Staphylococcal Major Autolysin (Atl) Is Involved in Excretion of Cytoplasmic Proteins

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Many microorganisms excrete typical cytoplasmic proteins into the culture supernatant. As none of the classical secretion systems appears to be involved, this type of secretion was referred to as “nonclassical protein secretion.” Here, we demonstrate that in Staphylococcus aureus the major autolysin plays a crucial role in release of cytoplasmic proteins. Comparative secretome analysis revealed that in the wild type S. aureus strain, 22 typical cytoplasmic proteins were excreted into the culture supernatant, although in the atl mutant they were significantly decreased. The presence or absence of prophages had little influence on the secretome pattern. In the atl mutant, secondary peptidoglycan hydrolases were increased in the secretome; the corresponding genes were transcriptionally up-regulated suggesting a compensatory mechanism for the atl mutation. Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a cytoplasmic indicator enzyme, we showed that all clinical isolates tested excreted this protein. In the wall teichoic acid-deficient tagO mutant with its increased autolysis activity, GAPDH was excreted in even higher amounts than in the WT, confirming the importance of autolysis in excretion of cytoplasmic proteins. To answer the question of how discriminatory the excretion of cytoplasmic proteins is, we performed a two-dimensional PAGE of cytoplasmic proteins isolated from WT. Surprisingly, the most abundant proteins in the cytoplasm were not found in the secretome of the WT, suggesting that there exists a selection mechanism in the excretion of cytoplasmic proteins. As the major autolysin binds at the septum site, we assume that the proteins are preferentially released at and during septum formation.

Proteome analyses were carried out in staphylococci to study the expression of proteins under endo- and anoxic growth conditions (1, 2), growing and nongrowing cells (3), or global regulator mutants such as agr, orB, and clpC (4–6). In most of the studies cytoplasmic proteins were addressed, and only few were focused on the extracellular proteome or secretome. The latter studies showed that in the culture supernatants of Bacillus subtilis and Staphylococcus aureus, a number of typical cytoplasmic proteins, which lack a signal sequence, were present (5, 7, 8). It is generally assumed that the release of such cytoplasmic proteins is due to cell lysis, although specific export processes for excretion of cytoplasmic proteins could not be excluded.

In B. subtilis, various possibilities have been discussed about how such cytoplasmic proteins could be secreted (8). It was speculated that prophage-encoded holins could form pores in the membrane through which the lytic enzymes of bacteriophages, which usually lack a signal peptide (SP),2 gain access to the cell wall (CW) (9). However, elimination of several prophages from a strain had no effect on the appearance of cytoplasmic proteins in the extracellular proteome (10). Thus, cytoplasmic proteins do not seem to leave the cytoplasm in B. subtilis via prophage-encoded holins, as proposed for Lactococcus lactis (11). Another long held assumption was that they are released by cell lysis; in particular, some of the proteins were highly abundant in the cytoplasmic proteome of B. subtilis (12).

In S. aureus, various specialized protein secretion systems have been described, including the Ess (ESAT-6 secretion system) (13), the TAT secretion system (14), and ATP-cassette transporters involved in antibiotic efflux or secretion of peptide antibiotics (15, 16). However, apparently none of these well known secretion machineries appear to be involved in the secretion of cytoplasmic proteins. Because no pattern of specific signal sequences or other peptide motifs could be identified with the proteins present in the culture supernatants, the existence of a novel, “nonclassical protein secretion” mechanism was postulated (17).

Here, we addressed the question of whether prophages and/or autolysins might play a role in excretion of cytoplasmic proteins. For S. aureus, it is well known that cell lysis is caused by induction of prophages, the major autolysin, or by the addition of β-lactam antibiotics. Most clinical S. aureus isolates possess prophages, and many of them carry virulence genes. The staphylococcal strain used in this study, S. aureus SA113, is a derivative of S. aureus NCTC8325, which is lysogenic for the three functional phages φ11, φ12, and φ13 (18). To study the effect of prophages in excretion of cytoplasmic proteins, S. aureus 8325–4, a derivative of NCTC8325 (RN1), is the strain of 2

The abbreviations used are: SP, signal peptide; Atl, major autolysin; CW, cell wall; CHAP, Cys-His-dependent amidohydrolases/peptidases; MV, membrane vesicle.

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TABLE 1
Bacteria used in this study

The abbreviations used are as follows: Spc, spectinomycin; Cm, chloramphenicol; Erm, erythromycin; and superscript r indicates resistance.

| Strains               | Genotypes                          | Refs./Source |
|-----------------------|------------------------------------|--------------|
| S. aureus SA113       | rsbl⁻, agr⁻, tcaR⁻                   | 50           |
| S. aureus SA113 Δatl::spc | Δatl::spc complemented with pRC14, Spc⁻, Cm⁻ | 23           |
| S. aureus SA113 Δatl::spc (pRBatlE) | Δatl::spc with the empty vector pRB473, Spc⁻, Cm⁻ | 51           |
| S. aureus SA113 ΔtagO::erm | ΔtagO::erm, Erm⁻ | 52           |
| S. aureus 8325-4      | Δpca::erm                           | 19           |
| S. aureus SA113 Δpca::erm | Δpca::erm                           | 38           |
| S. aureus RN1         | rsbl⁻, agr⁻                         | 53           |
| S. aureus HG001       | rsbl⁻ repaired, tcaR⁻               | 38           |
| S. aureus HG002       | rsbl⁻, tcaR⁻ repaired               | 38           |
| S. aureus HG003       | HG001 derivative, rsbl⁻ and tcaR⁻ repaired | 38           |
| S. aureus Newman      | Clinical isolate                    | 54           |
| S. aureus USA300      | Clinical isolate                    | 55           |
| S. aureus UAMS-1      | Clinical isolate                    | 56           |
| S. aureus COL         | agr⁻                               | 57           |
| S. carnosus TM300     | Clinical isolate                    | 58           |
| S. epidermidis O-47   | Clinical isolate                    | 59           |

choice, because it has been UV-cured of all three prophages and is very useful for phage propagation (19).

The other well studied enzyme that is involved in cell lysis in S. aureus and Staphylococcus epidermidis is the major autolysin (Atl). It is the main peptidoglycan hydrolase in staphylococci. It represents a bifunctional protein composed of a propeptide region, an amidase, and an endo-β-N-acetylmuramidase domain. Atl is processed to generate two extracellular lytic enzymes, which are noncovalently attached to the staphylococcal cell surface (20, 21). Autolysis is markedly decreased in an atl mutant (22); the major lytic activity resides in the amidase (23, 24). Atl is sec-dependent exported, and it has been shown that wall teichoic acid plays a crucial role in targeting the Atl-derived autolysins to the septum region (22). Atl has various functions; it is involved in the separation of the daughter cells by hydrolyzing the amide bond between N-acetylmuramic acid and l-alanine (23); it acts directly as an adhesin by binding to fibronectin and vitronectin mainly via the peptidoglycan binding domains; it is involved in biofilm formation (21); and finally, atl mutants were attenuated in pathogenesis in an intravascular catheter-associated rat infection model (25).

We show here that the major autolysin (Atl) plays a crucial role in excretion of cytoplasmic proteins, that the excretion of cytoplasmic proteins appears to be a general phenomenon in clinical isolates of S. aureus and also S. epidermidis, and finally, that excretion of proteins is not random. It seems that there is a selection process at work.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Bacterial strains used in this study are listed in Table 1. Cells were grown in Basic medium (BM: 10 g of peptone; 5 g of yeast extract; 5 g of NaCl; 1 g of K2HPO4; 3H2O; 1 g of glucose; pH 7.2; per liter) or on BM-agar (B-medium supplemented with 15 g of agar per liter). Strain stocks were stored in freeze medium containing 65% (w/v) glycerol at −80 °C. Frozen strains were streaked for isolation onto BM-agar with and without antibiotic for the mutants and WT strains, respectively, and incubated overnight. Antibiotics were used at the following concentrations: spectinomycin 150 μg/ml; chloramphenicol 10 μg/ml, and erythromycin 10 μg/ml.

Cells were grown in B-medium as described before, harvested after 8 h of growth, and pelleted by centrifugation at 8000 × g for 30 min at 4 °C. For preparation of the extracellular fraction, supernatant proteins were extracted, pelleted, and dried as described earlier (5). Isolation of the cytoplasmic proteins was described previously (26). Cells were washed and disrupted using glass beads. Cytoplasmic proteins were precipitated, pelleted, washed with 100% ethanol, and dried.

Proteins were redissolved in an appropriate volume of buffer consisting of 8 M urea, 2 M thiourea, 4% CHAPS (w/v), 1% dithiothreitol (DTT), 0.7% Phormlyte (pH 3–10; GE Healthcare). All chemicals were purchased from Merck in the highest available grade unless stated otherwise. The total soluble protein concentration was measured by the Bradford method (27). Two-dimensional PAGE—Two-dimensional PAGE was performed as described previously (28, 29). 500 μg of protein were loaded onto commercially available IPG strips (24 cm, pH 3–10 nonlinear or pH 4–7 linear; Bio-Rad) in a Protean IEF cell (Bio-Rad). After rehydration, the voltage was progressively increased to 10,000 V. The strips received about 63 kVh. The maximum current was 50 μA per gel strip. For the second dimension, the equilibrated gel strips were applied to 15% (extracellular fraction) and 12% (intracellular fraction) polyacrylamide gels. A standard molecular weight marker from Anamed (Gross-Bieberau, Germany) was loaded. The resulting protein gels were stained with Colloidal Coomassie “Silver Blue” (0.12% Coomassie G-250, 10% ammonium sulfate, 10% phosphoric acid, and 20% methanol) as described by Candiano et al. (30) and scanned using a flatbed scanner (Powerlook 2100 XL, Umax, Willich, Germany).

Image Analysis—Three independent experiments were performed for each condition having three biological replicates.
The three resulting gels of the secretome of the WT SA113 were compared with the gels of the secretome of the atl mutant by image analysis using the Delta2D-software (DECODON, Greifswald, Germany). Protein spots showing significant differences in intensity between WT and mutant secretome were recognized, and their reproducibility of differential representation was confirmed. Protein spots were identified by mass spectrometry.

**Liquid Chromatography-Mass Spectrometry (MS) Analysis**—Tryptic in-gel digestion and peptide analysis were done as described previously (31), using an Ultimate nanoflow HPLC system (Dionex/LC Packings) coupled to a QSTAR Pulsar i Hybrid QqTOF mass spectrometer (Applied Biosystems/MDS Sciex) equipped with a nanoelectrospray ion source. MS data were searched using the Mascot search engine (Matrix Science, London, UK) against a specialized data base containing the sequence of S. aureus N315; this data base also contained common contaminants as well as reversed versions of the sequences. Data related to the same gel were combined into a single peak list and subsequently processed as combined searches. The software packet MS Quant was used for assigning the resulting sequence information to the respective two-dimensional PAGE signals. Following the approach of MS Quant, peptides were classified into categories. Three groups were formed by applying probability values of different stringency as follows: group A, p < 0.01, score >29; group B, p < 0.05 and p > 0.01, score >22; and group C, p < 0.1 and p > 0.05, score >19. The scores corresponded to the mascot ion score for different false-positive probabilities. Protein identification was defined as valid if the sum of all associated peptide scores was greater or equal to the score of group B plus the score of group C, i.e. 41. Furthermore, the observed molecular weight and pI had to be close to the predicted protein apart from protein fragments.

**Northern Blot Analyses**—The cells of the WT S. aureus SA113, the isogenic atl mutant, and the complemented strain were grown in B-medium as described earlier and were harvested after 3, 6, and 8 h of growth. For isolation of total RNA from S. aureus, the acid-phenol method (32, 33) was used with some modifications as described by Fuchs et al. (1). Digoxigenin-labeled RNA probes of the corresponding genes were prepared by in vitro transcription with T7 RNA polymerase by using a PCR fragment as template (32), which was generated by using chromosomal DNA of S. aureus SA113 and the respective oligonucleotides listed in supplemental Table 1. The reverse oligonucleotides contained the T7 RNA polymerase recognition site sequence at the 5’ end. For Northern blot analyses, equal amounts of total RNA (10 μg for each Northern blot, for spa, hla, and aaa only 5 μg of total RNA were loaded) were separated under denaturing conditions in a 1% agarose gel containing 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 1.85% formaldehyde, pH 7.0. The gel was blotted onto a nylon membrane with 20× SSPE (3 M NaCl, 0.2 M NaH2PO4, 0.02 M EDTA, pH 7.4) by using a vacuum blower. After 4 h of transfer, the RNA was fixed to the membrane by UV cross-linking for 1 min. The membrane was stained with methylene blue to visualize 16 and 23 S rRNA bands to control for successful blotting and the presence of the comparable amounts of total RNA in each lane.

The digoxigenin-labeled RNA probes were used for gene-specific hybridization according to the manufacturer’s instructions (Roche Applied Science). Hybridization signals were detected using the Lumi-Film chemiluminescent detection film (Roche Applied Science).

**Preparation of Protein Extracts for Western Blot Analysis**—Cells (Table 1) were grown for 2, 4, 6, 8, 14, 24, and 48 h in B-medium and then pelleted by centrifugation at 5000 rpm for 30 min at 4 °C. Proteins in the supernatant were isolated as described above. The resulting protein pellet was dried and resuspended in an appropriate volume of buffer consisting of 8 M urea and 2 M thiourea. The amount of protein in the samples was determined by the method described by Bradford (27). For determination of GAPDH in the supernatant of different S. aureus WT strains, the cells were pelleted, and the supernatant proteins were concentrated using StrataClean™ resin (Stratagene, Heidelberg, Germany) prior to gel electrophoresis.

**Western Blot Analyses**—The same amount of protein per lane was separated on 15% SDS-PAGE according to Schagger and von Jagow (34) and electrophoretically transferred to nitrocellulose membrane (Protran, nitrocellulose membrane, Schleicher & Schuell BA83) (1 h 20 min, 350 mA), buffered in semi-dry transfer buffer using a Trans-Blot SD Semi-Dry Transfer Cell from Bio-Rad). After blotting, the membrane was treated and washed as described previously (35). To block unspecific interactions with protein A, the membrane was incubated for 2 h with 5% goat serum (Sigma) and diluted in TBS buffer containing 0.3% bovine serum albumin (BSA, Carl-Roth, Karlsruhe, Germany). For the detection of GAPDH, the membrane was incubated for 1 h with the specific rabbit polyclonal anti-GapA antibody diluted 1:15,000 in TBS buffer containing 0.3% BSA. Generation of the anti-GapA antibody has been described previously (36). The specific bound anti-GapA was detected by incubation with anti-rabbit alkaline phosphatase-labeled secondary antibody produced in goat (Sigma) diluted 1:20,000 in TBS buffer containing 0.3% BSA. For the chemiluminescent detection, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate ready-to-use solution (Sigma) was used following the manufacturer’s instructions.

**Determination of Extracellular Proteases on Casein Plate**—Cells of the WT S. aureus SA113, its isogenic atl mutant, and the complemented strain were grown in B-medium agar plate overnight and resuspended in B-medium, and equal amounts of cells in a volume of 5 μl was dropped on a casein plate (B-medium agar plate + 1% milk powder as casein source) and incubated for 18 h at 37 °C.

**Definition of Functional Domains of Alternative Peptidoglycan Hydrolase Proteins**—For definition of lengths and positions of different domains of the alternative peptidoglycan hydrolases, the publicly available annotation tool from the Sanger Institute was used.

### RESULTS

**In the Atl Mutant the Amount of Excreted Cytoplasmic Proteins Was Significantly Decreased**—It is well known that many bacteria release into the culture supernatant typical cytoplasmic proteins lacking an SP. It is commonly believed that such proteins are released “accidentally” by some unspecific autoly-
sis processes. On the other hand, one cannot exclude that a previously unidentified mechanism underlies this protein excretion. With regard to lytic processes, an obvious mechanism for cell lysis could be induction of prophages or unbalanced autolysis.

To investigate the contribution of lytic processes in release of cytoplasmic proteins, we carried out a comparative secretome (total proteins released to the bacterial culture supernatants) analysis. We compared the secretome of SA113 (NCTC8325 lineage) carrying three prophages (\(\phi 11\), \(\phi 12\), and \(\phi 13\)) with two mutants; one was affected in phage lysis and the other in the major autolysis. The two mutants were 8325-4, which is cured of the prophages (19), and SA113 \(\text{at}l\) mutant, in which the major autolysin gene \(\text{at}l\) has been deleted (23). The \(\text{at}l\) deletion and the \(\text{at}l\) complementing plasmid are illustrated in supplemental Fig. 1. A comparison of the secretome of SA113 with that of 8325-4 revealed virtually no differences in the general protein pattern; the released cytoplasmic proteins were almost the same, indicating that the presence of prophages had no marked influence on release of cytoplasmic proteins. None of the prevailing opinions on the role of prophage in the release of cytoplasmic proteins was therefore relevant, at least with these strains.

However, the secretome pattern of the \(\text{at}l\) mutant versus WT was drastically altered (Fig. 1B). For better comparison of the protein spots of the two-dimensional PAGE, the gels of SA113 and the \(\text{at}l\) mutant were overlaid using Delta2D software. Proteins with higher abundance in SA113 are marked in Fig. 1A, and proteins with higher abundance in the \(\text{at}l\) mutant are marked in Fig. 1B. The most striking result was that the secretome of SA113 contained 22 typical cytoplasmic proteins (Fig. 1A, labeled in blue) in much higher abundance than that of the \(\text{at}l\) mutant. All these cytoplasmic proteins lack a typical SP and are supposed to be localized in the cytoplasm. Among the excrated cytoplasmic proteins in the SA113 were chaperones, the DNA-dependent RNA polymerase RpoB, proteins which are involved in translation, in glycolysis, TCA cycle, and several other metabolic functions and pathways. They are listed in Table 2.

To answer the question whether only highly expressed cytoplasmic proteins were excreted, we performed a two-dimensional PAGE of cytoplasmic proteins isolated from SA113. In blue were those proteins labeled that were also found in the secretome and in black those proteins that were not found in the secretome (Fig. 1C). As one can see, there are highly aban-
doned cytoplasmic proteins that were not excreted as follows: Fhs (formyltetrahydrofolate synthetase), GuaB (IMP dehydrogenase, part of a four-gene operon consisting of xpt, pbuX, guaB, and guaA in \(S. aureus\)), SA0802 (hypothesis protein, NADH dehydrogenase), KatA (catalase), GlpA (glycerol-3-phosphate dehydrogenase subunit A), PdhD (dihydrolipoamide dehydrogenase component of pyruvate dehydrogenase E3), SucC (succinyl-CoA synthetase, \(\beta\) subunit), or EF-Ts (elongation factor Ts). All these typical cytoplasmic proteins are in very high abundance in the cytoplasm and still not found in the secretome.

Besides the typical cytoplasmic proteins, three CW-anchored proteins (possessing an LPXTG motif) were in higher abundance in the secretome of WT compared with the \(\text{at}l\) mutant. The CW-anchored proteins were SdrD (Ser-Asp-rich fibrinogen- and bone sialoprotein-binding protein), Spa (immunoglobulin G-binding protein A), and SA0129 (protein with unknown function).

There were also a few secreted proteins (with typically SP) present in larger quantity in the secretome of SA113 compared with the \(\text{at}l\) mutant. Among them were \(\alpha\)-hemolysin (Hla), glycerol ester hydrolase (Geh), and protein SA0570 with unknown function.

Some of the higher abundant proteins in the secretome of SA113 were proteolytically processed, such as Geh, Lip, IsaA, metalloprotease aureolysin (Aur), N-acetylumuramyl-L-alanine amidase SA2437 (Aly), or of the major autolysin (Atl) itself (Fig. 1A and Table 2). Indeed, SA113 and the complemented \(\text{at}l\) mutant showed proteolytic activity on casein plates, although the \(\text{at}l\) mutant does not (Fig. 2). Most likely, the proteolytic activity is due to the Zn\(^{2+}\) metalloprotease, aureolysin, as other protease spots were not found in the secretome.

In the \(\text{at}l\) Mutant Secondary Peptidoglycan Hydrolases Were Overexpressed at Transcript and Protein Levels—Although the secretome of SA113 was characterized by a high content of cytoplasmic proteins, the secretome of the \(\text{at}l\) mutant was distinguished by a predominance of secreted proteins, particularly peptidoglycan hydrolases. Of the 11 highly abundant proteins, at least 8 represented known or putative peptidoglycan hydrolases, for example the immunodominant antigen (IsaA), peptidoglycan hydrolase (LytM), autolysin precursor SA2437 (Aly), transglycosylase (SceD), and some secretory antigen precursor SsaA homolog proteins such as SsaA (SA2093), SA0620, and SA2097 (Fig. 1B and Table 3). Although these proteins have a typical SP, the elastin-binding protein (EbpS) has no SP but is described to be localized in the membrane (37). All of these proteins carry domains typical for peptidoglycan hydrolases such as Cys-His-dependent amidohydrolases/peptidases (CHAP), LysM, amidase, glucosaminidase, or transglycosidase domains (Table 4).

The differently increased content of proteins in WT and \(\text{at}l\) mutant strains raises the question of whether the corresponding genes were transcriptionally up-regulated. Therefore, transcript amount was determined by Northern blotting at three different time points (Fig. 3 and Table 4). The transcripts for two cytoplasmic proteins GAPDH (Gap) and enolase (Eno) showed little difference in Northern blots, although these two proteins were increased in the secretome of SA113 (Figs. 1A and 3A).

Transcription of the \(\text{hla}\) (\(\alpha\)-hemolysin gene), \(\text{spa}\) (protein A gene), and \(\text{geh}\) (glycerol ester hydrolases gene) were clearly repressed in the \(\text{at}l\) mutant. All three SP-containing proteins were highly abundant in the WT secretome compared with the \(\text{at}l\) mutant (Figs. 1A and 3A).

The most striking result of the transcriptional analyses was, however, that the majority of the up-regulated genes in the \(\text{at}l\) mutant encoding peptidoglycan hydrolases. These were the CHAP domain containing proteins (SA2093, SA2097, SA2353, SA0620, SA0710, SA2332, Aly, Aaa, and LytN) as well some other the alternative peptidoglycan hydrolases (SA2100, LytM, SceD, IsaA, and LytH). The Northern blots of eight alternative peptidoglycan hydrolases are shown in Fig. 3B. The up-regula-
tion and enhanced production of these CW hydrolases suggest that the \( \text{atl} \) mutant tries to compensate for the severe defect in cell separation because of the \( \text{atl} \) mutation.

These data lead to the conclusion that there was altered expression of global regulators, which are known to control autolysis activity in \( S. \text{aureus} \). We therefore analyzed the transcription of \( \text{lrgAB} \), \( \text{walKR} \), \( \text{lytRS} \), \( \text{arlRS} \), \( \text{cidAB} \), and \( \text{mgrA} \). Interestingly, none of these regulatory proteins showed differences on a transcriptional level.

GAPDH, as a Cytoplasmic Reporter Protein, Was Hardly Detectable in the Culture Supernatant of the \( \text{atl} \) Mutant—To substantiate the results of the secretome analysis, we followed the presence of one of the cytoplasmic proteins (GAPDH) in the culture supernatants by Western blotting using \( \alpha \)-GapA rabbit

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FIGURE 1. Differential two-dimensional PAGE of secretomes (A and B) of \( \text{SA113} \) and its \( \text{atl} \) mutant and of the intracellular protein fraction of \( \text{SA113} \) (C). Cells were grown in B-medium and harvested after 8 h of growth. 500 \( \mu \text{g} \) of proteins were separated by two-dimensional PAGE in a pH range from 3 to 10 or 4 to 7 and stained with Colloidal Commassie “Silver Blue.” Secretome of the WT \( S. \text{aureus} \) \( \text{SA113} \) (green staining of protein spots) and secretome of the \( \text{atl} \) mutant (red staining of protein spots) were overlaid using Delta2D software. A, proteins with higher abundance in the WT are indicated by arrows; typically cytoplasmic proteins are labeled in blue. B, proteins with higher abundance in the \( \text{atl} \) mutant are indicated by arrows; proteins with potential peptidoglycan hydrolase activity are labeled in red. C, proteins that were also excreted are labeled in blue; proteins that were not found in the secretome are labeled in black.
Protease activity is indicated by an antibody (Fig. 4). Over a time range from 2 to 48 h, GAPDH (~36 kDa) was clearly detectable in the supernatant of SA113 and the complemented atl mutant (with atl expressing plasmid pRC14 (21)); although in all strains that lack a functional atl, it was hardly detectable. GAPDH was also excreted in 8325-4 (lacking prophages), corroborating the secretome results above, and in the spa mutant that lacks protein A, an IgG-binding protein.

We also were interested whether the wall teichoic acid tagO mutant has more GAPDH in the supernatant. This mutant shows an increased cell lysis because the septum-specific binding of Atl is relieved, and binding occurs all around the cell wall (22). In the tagO mutant, GAPDH was even in higher amounts in the supernatant as in the WT (Fig. 4B).

All Clinical Isolates Tested Excreted GAPDH—We also addressed the question of how widespread is the phenomenon of cytoplasmic protein excretion in S. aureus. A number of clinical isolates as well as strains differing in global regulators (38) were tested for the presence of GAPDH in the culture supernatants by Western blotting (Fig. 4C). All S. aureus strains tested excreted GAPDH. Somewhat higher amounts were present in SA113, HG001 (rsbU repaired), and HG003 (rsbU/land tcaR repaired), although in USA300 GAPDH excretion appeared a bit delayed. GAPDH was also excreted in S. epidermidis O-47 and a little bit in Staphylococcus carnosus TM300.

**DISCUSSION**

Release of typical cytoplasmic proteins into the culture supernatant is not restricted to individual species. It has been observed in Gram-positive and -negative bacteria (S. aureus, B. subtilis, L. monocytogenes, and E. coli) and also eukaryotic cells. In particular, glycolytic enzymes, chaperones, translation factors, or enzymes involved in detoxification of reactive oxygen were found in the supernatants by secretome analysis (8,
atl (Major Autolysin) Mutant

39–42). It is generally believed that these cytoplasmic proteins are simply expelled by some kind of cell lysis.

Here, we addressed the following question. What kind of cell lysis causes release of cytoplasmic proteins in  S. aureus, the prophage, or the autolysin-induced cell lysis? To study this comparative secretome, analysis with  S. aureus SA113, its prophage-less derivative 8325-4, and an atl mutant was carried out. First of all, it was surprising how many cytoplasmic proteins were present in the culture supernatant of the WT. The 22 proteins, which may trigger cell lysis upon induction with mutagens in WT atl mutant were tested whether transcription of some of the excreted cytoplasmic proteins in the atl mutant could be their decreased expression. Therefore, we tested whether transcription of some of the excreted cytoplasmic proteins in the atl mutant is due to proteolytic digestion.

An explanation for the decreased cytoplasmic proteins in the atl mutant could be their decreased expression. Therefore, we tested whether transcription of some of the excreted cytoplasmic proteins in the atl mutant was decreased in the extracellular proteases particularly aureolysin, which was reflected by the absence of proteolytic activity on casein plates; therefore, it is unlikely that the low amount of “extracellular” cytoplasmic proteins in the atl mutant was carried out. First of all, it was surprising how many cytoplasmic proteins were present in the culture supernatant of the WT. The 22 cytoplasmic proteins belonged to metabolic pathway enzymes, regulators, and chaperones, as well as proteins involved in translation. However, almost the same protein pattern revealed the prophage-cured mutant 8325-4, indicating that proophages, which may trigger cell lysis upon induction with mutagens in WT (43), do not significantly contribute to the release of cytoplasmic proteins, which is in line with the findings in  B. subtilis (10).

On the other hand, the secretome pattern of the atl mutant looked completely different in three respects. (a) The amount of typical cytoplasmic proteins was significantly decreased. (b) There was a pronounced predominance of alternative cell wall hydrolases with CHAP and/or LysM domains; all these proteins contain an SP and are supposed to be exported by the sec pathway. (c) The atl mutant secreted less extracellular proteases, particularly aureolysin, which was reflected by the absence of proteolytic activity on casein plates; therefore, it is unlikely that the low amount of “extracellular” cytoplasmic proteins in the atl mutant is due to proteolytic digestion.

By investigating GAPDH protein as a prototype of excreted cytoplasmic proteins, we could confirm in Western blots the decreased content in the supernatant. which may trigger cell lysis upon induction with mutagens in WT 8325-4, indicating that prophages, regulators, and chaperones, as well as proteins involved in translation was reduced in the prophage-cured mutant, GAPDH was hardly detectable in the culture supernatant over a time range of 2–48 h (Fig. 4A). If autolysis plays a crucial role in excretion of cytoplasmic proteins, then one would also expect an effect in the autolysis. How- ever, there was not much difference compared with the WT, which ruled out that increased transcription is responsible for the decreased content in the supernatant.

On the other hand, the secretome pattern of the atl mutant looked completely different in three respects. (a) The amount of typical cytoplasmic proteins was significantly decreased. (b) There was a pronounced predominance of alternative cell wall hydrolases with CHAP and/or LysM domains; all these proteins contain an SP and are supposed to be exported by the sec pathway. (c) The atl mutant secreted less extracellular proteases, particularly aureolysin, which was reflected by the absence of proteolytic activity on casein plates; therefore, it is unlikely that the low amount of “extracellular” cytoplasmic proteins in the atl mutant is due to proteolytic digestion.
to a decreased cross-reactivity of the anti-GapA<sub>S. aureus</sub> with GAPDH<sub>S. carnosus</sub> because the two proteins have only 87% sequence identity, whereas GAPDH<sub>S. aureus</sub> and GAPDH<sub>S. epidermidis</sub> have 93% sequence identity. However, it could also mean that the nonpathogenic <i>S. carnosus</i> simply excretes lower amounts of cytoplasmic proteins.

An important question is how discriminatory is the excretion of cytoplasmic proteins. Are these 22 cytoplasmic proteins preferentially released because they represent the most abundant proteins in the cytoplasm? To answer this question, we performed a two-dimensional PAGE of cytoplasmic proteins isolated from SA113 (Fig. 1C). To our great surprise, quite a number of very high abundant proteins in the cytoplasm were not found in the secretome of the SA113. Two conclusions can be drawn from this observation. (a) There is no simple correlation between the quantity of cytoplasmic proteins in the cytoplasm and their release to the extracellular environment. (b) There exists a selection procedure in the excretion of cytoplasmic proteins. This brings us back to the following questions. Why do microorganisms excrete cytoplasmic proteins, which is a loss of recourses and energy? Is the excretion of such proteins an accident or is it program? If there is a program involved, one can speculate that nonfunctional (e.g. misfolded) cytoplasmic proteins are recognized and withdrawn by excretion. For example, <i>E. coli</i> enolase was only excreted when it was covalently modified by its substrate 2-phosphoglycerate (44), suggesting a selective and specific export system. Furthermore, it has been observed in a <i>ptaA</i> foldase mutant in <i>B. subtilis</i> that the number of nonclassically secreted proteins was increased (45); however, as in this mutant cell lysis was also increased, it is not certain whether this was due to the increase of misfolded proteins. On the other hand, one could argue that the enhanced cell lysis is a rescue program to get rid of misfolded and therefore dangerous proteins.

As Atl binds preferentially at the septum site, we assume that the proteins are preferentially released at and during septum formation. This is the site with the highest CW biosynthetic activity and binding site of Atl where it resolves the interlinked murein layers of the daughter cells (22, 46). Therefore, the septum region could be the leaky site for cytoplasmic protein excretion.

Another mechanism for excretion of cytoplasmic proteins could be their release by membrane vesicles (MVs). In Gram-negative as well as in Gram-positive bacteria, it has been shown that in such MVs cytoplasmic proteins like GAPDH, PdhB, EF-Tu, RpsB, and GroEL can be transported to the medium (47–49). Indeed, in transmission electron microscopic images of staphylococcal strains during autolysis and particularly in the tagO mutant (22), one can see pleb-like structures on the cell surface that might be MVs. However, the list of proteins identified from <i>S. aureus</i>-derived MVs mentions 91 proteins of cytoplasmic, membrane, and secreted origin (48), which implies little specificity in contrast to our results. But it is quite possible that MVs and autolysis are somehow connected.

**CONCLUSION**

This study shows that the major autolysin plays a crucial role in excretion of cytoplasmic proteins. Although such an excretion/release by autolysis implies little differentiation, our
results suggest the existence of a selection procedure. There are still many questions open, such as the selectivity and the site of excretion, and more detailed knowledge regarding its mechanism is needed.

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