewind *et al.*, 1992).

Pro-Atl (P1) was identified as a 134 kDa species that immunoprecipitated with both anti-glucosaminidase and anti-amidase (Figure 1). No pro-Atl processing occurred during the first 5 min of the chase; then, the amount of radioactive pro-Atl decreased, with 10% remaining even after a 60 min chase. Another intermediate of Atl processing, designated P2 (115 kDa), also reacted with both anti-amidase and anti-glucosaminidase, and presumably resulted from pro-Atl cleavage at residue 198, thereby removing the pro-peptide from the remainder of the polypeptide (Figure 1A). A third intermediate species (P3, 80 kDa) was precipitable with anti-amidase but not with

anti-glucosaminidase and, therefore, probably represented the N-terminal cleavage fragment of pro-Atl after processing at residue 775 (Figure 1). Mature amidase first appeared 30 min after the addition of the chase and its concentration steadily increased until the 60 min time interval. In contrast, glucosaminidase was generated earlier (15 min) and its concentration increased further until 30 min of chase but then remained constant (Figure 1B). The relative abundance of the P3 precursor over the P2 molecule suggested that residue 775 was the preferred cleavage site of pro-Atl. Consistent with this notion is the observation that radiolabeled glucosaminidase was generated prior to amidase.

Staphylococcal cell division is completed within a 20 min period; however, pro-Atl processing was observed to require a longer time (>40 min). To initiate peptidoglycan hydrolysis and cell division at the equatorial surface ring, the enzymatically active pro-Atl species might be targeted to this site prior to its proteolytic processing. This was tested and, 3 min after the addition of the chase, pulse-labeled staphylococci were centrifuged and proteins in the culture medium were separated from those that sedimented with the cells. Almost all pro-Atl was found in the staphylococcal cell pellet (Figure 1C). To determine whether pro-Atl, amidase or glucosaminidase remained bound to the cell surface, pulse-labeled staphylococci were centrifuged at timed intervals and proteins in the culture medium were separated from those that were attached to the staphylococcal surface (Figure 2). Most pro-Atl remained bound to staphylococci at each time interval of the chase. Mature amidase appeared after 40 min chase

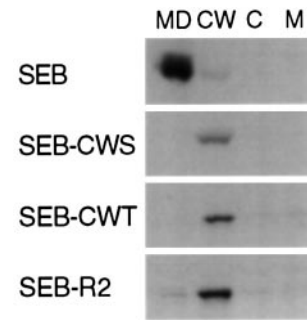


**Fig. 2.** Targeting of pro-Atl, amidase and glucosaminidase to the staphylococcal cell surface. **(A)** Staphylococcal cultures were pulse-labeled as described in the legend to Figure 1. At the indicated time intervals during the chase, culture aliquots were centrifuged to separate the staphylococcal cell pellet from the culture medium in the supernatant. Proteins in both samples were precipitated with TCA, immunoprecipitated with anti-amidase and analyzed by SDS-PAGE. **(B)** A similar experiment to that described in (A) but proteins were immunoprecipitated with anti-glucosaminidase. The migration of protein size markers during SDS-PAGE is indicated in kDa.

and was mostly attached to staphylococci (Figure 2A). Similarly, most pulse-labeled glucosaminidase sedimented with the staphylococci, and this distribution did not change during the 60 min chase (Figure 2B). Together, these results suggested that pro-Atl was targeted to the staphylococcal cell surface prior to its proteolytic cleavage. Furthermore, all three species, pro-Atl, amidase and glucosaminidase, remained largely bound to the cell surface during the 60 min observation period.

#### Repeat domains of Atl and their role in targeting of enterotoxin B fusions

Pro-Atl encompasses three repeat domains that are similar but not absolutely identical to one another. After cleavage at residue 775, the first two repeat domains (R1 and R2) remain linked to the C-terminal part of amidase, whereas the third domain (R3) is attached to the N-terminus of glucosaminidase (Oshida *et al.*, 1995). Repeat domains of other muralytic enzymes of Gram-positive bacteria previously have been shown to be required for their proper targeting to the cell wall (Wren, 1991; Joris *et al.*, 1992). For example, the six repeat domains at the C-terminal end of *Streptococcus pneumoniae* LytA function to target this muralytic enzyme to the bacterial surface via binding to choline within the pneumococcal teichoic acid moieties (Sánchez-Puelles *et al.*, 1990). We therefore suspected that the repeat domains of pro-Atl might be responsible for its targeting to the equatorial ring on the staphylococcal surface. If so, repeat domain fusions to the C-terminus of the normally secreted enterotoxin B (Seb) (Tweten and Iandolo, 1983) might direct the hybrid protein to the bacterial cell surface. Pulse-labeled staphylococci expressing various Seb constructs were fractionated into four compartments (Figure 3). The medium was separated from the bacterial cells by centrifugation (MD). The staphylococcal envelope was solubilized with lysostaphin (CW) and the resulting protoplasts were collected by



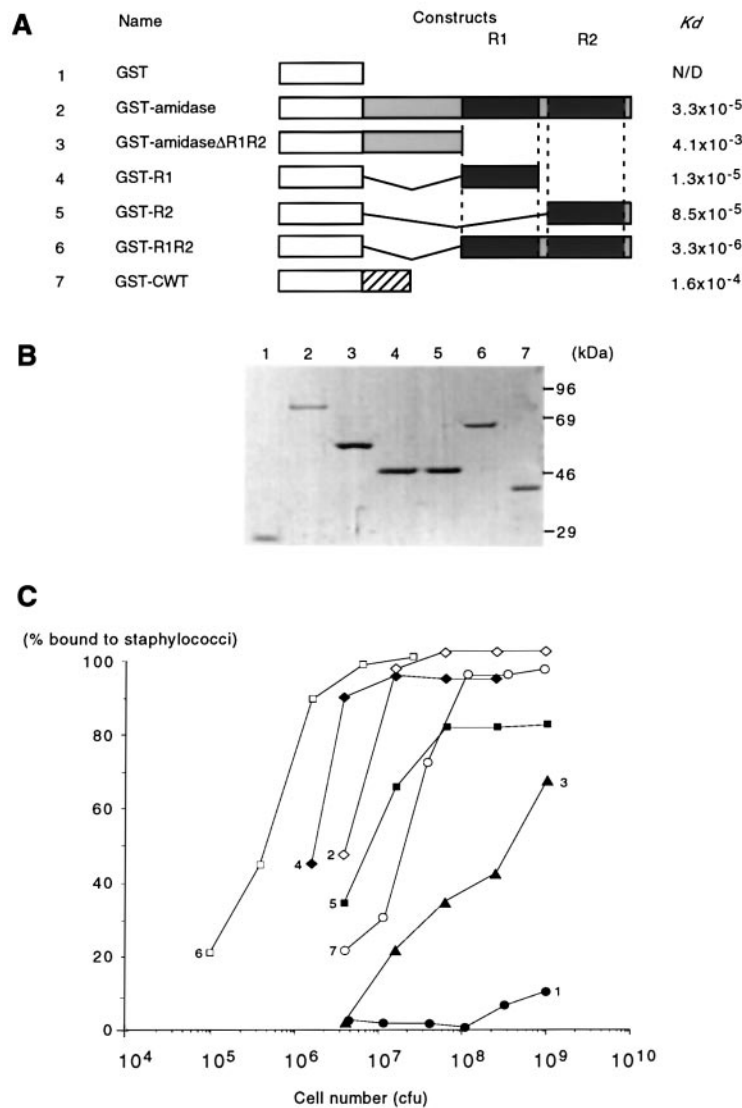
**Fig. 3.** The R2 repeat domain of Atl is sufficient to direct enterotoxin B (Seb) to the staphylococcal envelope. Cell fractionation of *S.aureus* OS2 strains expressing either wild-type Seb, or C-terminal fusions of Seb to the cell-wall sorting signal of protein A (Cws), the cell-wall targeting signal of lysostaphin (Cwt) or the R2 repeat domain of Atl (R2). Staphylococcal cultures were pulse-labeled with [<sup>35</sup>S]methionine for 2 min followed by a chase of non-radioactive amino acids for 5 min. Cultures were centrifuged to separate the medium (MD) from the staphylococcal cells. The cell wall was digested with lysostaphin (CW). The resulting protoplasts were sedimented by centrifugation, lysed and membranes (M) separated from the cytosol (C) by ultracentrifugation. Proteins in all fractions were precipitated with TCA, solubilized in hot SDS, immunoprecipitated with anti-Seb and analyzed by SDS-PAGE and fluorography.

centrifugation. After cell lysis, the cytoplasmic membrane (M) was sedimented by ultracentrifugation and separated from the cytosol (C) (Schneewind *et al.*, 1993). Seb-R2, containing the R2 repeat domain fused to the C-terminus of Seb, was found mostly in the solubilized cell-wall compartment (Figure 3), indicating that the repeat domain was sufficient to direct the hybrid protein to the cell-wall envelope. As controls for correct fractionation, wild-type Seb was located in the culture medium, whereas the peptidoglycan-linked Seb-Cws as well as the targeted Seb-Cwt were found in the solubilized cell wall compartment (Baba and Schneewind, 1996) (Figure 3).

#### Targeting of hybrid glutathione S-transferase to the staphylococcal surface

We asked if the repeat domains of amidase were necessary and sufficient for cell surface targeting of externally added protein. Towards this end, we constructed fusions of staphylococcal amidase to the C-terminal end of glutathione S-transferase (GST) (Smith and Johnson, 1988) (Figure 4A). GST fusions were expressed in *Escherichia coli*, purified by affinity chromatography and labeled at a unique kinase site of GST with [<sup>32</sup>P]ATP and cAMP-dependent heart muscle kinase while bound to glutathione-Sepharose (Kaelin *et al.*, 1992). The beads were washed and hybrid GST was eluted with glutathione prior to SDS-PAGE followed by Coomassie Blue staining (Figure 4B).

To measure binding of GST fusions to the staphylococcal surface, ~10 000 c.p.m. of labeled polypeptide (between 10 and 100 pmol) were incubated with various numbers of staphylococcal cells (Figure 4C). Bound polypeptide sedimented with the cells during a brief centrifugation step. The depletion of GST fusions from the medium was measured by scintillation counting and plotted against the number of added staphylococci. Fifty percent depletion of GST-amidase (10 pmol) was observed when 10<sup>7</sup> c.f.u. of *S.aureus* OS2 were added during the sedimentation assay. As a control, GST alone could not be sedimented by increasing the number of added staphylococci. A fusion

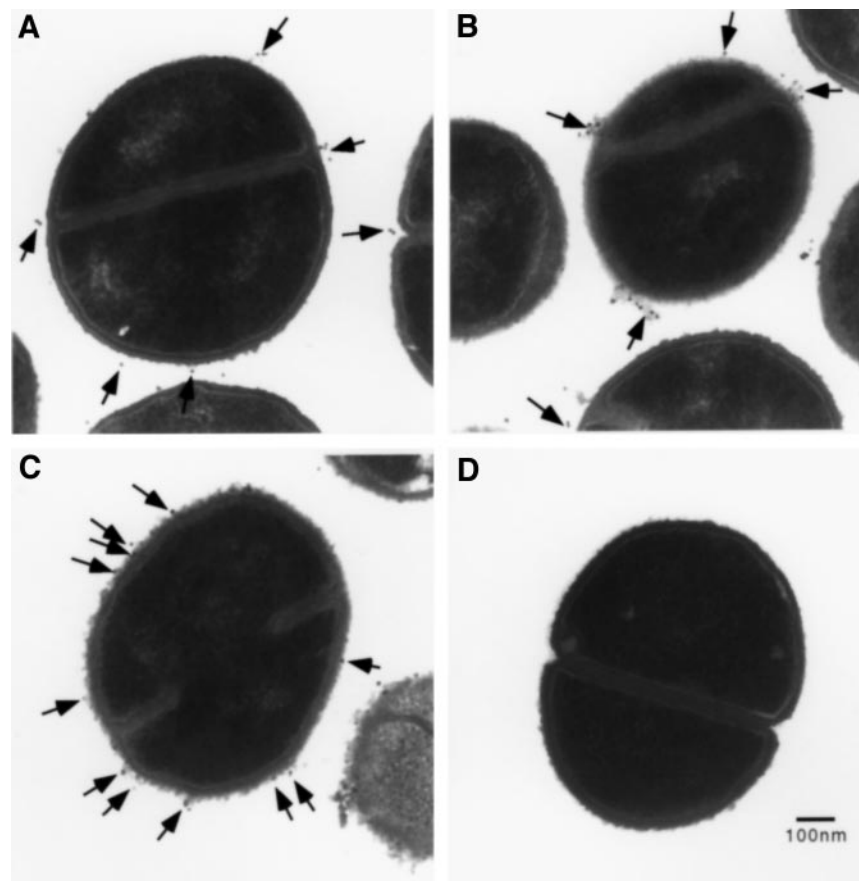


**Fig. 4.** Targeting of GST fusion proteins to the staphylococcal surface. (A) Fusions of staphylococcal amidase sequences to the C-terminus of GST. Hybrid polypeptides comprised either full-length amidase, the catalytic domain or the R1 and R2 repeat domains, alone or in combination. The relative affinity of each fusion protein for  $10^8$  staphylococcal c.f.u. was determined and is indicated as the dissociation constant ( $K_d$ ). (B) Coomassie Blue stained SDS-PAGE analysis of purified GST fusion proteins (1  $\mu$ g each), lanes (1) GST, (2) GST-amidase, (3) GST-amidase $\Delta$ R1R2, (4) GST-R1, (5) GST-R2, (6) GST-R1R2 and (7) GST-Cwt. (C) Binding of GST fusion proteins to staphylococci. One microgram of radiolabeled GST fusion protein was added to various numbers of staphylococci (c.f.u.), incubated for 10 min, and centrifuged to co-sediment bound fusion protein with the cells. Aliquots of the supernatant were analyzed by scintillation counting and plotted against the number of added cells (log scale). Symbols indicate GST-amidase ( $\diamond$ ), GST-amidase $\Delta$ R1R2 ( $\blacktriangle$ ), GST-R1R2 ( $\square$ ), GST-R1 ( $\blacklozenge$ ), GST-R2 ( $\blacksquare$ ), GST-Cwt ( $\circ$ ) and GST alone ( $\bullet$ ).

(GST-amidase $\Delta$ R1R2) comprising the enzymatic domain of amidase without its C-terminal repeat domains (R1 and R2) required  $\sim 10^9$  c.f.u. for 50% depletion. This weak interaction may be explained as the binding of enzyme to substrate, i.e. amidase to peptidoglycan. Fusion of either single or both R1 and R2 repeat domains to GST resulted in polypeptides that also bound the staphylococcal surface, similarly to GST-amidase.

Our measurements indicated that binding of GST-amidase to staphylococci was saturated at  $10^8$  molecules per c.f.u. The affinity of GST fusions for the repeat domain receptor was determined by adding increasing amounts of fusion protein to  $2 \times 10^8$  c.f.u. and measuring the amount of bound and unbound polypeptide after a brief centrifugation step. GST-amidase ( $K_d$   $3.3 \times 10^{-5}$ /M) bound with lesser affinity to the staphylococcal cell surface than GST-R1,

GST-R2 or GST-R1R2, respectively ( $K_d$   $1.3 \times 10^{-5}$ /M,  $8.5 \times 10^{-5}$ /M,  $3.3 \times 10^{-6}$ /M) (Figure 4A). The  $K_d$  value of GST-R1R2 revealed an additive effect on the affinity of the two repeat domains for their staphylococcal surface receptor as compared with fusions bearing a single repeat domain. To compare the affinity of the Atl repeat domain for its surface receptor with that of another targeting signal, we employed lysostaphin, a bacteriocin secreted by *S.simulans* biovar *staphylolyticus* (Schindler and Schuardt, 1964; Heinrich *et al.*, 1987; Recsei *et al.*, 1987) which binds to its receptor on the surface of *S.aureus* cells (Baba and Schneewind, 1996). A GST fusion harboring the targeting signal of lysostaphin bound to staphylococci with lesser affinity ( $K_d$   $1.6 \times 10^{-4}$ /M) than the GST repeat domain fusions to their receptor. Together, these results suggest that the R1, R2 and R3 repeat



**Fig. 5.** Repeat domains direct GST fusion proteins to the equatorial ring on the staphylococcal surface. Purified GST fusion protein was added to staphylococci, cells were washed, and incubated first with anti-GST and then with protein A–colloidal gold conjugate (10 nm particles). Samples were fixed with glutaraldehyde embedded in paraffin, thin sectioned and viewed under a transmission electron microscope. Panels show representative pictures of staphylococci that were incubated with either GST–amidase (A), GST–R1R2 (B), GST–Cwt (C) or GST alone (D), respectively.

domains of amidase and glucosaminidase are necessary and sufficient for the targeting of these enzymes to their staphylococcal surface receptors.

#### **Targeting of GST fusion proteins to the equatorial surface rings of staphylococci**

Binding of GST fusion proteins to the staphylococcal surface does not definitively demonstrate protein targeting to the equatorial surface ring. The precise location of GST fusion proteins bound to the staphylococcal surface was visualized by electron microscopy (Yamada *et al.*, 1996). Staphylococci were incubated with GST fusion proteins, stained with anti-GST and protein A–gold particle conjugate, fixed with glutaraldehyde, thin sectioned and viewed under transmission electron microscopy. Previous work demonstrated that Atl staining of the equatorial surface rings occurs at four distinct sites of sectioned staphylococci, each separated by a 90° angle on a circle representing the staphylococcal surface (Yamada *et al.*, 1996). Two of these sites occur at the septum of dividing cells and reveal the septal surface ring. The other two clusters of Atl binding represent a second perpendicular surface ring that marks the future cell division site. When investigated for bound GST–amidase as well as GST–R1R2, protein A–gold particles were identified in clusters at four sites on the staphylococcal surface, each separated by ~90° (Figure 5). An average of 6.8 gold particles was observed

**Table I.** Distribution of GST fusion proteins on the staphylococcal surface<sup>a</sup>

| Fusion protein | Gold particles/cell | Gold particles at division septum (%) |
|----------------|---------------------|---------------------------------------|
| GST–amidase    | 6.83                | 33%                                   |
| GST–R1R2       | 6.77                | 33%                                   |
| GST–Cwt        | 10.53               | 7%                                    |
| GST            | 1.23                | 4%                                    |

<sup>a</sup>*Staphylococcus aureus* OS2 cells were incubated with GST fusion protein, followed first by anti-GST and then by protein A–conjugated gold particles, fixed with glutaraldehyde and embedded for thin sectioning. Samples were viewed under a transmission electron microscope, photographed, and cells displaying septa were analyzed for the number of surface-located gold particles per cell as well as the number of gold particles at the cell division septum. Data were collected from 25–45 dividing cells.

on the cell surface, and 33% of these particles were located immediately adjacent to the cell division septum (Table I). (Only cells displaying a division septum were included in these measurements.) These observations are in agreement with the expected localization (50%) of gold particles to the septal surface ring, indicating that the repeat domains direct fusion proteins to both equatorial surface rings. As control, few protein A–gold particles were found on the cell surface when GST alone was

incubated with staphylococci (on average one gold particle per cell). The binding of GST-Cwt to the lysostaphin receptor was also measured by the deposition of protein A-gold particles on the staphylococcal surface (10.5 particles per cell). In contrast to the defined and regular staining observed for GST-amidase and GST-R1R2, GST-Cwt was found deposited irregularly on staphylococcal cells (Figure 5C and Table I); 7% of all particles were located adjacent to the cell septum, indicating that the lysostaphin receptor is not targeted to the equatorial surface rings.

## Discussion

Staphylococcal cell division requires localized hydrolysis as well as *de novo* biosynthesis of the thick cell wall peptidoglycan layer (Giesbrecht *et al.*, 1976). The mechanisms by which the responsible enzymatic machines, either penicillin-binding proteins or cell wall hydrolases, are directed to the future cell division sites have thus far remained obscure (Shockman and Barrett, 1983; Shockman and Hölftje, 1994). Recent work on staphylococcal amidase and glucosaminidase revealed that both enzymes were localized to an equatorial ring on the cell surface that marked the future division site (Yamada *et al.*, 1996). This observation suggested that each enzyme contained targeting information that directed them to a specific surface receptor which was positioned at the cell equator. Presumably, these targeted muralytic enzymes do not hydrolyze the peptidoglycan randomly, but separate dividing cells by cleaving the cell wall at designated sites (Giesbrecht *et al.*, 1992).

Most Gram-positive bacteria are surrounded by a structurally similar, if not identical, peptidoglycan layer (Schleifer and Kandler, 1972). Thus, targeting of muralytic enzymes cannot be achieved by simple enzyme-substrate interactions but requires specific surface receptors (Shockman and Hölftje, 1994). For example, choline within teichoic acid moieties serves as a receptor for the LytA enzyme of *S.pneumoniae* (Sánchez-Puelles *et al.*, 1986). Other bacterial surface receptors have not been characterized thus far; however, their identification might provide us with an understanding of topological arrangements within the bacterial cell wall. Muralytic enzymes typically harbor sequence elements that direct them to the peptidoglycan of specific species (Sánchez-Puelles *et al.*, 1990; Joris *et al.*, 1992). These enzymes generally are exported from the cytosol and secreted into the surrounding medium. Under physiological conditions, i.e. in the presence of mixed bacterial populations, muralytic enzymes need to distinguish between the surface receptors of different microbial species. Thus, it seems likely that the targeting mechanisms of murein hydrolases employ species-specific receptors for either physiological cell-wall turnover or the bacteriolytic killing of competing microorganisms (Baba and Schneewind, 1998).

This work provides a rationale for the purification of a staphylococcal surface receptor to which the repeat domains of pro-Atl bind. Because of the equatorial distribution of the Atl enzyme on the staphylococcal cell (Yamada *et al.*, 1996), it is probable that the surface receptor is distributed in the same manner. The abundance of repeat domain-binding sites ( $10^8$  per c.f.u.) suggests

that either the receptor may be a major component of the staphylococcal envelope or that each receptor molecule may contain multiple binding sites. Characterization of the receptor might reveal a mechanism with which staphylococci mark their future cell division site. Because the plane of division changes for every newborn cell that presumably still contains at least half of its parental cell wall envelope, this could mean that receptors for the repeat domains are turned over to prevent hydrolysis at old cell division sites. Hence, targeting of pro-Atl, amidase or glucosaminidase to the cell surface might be a cyclical event in which all bound molecules are solubilized by their own enzymatic activities.

## Materials and methods

### Bacterial strains and plasmids

*Staphylococcus aureus* strain OS2 (Schneewind *et al.*, 1992) was used for all experiments involving staphylococci. The *E.coli* strains XL-1 Blue (Bullock *et al.*, 1987) and BL-21 (Studier *et al.*, 1990) were employed for molecular cloning and protein purification, respectively. To construct GST fusions, DNA fragments specifying the respective polypeptide sequences were amplified with PCR and *S.aureus* OS2 chromosomal template DNA. Primers were Bam-AM (5'-AAGGATCCGTTTCAGCACAAACCAAGATCA-3') and AM-Eco (5'-AAGAATCTTATTTTACAGCTGTTTTGGTTGT-3') for GST-amidase, Bam-A and ΔAM-Eco (5'-AAGAATCTTAACCAAGTTGATGGTTTCGACGGT-3') for GST-amidaseΔR1R2, Bam-R1 (5'-AAGGATCCAAATTAAACAGTTGCTGCAAACA-3') and R1-Eco (5'-AAGAATCTTATAAATATGCTTTACTTACCAACCA-3') for GST-R1, Bam-R2 (5'-AAGGATCCAATAAATTAACAGTTTCATCATTAATA-3') and AM-Eco for GST-R2, and Bam-R1 and AM-Eco for GST-R1R2. Each PCR product was first cloned into pCR-Blunt (InVitrogen), excised via the abutted *Bam*HI and *Eco*RI sites, and inserted into the corresponding sites of pGEX-2TK (Pharmacia). The Seb-R1 and Seb-R2 fusions were also constructed by PCR amplification using pGST-amidase template DNA and the primers R-Bam (5'-AAGGATCCTTATAAATATGCTTTACTTACCAACCA-3') and Kpn-R2 (5'-AAGGTACCAATAAATTAACAGTTTCATTAATA-3'). After cloning into pCR-Blunt, the fragment was released by *Kpn*I and *Bam*HI digestion, and cloned into the corresponding sites of pSEB-SPA<sub>Kpn</sub> (Schneewind *et al.*, 1993) to generate pSeb-R2 which was electroporated into *S.aureus* strain OS2.

### Pulse-chase experiments and immunoprecipitation

Staphylococcal cultures were grown overnight in chemically defined medium, diluted into modified minimal medium 4 and grown at 37°C to OD<sub>600</sub> 0.5. Culture aliquots were pulse-labeled with [<sup>35</sup>S]methionine and all further incorporation of radioactivity into polypeptides quenched by the addition of chase (50 μl of 10% casamino acids, 20 mg/ml methionine to 1 ml of culture). At timed intervals, culture aliquots were precipitated with 5% TCA, protein pellets washed with acetone, dried and solubilized in 30 μl of 4% SDS, 0.5 M Tris-HCl, pH 8.0 and boiled for 10 min. Insoluble staphylococci were sedimented by centrifugation at 15 000 g for 5 min. Twenty microliters of the soluble supernatant was added to 1 ml of immunoprecipitation reaction containing rabbit antiserum diluted 1:500 into RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 8.0). After incubation for 1 h at room temperature, the immune complexes were harvested on protein A-Sepharose 4B beads (10 μl bed volume, Sigma), washed with RIPA buffer and eluted with 20 μl of sample buffer prior to SDS-PAGE and fluorography.

### Staphylococcal cell fractionation

To separate the surrounding medium from the cell pellet, 1 ml of pulse-labeled staphylococci were sedimented by centrifugation at 13 000 g for 3 min. Protein in the supernatant was removed and precipitated with 5% TCA. The cell sediment was boiled directly in 20 μl of 0.5 M Tris-HCl, 4% SDS. Fractionation of staphylococci into medium, cell wall, membrane and cytosol compartments followed a previously established procedure (Schneewind *et al.*, 1993).

### Targeting of GST fusion proteins to *S.aureus* cells

The *E.coli* BL21 strains expressing hybrid GST proteins were grown in 2 l of LB medium to mid-log phase at 37°C, induced with 1 mM

of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) and incubated for an additional 2 h. Cells were harvested by centrifugation, suspended in 8 ml of F buffer [20% sucrose, 50 mM Tris-HCl, 1 mM dithiothreitol (DTT), 10 mM EDTA pH 8.0 and 0.1 mg/ml of lysozyme], incubated for 30 min on ice and then disrupted in a French pressure cell at 6000 p.s.i. Unbroken cells and cellular debris were removed by centrifugation for 5 min at 15 000 g and the supernatant was subjected to affinity chromatography. One microliter of glutathione-Sepharose 4G (Pharmacia) was equilibrated with T buffer (50 mM Tris-HCl pH 8.0, 150 mM of NaCl, 15% glycerol) prior to loading of crude soluble *E. coli* extracts. The column was washed with 30 ml of T buffer.

Fusion proteins were radiolabeled on the glutathione-Sepharose 4G beads by removing 150  $\mu$ l of charged glutathione-resin and suspending the beads in 20 mM Tris-HCl pH 7.5, 1 mM DTT, 12 mM MgCl<sub>2</sub>, 100 mM NaCl, 500 nM of cAMP, 1  $\mu$ l of [ $\gamma$ -<sup>32</sup>P]ATP (6000 Ci/mmol, 10 mCi/ml) and 50 U of cAMP-dependent heart muscle kinase (Sigma) (Blanar and Rutter, 1992) in a total volume of 600  $\mu$ l and incubated at 37°C for 2 h. Radiolabeled protein was eluted in 300  $\mu$ l of 20 mM reduced glutathione, 10% glycerol, 100 mM Tris-HCl pH 8.0, 120 mM NaCl. One microgram of protein (60 000–90 000 c.p.m.) in ~4  $\mu$ l was added to 150  $\mu$ l of buffer GT (10% glycerol and 50 mM Tris-HCl, pH 7.0) containing a suspension of *S. aureus* OS2 cells and incubated for 10 min at room temperature. Cells were sedimented by centrifugation and the amount of radioactivity depleted from the supernatant was measured by liquid scintillation counting (Beckman LS-6500).

#### Determination of $K_d$ values for GST fusion protein-cell binding

GST-amidase fusion protein (0.4–100  $\mu$ g) was mixed with  $2.5 \times 10^8$  cells in 150  $\mu$ l of buffer GT, incubated, centrifuged and protein depletion from the supernatant measured as described above. Assuming that bound fusion protein forms an equimolar complex with its presumed receptor, the  $K_d$  value was calculated from the slope of a Scatchard plot where the ratio of [bound protein]/[free protein] was plotted against that of [bound protein] (Creighton, 1993). The number of staphylococcal cells required for 50% sedimentation of 1  $\mu$ g of labeled fusion protein was deduced from the graph shown in Figure 4C. The number of repeat domain receptor molecules per cell was determined for GST-amidase. Fifty percent co-sedimentation of 1  $\mu$ g of GST-amidase (88 kDa protein;  $0.5 \mu\text{g} = 3.7 \times 10^{-8} \text{ M} = 3.4 \times 10^{12}$  molecules) required  $5.0 \times 10^6$  cells, indicating that each cell may display as many as  $6.2 \times 10^8$  repeat domain receptor molecules.

#### Preparation of samples for transmission electron microscopy

Purified GST, GST-Cwt, GST-R1R2 or GST-amidase (0.5  $\mu$ g) were incubated in phosphate-buffered saline (PBS) for 5 min at room temperature with  $10^8$  cells of *S. aureus* strain OS2. After washing with PBS twice, cells were suspended in PBS with 1:25 diluted rabbit anti-GST and incubated for 20 min at room temperature. Cells were washed and incubated with 1:25 diluted protein A-colloidal gold conjugate (10 nm particles, Sigma) for 30 min. After several washes, samples were fixed with glutaraldehyde, embedded and viewed under a transmission electron microscope.

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