A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor

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Summary

Staphylococcus aureus and Staphylococcus epidermidis can cause serious chronic and recurrent infections that are difficult to eradicate. An important pathogenicity factor in these infections caused by S. aureus is its ability to be internalized by non-professional phagocytes thereby evading the host immune system and antibiotic treatment. Here, we report a novel mechanism involved in staphylococcal internalization by host cells, which is mediated by the major autolysin/adhesins Atl and AtlE from S. aureus and S. epidermidis respectively. In a flow cytometric internalization assay, atl and atlE mutants are significantly reduced in their capacities to be internalized by endothelial cells. Moreover, pre-incubation of endothelial cells with recombinant Atl dose-dependently inhibited internalization. As putative Atl-host cell receptor, the heat shock cognate protein Hsc70 was identified by mass spectrometry. The importance of Hsc70 in internalization was demonstrated by the inhibition of S. aureus internalization with anti-Hsc70 antibodies. In conclusion, this novel Atl- or AtlE-mediated internalization mechanism may represent a ‘back-up’ mechanism in S. aureus internalization, while it may represent the major or even sole mechanism involved in the internalization of coagulase-negative staphylococci and thus may play an important role in the pathogenesis of chronic and relapsing infections with these serious pathogens.

Introduction

Despite the fact that staphylococci are ubiquitous commensals of the skin and mucous membranes of humans, Staphylococcus aureus and the coagulase-negative Staphylococcus epidermidis are among the most leading causes of serious and life-threatening infections, such as endocarditis, osteomyelitis, pneumonia and sepsis (De Wit et al., 1993; Lowy, 1998; Carek et al., 2001; Ziebuhr, 2001; Karlowsky et al., 2004). Infections due to S. epidermidis typically are more subacute or even chronic and require a predisposed or immunocompromised host, such as patients with indwelling medical devices, like prosthetic heart valves and orthopaedic implants (Götz and Peters, 2000; Lentino, 2003). In contrast, S. aureus usually causes more acute infections associated with the colonization of the host tissue. However, S. aureus is also a common cause of foreign body-associated infections and known to cause persisting and relapsing infections, such as chronic osteomyelitis (von Eiff et al., 1997, von Eiff et al., 2000, Lentino, 2003). Similarly, S. epidermidis has been reported to cause chronic and recurrent bone diseases (De Wit et al., 1993; Carek et al., 2001).

The ability of S. aureus to be internalized by host cells is considered one of the most critical pathogenicity factors in persisting and relapsing infections. Although recognized as an extracellular pathogen for a long time, it is now widely accepted that S. aureus is internalized by a variety of non-professional phagocytes, such as endothelial cells (Ogawa et al., 1985; Menzies and Kourteva, 1998), epithelial cells (Bayles et al., 1998; Dziewanowska et al., 1999; Sinha et al., 1999), fibroblasts (Usui et al., 1992) and osteoblasts (Ellington et al., 1999; Jevon et al., 1999). Very recently, first reports demonstrated that also coagulase-negative staphylococci are internalized, i.e. S. epidermidis strains are internalized by bone cells (Khalil et al., 2007) and Staphylococcus saprophyticus is internalized by a human urinary bladder carcinoma cell line (Szabados et al., 2008). It has been postulated that the
internalized staphylococci can evade the host immune system as well as antibiotic treatment by ‘hiding’ within host cells, thus explaining why some staphylococcal infections are extremely difficult to eradicate.

The adherence of the staphylococci to the host cells represents the prerequisite for their internalization. Staphylococcal adhesins can be divided into different classes: Surface proteins of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family exhibit a number of common structural features, such as a signal sequence at the N-terminus, a region with different binding specificities for one or more ligands, various repeated sequences, and the C-terminal motif LPXTG, which is responsible for the covalent linkage to the cell wall peptidoglycan (Patti et al., 1994; Clarke and Foster, 2006). Well-studied members of the MSCRAMMs are the fibronectin (Fn)-binding proteins (FnBPs), which confer binding to Fn, fibrinogen (Fg) and human host cells (Flock et al., 1987; Jönsson et al., 1991; Peacock et al., 1999; Wann et al., 2000). Moreover, the FnBPs (FnBPA and FnBBP) are thought to be the major adhesins involved in S. aureus internalization by host cells. The FnBP-mediated mechanism of S. aureus internalization requires Fn as bridging molecule and the α5β1 integrins as the host cell receptor resulting in signal transduction, tyrosine kinase activity and cytoskeletal rearrangements (Dziewanowska et al., 1999; Sinha et al., 1999; Fowler et al., 2000). The FnBPs also bind to the heat shock protein 60 (Hsp60), which could act as a co-receptor in the FnBP-mediated uptake of S. aureus (Dziewanowska et al., 2000).

Another class of staphylococcal adhesins is represented by the autolysin/adhesins first described by us and others (Heilmann et al., 1997; Hell et al., 1998). These non-covalently bound proteins are associated to the surface by ionic or hydrophobic interactions and have both enzymatic (peptidoglycan-hydrolytic) and adhesive functions. In general, peptidoglycan hydrolases or autolysins are thought to play important roles in cell-wall turnover, cell division, cell separation and antibiotic-induced lysis of bacteria (Vollmer et al., 2008). As the first member of the autolysin/adhesins, we characterized the 148 kDa Atl from S. epidermidis, which mediates attachment to polystyrene, biofilm formation, and adherence to the host cells, thus explaining why some staphylococcal infections are extremely difficult to eradicate.

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within the C-terminal 232 aa domain. InlB promotes bacterial entry into a variety of cultured cell lines (Dramsi et al., 1995; Braun et al., 1998). Ami is a 102 kDa surface-associated autolysin, whose N-terminal domain shows similarity to the AM domains in Atl and AtlE (Braun et al., 1997). The C-terminal domain of Ami contains eight GW repeats that mediate binding to the bacterial cell surface and contribute also to the adherence of L. monocytogenes to eukaryotic cells as well as to virulence in vivo (Braun et al., 1997; Milohanic et al., 2000; Milohanic et al., 2001). The GW repeats of InlB and Ami have been shown to attach to the bacterial surface via lipoteichoic acid (Jonquieres et al., 1999; Milohanic et al., 2001). More recently, another surface-associated autolysin, the 64 kDa Auto of L. monocytogenes that contains four GW repeats within its C-terminal domain has been described to promote entry of the bacteria into eukaryotic cells and to contribute to virulence in vivo (Cabanes et al., 2004).

Based on these and our earlier findings, we hypothesized that the major staphylococcal autolysin/adhesins Atl from S. aureus and AtlE from S. epidermidis and more specifically their GW repeats might not only be involved in surface association and biofilm formation (Heilmann et al., 1997; Baba and Schneewind, 1998; Biswas et al., 2006), but also in adherence to and internalization by human host cells. In this report, we provide evidence that Atl and AtlE indeed contribute to S. aureus and S. epidermidis internalization by human host cells respectively. Moreover, we identified the heat shock cognate 71 kDa protein (Hsc70) as a putative host cell receptor recognizing Atl. Thus, we identified a novel mechanism of staphylococcal internalization by non-professional phagocytes that is not restricted to S. aureus, but is employed also by pathogenic coagulase-negative staphylococci and thus may play a critical role in the pathogenesis of chronic and recurrent infections with these important pathogens.

Results

Internalization of staphylococcal strains lacking surface-exposed FnBPs

To evaluate the potential of staphylococcal strains that lack surface-exposed FnBPs (FnBPA and FnBPB) for the internalization by human host cells, we performed flow cytometric internalization assays by using the endothelial cell line EA.hy 926. We analysed S. aureus strain Newman, which produces truncated versions of FnBPA and FnBPB due to point mutations that create a stop codon and thus FnBPs lacking the C-terminal anchor region (Grundmeier et al., 2004), the fnbA fnbB double knockout mutant of S. aureus 8325-4 (DU5883) (Greene et al., 1995), and the S. epidermidis strain O-47 (Heilmann et al., 1996). S. epidermidis O-47 does not seem to possess fnbA/ fnbB-homologous genes, because in a commercial S. aureus identification latex agglutination test (Pastorex Staph-Plus), S. epidermidis O-47 appeared negative (not shown). This test was used to control Staphylococcus carnosus expressing fnbA or fnbB for the production of the respective proteins, which in contrast to the S. carnosus wild type gave a positive result (Grundmeier et al., 2004). As a positive control, the highly invasive and functional FnBP-producing strain S. aureus Cowan 1 was used, whose internalization was set to 100% as described previously (Grundmeier et al., 2004). As a negative control, the apathogenic strain S. carnosus TM300 was used.

Flow cytometric analysis of staphylococcal internalization revealed that the S. aureus strains Newman and DU5883 were internalized by EA.hy 926 cells albeit to a lesser extent than S. aureus Cowan 1 (28.2 ± 2.1% internalization for Newman and 8.6 ± 1.1% internalization for DU5883) (Fig. 1A). S. epidermidis O-47 internalization was in the range of 13.6 ± 4.3% and thus was in between the S. aureus strains that lack surface-exposed FnBPs (Fig. 1A). For comparison, flow cytometry revealed 2.3 ± 0.2% internalization for S. carnosus TM300. Thus, pathogenic staphylococcal strains that lack surface-exposed FnBps are internalized to a certain degree albeit to a lesser extent than S. aureus strains expressing functional FnBPs.

The internalization of S. epidermidis O-47 by EA.hy 926 cells resulting in intracellularly located bacterial cells was also demonstrated by transmission electron microscopy (Fig. 1B–E). Intracellularly located S. epidermidis cells reside within the cytoplasm (Fig. 1B and C) or are surrounded by lysosomal membranes (Fig. 1D). Figure 1E shows an early-stage endocytosed S. epidermidis cell that is surrounded by protuberances of the endothelial cell. The intracellular structure of the endothelial cells appeared not to be disturbed by the uptake of S. epidermidis.

The major staphylococcal autolysin/adhesins Atl and AtlE mediate internalization

To test our hypothesis that the major autolysin/adhesins Atl and AtlE are involved in staphylococcal internalization by human host cells, we analysed the atl-deficient mutant S. aureus SA113atl (Biswas et al., 2006) and the atlE-deficient mutant S. epidermidis Mut1 (O-47atlE) (Heilmann et al., 1997) for their capabilities to be internalized by EA.hy 926 cells in flow cytometric internalization assays. Both the SA113atl and the O-47atlE mutant were significantly impaired in their abilities to be internalized by EA.hy 926 cells in comparison with their
Fig. 1. Internalization of staphylococcal strains lacking surface-exposed FnBPs by endothelial EA.hy 926 cells.

A. The internalization of FITC-labelled *Staphylococcus* strains by adherent EA.hy 926 cells was assessed by flow cytometry and computed in relation to *S. aureus* strain Cowan 1, which was set to 100% internalization. *S. aureus* Newman and DU5883 as well as *S. epidermidis* O-47, which lack surface-exposed FnBPs, were internalized by the endothelial cells albeit to a markedly lesser extent than the highly invasive *S. aureus* Cowan 1. However, internalization levels were 12-fold (Newman), fourfold (DU5883) or sixfold (*S. epidermidis* O-47) higher than that of *S. carnosus* TM300. Data are shown as the mean of five independent experiments.

B–E. Transmission electron micrographs of *S. epidermidis* O-47 internalized by endothelial EA.hy 926 cells. Bacteria were incubated with EA.hy 926 cells for 3 h. Subsequently, cells were fixed and processed for electron microscopy. Internalized *S. epidermidis* O-47 cells reside within the cytoplasm (B and C) or are surrounded by lysosomal membranes (D). (E) shows an early endocytosed *S. epidermidis* cell. Scale bars: 1 μm.
wild-type strains: 83.2 ± 10.7% of the S. aureus SA113 wild-type cells were detected intracellularly, whereas only 39.6 ± 12% of the SA113atl mutant cells were internalized (Fig. 2A). Thus, the insertional inactivation of atl in strain SA113 caused a significant reduction in internalization by ~52%. Similar results were obtained for S. epidermidis: Only 1.8 ± 0.5% of O-47atlE mutant cells could be detected intracellularly in contrast to 16.6 ± 4.1% of internalized wild-type O-47 cells, which relates to a significant reduction in internalization by ~89% with the O-47atlE mutant (Fig. 2A). To ensure that the reduced uptake of the SA113atl or O-47atlE mutant was indeed due to the absence of Atl or AtlE and not due to the pronounced cell clumps formed by the mutants because of their defect in cell separation, we put special emphasis on sonication to disrupt all cell clusters. Successful cell
separation, which was maintained during the assay due to the fixation of bacteria, was verified by transmission light microscopy (not shown).

Both the SA113atI and the O-47atI atIE mutant could be complemented for the production of the autolysin/adhesins Atl and AtIE by the plasmids pCXatI and pRC14 respectively. Plasmid pCXatI encodes the atI gene under a xylose-inducible promoter and the resulting strain S. aureus SA113atI (pCXatI) was demonstrated to produce surface-exposed Atl by SDS-PAGE and zymographic analysis (data not shown). Plasmid pRC14 encodes the atIE gene under the control of its own promoter (Heilmann et al., 1997). The internalization levels of the complemented SA113atI and O-47atI atIE mutants were significantly higher than those of the respective mutants. However, in contrast to the complemented S. epidermidis O-47atI atIE mutant [O-47atI (pRC14): internalization of 23.9 ± 4.3% cells], whose internalization level was even more pronounced than that of the O-47 wild-type strain, the internalization level of the complemented S. aureus SA113atI atI mutant [SA113atI (pCXatI): internalization of 69.2 ± 4.7% cells] did not entirely reach that of the SA113 wild-type strain (Fig. 2A). The internalization levels of SA113atI and O-47atI atIE carrying the control vector pCA44 (Heilmann et al., 1996) and pTC13 (Heilmann et al., 1997), respectively, were comparable with the internalization levels of plasmid-less mutant strains (Fig. 2A). These experiments indicated that the autolysin/adhesins Atl and AtIE play a significant role in staphylococcal internalization.

We then sought to find out whether Atl is sufficient for entry and confers the capability for internalization to S. carnosus TM300. For this, the plasmid pCXatI carrying the atI gene was introduced into S. carnosus TM300. The resulting strain S. carnosus (pCXatI) was demonstrated to produce surface-exposed Atl by SDS-PAGE and zymographic analysis (data not shown) and analysed for its internalization by EA.hy 926 cells. The expression of the atI in S. carnosus TM300 did not significantly increase the level of internalization: 2.5 ± 0.3% of S. carnosus (pCXatI) cells were internalized versus 2.1 ± 0.6% S. carnosus (pCX19) cells (Fig. 2A). However, pre-incubation of S. carnosus wild-type cells with 0.5 or 5 μg ml⁻¹ recombinantly expressed and purified Atl (rAtl) (see below) and subsequent flow cytometric analysis led to the detection of an increased number of internalized S. carnosus cells of approximately 2.5-fold with 0.5 μg ml⁻¹ rAtl (5.2 ± 0.3%) or threefold with 5 μg ml⁻¹ rAtl (6.6 ± 0.5%) (Fig. 2A), which however was not statistically significant.

To further support a role for Atl in S. aureus internalization, we analysed whether Atl dose-dependently and significantly inhibited internalization of S. aureus Cowan 1 (Fig. 2B). Inhibition was approximately 10% at 5 nM rAtl (89.8 ± 1.2% internalized S. aureus cells), 30% at 10 nM rAtl (70.3 ± 2.8% internalized S. aureus cells) and almost 50% at 25 nM rAtl (54.3 ± 2% internalized S. aureus cells) and reached a maximum at 50 nM rAtl (51.43 ± 2.4% internalized S. aureus cells). In contrast, pre-incubation with 500 nM of recombinantly expressed and purified mouse dihydrofolate reductase (rDHFR) as a negative control had no effect (Fig. 2B).

Atl mediates internalization with the functional domain being located within the N-terminal AM-R1/R2/R3 domain

To dissect the functional domains involved in internalization, we analysed the O-47atI atIE mutant complemented with plasmids that encode different subdomains of AtlE, i.e. strains O-47atI (pCC15) (am-R1/R2/R3) and O-47atI (pRC20) (am-R1/R2) (Heilmann et al., 1997). Plasmid pCC15 encodes the AM-R1/R2/R3 domain and plasmid pRC20 encodes the AM-R1/R2 domain of AtlE (see also Fig. 3A). Both plasmids were able to complement the O-47atI atIE mutant for biofilm formation (Heilmann et al., 1997). The SA113atI mutant could be complemented with plasmid pRC14 before and it was demonstrated that Atl and AtIE-related functions are interchangeable (Biswas et al., 2006). Moreover, we transformed the SA113atI mutant with the plasmid pCC15. All strains were analysed for their uptake by endothelial cells in the flow cytometric internalization assay. The complemented mutant strain O-47atI (pCC15) revealed a similar capacity for internalization by endothelial cells as the S. epidermidis O-47 wild-type, i.e. 14.7 ± 3.3% internalized cells. S. epidermidis O-47atI (pRC20) was internalized almost as efficiently (9.7 ± 3.1% internalized cells), which however did not reach statistical significance (Fig. 2A). Thus, the AM-R1/R2/R3 domain of AtlE was sufficient to reach the wild-type internalization level.

A similar result was obtained for S. aureus: The number of internalized bacteria with SA113atI (pRC14) (73 ± 16.3% internalized cells) and SA113atI (pCC15) (79 ± 7.8% internalized cells) was almost as high as that of the wild-type S. aureus SA113 strain (Fig. 2A). Thus, the AM-R1/R2/R3 domain of AtlE was sufficient to increase internalization of the S. aureus SA113atI mutant to the wild-type level.

In contrast, the uptake of S. carnosus TM300 harbouring plasmid pRC14 (2.4 ± 0.3% internalized cells), pCC15 (3.1 ± 1.2% internalized cells) or pRC20 (2 ± 0.2% internalized cells) by endothelial cells was very low and comparable with the uptake of the S. carnosus
TM300 wild type and S. carnosus TM300 (pCXatt) (not shown and Fig. 2A).

Expression and purification of 6 Histidine (His)-rAtl and 6 His-rAtl-subdomains in Escherichia coli

To analyse the adherence properties of Atl to plasma and extracellular matrix proteins and to endothelial cells, we expressed atl (without signal peptide) in Escherichia coli with an N-terminal His-tag [rAtl: aa A-30 to K-1256; 1227 aa with a molecular mass of 134 kDa]. Moreover, to dissect the functional domains within Atl, we also recombinantly expressed different His-tagged atl-subdomains, such as the amidase (AM) domain (rAM: aa A-30 to Q-420; 391 aa with 43 kDa), the AM domain containing the repeats R1 and R2 (rAM-R1/R2: aa A-30 to Q-786; 757 aa with 82 kDa), the glucosaminidase domain (GL) containing the repeat R3 (rGL-R3: aa T-787 to K-1256; 470 aa with 52 kDa), and the repeats R1, R2 and R3 (rR1–R3: aa G-418 to T-941; 524 aa with 56 kDa) and purified the respective proteins from E. coli by Ni-NTA affinity chromatography (Fig. 3A and B-I). To examine if rAtl and the enzymatic rAtl-subdomains purified from E. coli are bacteriolytically active, we performed zymographic analyses: SDS-PAGE was performed with the separation gel containing heat-inactivated S. carnosus or Micrococcus luteus cells as a substrate for bacteriolytic enzymes. Pronounced bacteriolytic activity was found with rAtl with a clearing zone ranging from approximately 70 kDa to 135 kDa (lanes 2), rAM-R1/R2 (lanes 3) and rAM (lanes 6) with S. carnosus as well as with M. luteus cells as a substrate. The arrow indicates lytic activity with rGL-R3 (lanes 4) that was visible with M. luteus cells and to a much lesser extent also with S. carnosus cells. As expected, rR1–R3 (lanes 5) did not show any lytic activity. The sizes of marker proteins (kDa) are indicated on the left.

Fig. 3. Model of Atl and AtlE, and bacteriolytic enzyme profiles of rAtl and different rAtl-subdomains. A. Schematic map of the structural organization of the autolysin/adhesins Atl from S. aureus Newman (137 kDa) and AtlE from S. epidermidis O-47 (148 kDa) and the different Atl-subdomains that were purified as 6 His-tag fusion proteins from E. coli. B. (I) SDS-PAGE (10% separation gel) of rAtl and rAtl-subdomains and corresponding zymograms to detect bacteriolytic activity against S. carnosus (II) and M. luteus cells (III). The lanes 2–6 each contain 4 µg of the respective protein. Bacteriolytic activity is visible as a clear zone after staining the gel with methylene blue and was found with rAtl (lanes 2), rAM-R1/R2 (lanes 3) and rAM (lanes 6) with S. carnosus as well as with M. luteus cells as a substrate. The arrow indicates lytic activity with rGL-R3 (lanes 4) that was visible with M. luteus cells and to a much lesser extent also with S. carnosus cells. As expected, rR1–R3 (lanes 5) did not show any lytic activity. The sizes of marker proteins (kDa) are indicated on the left.

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posed endothelial cell receptor for Atl, we performed pull-Atl-dependent staphylococcal uptake. To identify the sup-
to internalization, we assumed a receptor-mediated
Because Atl bound to endothelial cells and contributed
Identification of Hsc70 as putative endothelial
The amidase domain containing two repeats (rAM-R1/R2)
mediated adherence to plasma proteins and endothelial cells.
Binding was assessed as arbitrary units in an ELISA adherence
Fig. 4. The amidase domain containing two repeats (rAM-R1/R2)
mediated adherence to plasma proteins and endothelial cells.
(Arbitrary units) of Atl, rAM-R1/R2, and rAM.
Values greater than 0.2 arbitrary units were defined as
rAtl and rAM-R1/R2 efficiently bound to immobilized
human Fn and EA.hy 926 endothelial cells and to a lesser extent
also to Fg and Vn, whereas the adhesive features of the other
subdomains were significantly less pronounced. Binding of
recombinant His-tagged fusion proteins to the control protein BSA
was generally very low and therefore regarded as negative. Results
are shown as the mean of five independent experiments.

56 kDa). Thus, the gene products of the cloned atl and the
atl-subdomains seem to be functional.

**Atl mediates adherence with the functional binding domain being located within the N-terminal AM-R1/R2 domain**

The potential of rAtl and the different rAtl-subdomains to
bind to immobilized Fg, Fn and Vn, as well as to EA.hy
926 cells was analysed in an ELISA adherence assay. As
a negative control, binding to bovine serum albumin
(BSA) was assessed. We found that rAtl and rAM-R1/R2
most efficiently bound to immobilized human Fn and
EA.hy 926 cells (Fig. 4). Pronounced adherence of rAtl
and rAM-R1/R2 to immobilized Fg and Vn was also
detected. rAM and rR1–R3 showed moderate or low
binding to Fn and endothelial cells and rAM also to Fg. In
contrast, binding of rGL-R3 to plasma proteins or cells
was negligible. Thus, we conclude that Atl mediates
binding to the extracellular matrix and plasma proteins Fg,
Fn and Vn as well as to endothelial cells and that the
predominant functional binding domain is located within
the N-terminal AM-R1/R2 domain.

**Identification of Hsc70 as putative endothelial cell receptor**

Because Atl bound to endothelial cells and contributed
to internalization, we assumed a receptor-mediated
Atl-dependent staphylococcal uptake. To identify the sup-
posed endothelial cell receptor for Atl, we performed pull-
down assays. Using whole cell lysates of EA.hy 926 cells,
we eluted an Atl-binding protein component of approxi-
mately 70 kDa with rAtl as well as with rAM-R1/R2
coupled to the column. The respective protein band was
excised from a polyacrylamide gel (10% separation gel)
and subjected to matrix-assisted laser desorption/ionization
time-of-flight (MALDI-TOF) mass spectrometry. The signals obtained for the protein band were assigned
to the human Hsc70 (Accession No.: P11142; synonyms:
HSP7C, Hsp73), which is encoded by the gene HSPA8
(not shown).

**Inhibition of S. aureus internalization by monoclonal anti-Hsc70 antibodies**

It has been shown before that Hsc70 is not only found in
the cytoplasm, but is also present on the surface of a
human cell, which would be the prerequisite to act as a
receptor. To prove that cell surface-located Hsc70 may act
as a receptor involved in the internalization of staphylo-
cocci, we performed flow cytometric internalization assays
and analysed if a monoclonal antibody raised against
Hsc70 would be able to reduce internalization of S.
aureus Cowan 1, SA113 and Newman, as well as of S.
epidermidis O-47. As a control, the mutant strains
SA113atl and O-47atlE were included in the study. For
this, EA.hy 926 cells were pre-incubated with anti-Hsc70
(6 μg ml<sup>-1</sup>) or with an unrelated control immunoglobulin G
(λG) (6 μg ml<sup>-1</sup>) prior to infection with the staphylococci.
Indeed, pre-incubation with anti-Hsc70 led to a pro-
nounced decrease in S. aureus as well as in S. epidermi-
dis internalization, which was 23% with strain Cowan 1
(77.4 ± 6.8% versus 100%), 21% with strain SA113
(63.5 ± 11.7% versus 80.1 ± 11.7%), 50% with strain
Newman (11.8 ± 3.2% versus 23.8 ± 3.9%) and 64% with
S. epidermidis O-47 (6.4 ± 2% versus 17.6 ± 7.4%)
(Fig. 5). In contrast, pre-incubation with the anti-Hsc70
antibody had no effect on the internalization of the
SA113atl and the O-47atlE mutant. Similarly, pre-
incubation with the control λG had no effect (Fig. 5).
Therefore, we conclude that Hsc70 is involved in staphy-
lcoccal internalization by acting as a host cell receptor
for Atl or AtlE.

**Atl directly interacts with Hsc70**

To prove that Atl binds to Hsc70, we performed ligand
affinity blot analyses and ELISA adherence assays. The
interaction between Atl and Hsc70 may be direct or indi-
rect with extracellular matrix and plasma proteins, i.e. Fg,
Fn or Vn acting as bridging molecules. We found that
rAM-R1/R2 indeed bound to Hsc70 (Fig. 6A-II). This
binding could not be further increased by the addition of
Fn (Fig. 6A-III) or fetal bovine serum (FBS) (Fig. 6A-IV) in

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the assay. Thus, we conclude that the interaction is direct and not mediated via Fn or another plasma protein acting as a bridging molecule. In the negative controls, we did not detect considerable binding to BSA (not shown) or without a ligand (Fig. 6A-V).

Efficient binding of rAtl and rAM-R1/R2 to immobilized Hsc70 was confirmed in ELISA adherence assays, which was comparable to the binding to the control protein Fn (Fig. 6B). No significant binding was observed to BSA or in the negative controls (without first antibody) (Fig. 6B).

Surface abundance of Hsc70 is stimulated upon infection with S. aureus

It has been reported that phagocytosis of S. aureus induces a selective stress response in monocytes/macrophages; besides the release of two scavenging enzymes, the synthesis of human heat shock protein 70 (Hsp70) is upregulated (Kantengwa and Polla, 1993). We therefore analysed whether the expression of surface-associated human Hsc70 is stimulated upon infection with S. aureus. For this, we infected EA.hy 926 cells with different S. aureus strains and measured the number of cells carrying Hsc70 molecules on the cell surface 60 min after the infection by flow cytometry. Only a small fraction of non-infected EA.hy 926 cells contained Hsc70 molecules on their surface (0.4 ± 0.3%) (prior infection) (Fig. 7). The number of EA.hy 926 cells carrying Hsc70 molecules on their surface significantly increased 60- to 90-fold upon infection with S. aureus strains (Cowan 1: 27 ± 5.7%; SA113: 35.5 ± 3.3%; Newman: 23.5 ± 2.6%) (Fig. 7). In the negative controls, EA.hy 926 cells infected with S. aureus strains were incubated with an unrelated IgG. A slightly higher signal that was observed in
the negative controls was not statistically significant (Fig. 7).

**Discussion**

The internalization of *S. aureus* by human non-professional phagocytes, such as endothelial cells, is considered one of the most critical factors in the development of chronic infections and long-term persistence. Previously, internalization has only been reported for the staphylococcal species *S. aureus* and it was speculated that *S. epidermidis* cells may not be internalized at all (Sinha *et al*., 1999). Moreover, the internalization mechanism mediated by the *S. aureus* FnBPs, Fn as a bridging molecule, and α3β1-integrins as host cell receptor was regarded to be exclusive (Sinha *et al*., 1999). However, it has been reported that the FnBPs also interact with heat shock protein 60 (Hsp60), which may act as a co-receptor in FnBP-mediated uptake of *S. aureus* (Dziwanowska *et al*., 2000). Moreover, very recently it has been reported that coagulase-negative staphylococci, i.e. *S. epidermidis* and *S. saprophyticus* are also internalized by different host cells, such as osteoblasts and epithelial cells (Khalil *et al*., 2007; Szabados *et al*., 2008).

Here, we analysed the capacity of staphylococcal strains that lack surface-exposed FnBPs, such as the *S. aureus* strain Newman (Grundmeier *et al*., 2004) and a fnbA/fnbB knockout mutant (DU5883) as well as *S. epidermidis* O-47 for their internalization by endothelial cells and found that these strains show a pronounced higher capacity (12-fold with strain Newman, fourfold with strain DU5883 or sixfold with *S. epidermidis* O-47) to be internalized compared with the apathogenic *S. carnosus* TM300 albeit internalization with these strains was clearly lesser than that of the highly internalized strain Cowan 1 (3.5-fold lower with strain Newman, 11-fold lower with strain DU5883 or sevenfold lower with *S. epidermidis* O-47) (Fig. 1A). Additionally to our knowledge, the uptake of the coagulase-negative *S. epidermidis* by endothelial EA.hy 926 cells was visualized by transmission electron microscopy for the first time (Fig. 1B–E).

Recently, another *S. aureus* adhesin, the extracellular adherence protein Eap, has been found to enhance *S. aureus* internalization and to partially compensate for the loss of FnBPs (Haggar *et al*., 2003). However, both the FnBP- as well as the Eap-mediated mechanisms do not seem to play a role in *S. epidermidis* internalization, because a database search revealed that the *S. epidermidis* genomes sequenced encode neither eap- nor fnb-homologous genes. A complete different mechanism involved in *S. epidermidis* internalization was further supported by the observation of Khalil *et al*., who found that in contrast to *S. aureus* (Dziwanowska *et al*., 1999; Sinha *et al*., 1999; Fowler *et al*., 2000), the internalization of *S. epidermidis* could not be inhibited by D1–D4 fragments, which contain the Fn-binding activity of the *S. aureus* FnBPs, or anti-α3β1 antibodies (Khalil *et al*., 2007).

Previously, we and others found that staphylococcal autolysin/adhesins, such as Atl and Aas from *S. saprophyticus*, possess adhesive functions besides their roles in cell separation (Heilmann *et al*., 1997; Hell *et al*., 1998). These autolysin/adhesins contain six centrally located GW repeats, which are similar to the GW repeats of the *L. monocytogenes* surface proteins InIB, Ami and Auto. Because InIB, Ami and Auto have functions in adherence to and/or invasion of eucaryotic host cells, we hypothesized similar functions for the staphylococcal autolysin/adhesins Atl and AtIE. In our flow cytometric internalization assay, we indeed found a significantly reduced uptake of the *S. aureus* SA113atl and *S. epidermidis* O-47atIE mutants compared with the respective wild-type strains. Complementation of the SA113atl and O-47atIE mutant with the plasmid-encoded *atl* and *atIE* gene, respectively, restored their capacity for
internalization indicating a significant role for Atl and AtlE in internalization (Fig. 2A). Moreover, the SA113atf mutant could also be complemented by the atlE gene further indicating that Atl- and AtlE-related functions are interchangeable as it was suggested before (Biswas et al., 2006). A significant role for Atl in internalization was further supported by the observed inhibition of bacterial uptake upon pre-incubation of the endothelial cells with rAtl. Analogously, pre-treatment of eukaryotic cells with purified InlB before the infection inhibited the entry of L. monocytogenes (Braun et al., 1998).

In contrast to the complemented atl and atlE mutant strains, heterologous expression of the atl or the atlE gene in S. carnosus did not lead to an increased internalization by endothelial cells. The same was observed, when auto or inlB from L. monocytogenes were heterologously expressed in the apathogenic and non-invasive Listeria innocua, which did not allow L. innocua to invade eukaryotic cells (Dramsi et al., 1995; Cabanes et al., 2004). However more recently, it was demonstrated that L. innocua was able to invade cultured host cells, when high amounts of InlB were added externally to L. innocua (Braun et al., 1998). Thus, the authors concluded that rather the concentration of surface-exposed InlB was too low to support bacterial entry upon heterologous expression of InlB in L. innocua than an additional factor of L. monocytogenes was missing. Similarly, when rAtl was added externally to S. carnosus cells, we observed an increased uptake of S. carnosus that however was not statistically significant. Therefore, we assume that either an additional S. aureus or S. epidermidis factor might be involved in the Atl- or AtlE-mediated internalization, or that an even higher concentration of externally added rAtl would be necessary to reach a statistically significant internalization level. Alternatively or additionally, when atl or atlE is expressed in S. carnosus, the resulting surface exposure of Atl or AtlE may not favour a proper function in internalization. Our previous work demonstrated that cleavage products with different molecular masses were found on the surface of S. carnosus cells upon heterologous expression of the atlE gene indicating a different processing and surface exposure and thus supporting this idea (our unpublished results). In the genome of S. carnosus TM300, an Atl homologue [AtlCT (Bph; bifunctional peptidoglycan hydrolase precursor; EC:3.5.1.28)] was identified that shows a similar organization as Atl or AtlE (Rosenstein et al., 2009). It is conceivable that the expression of AtlCT could largely block the binding sites for the heterologously produced Atl or AtlE, a problem that might be overcome, when higher concentrations of rAtl are added exogenously. A prerequisite for this scenario would be that AtlCT is produced by S. carnosus TM300, but does not favour internalization (see below).

The ability of a staphylococcal strain to bind to extracellular matrix and plasma proteins and to host cells determines its capacity for tissue colonization. Therefore, we analysed the potential contribution of Atl to these processes. We found that rAtl bound to Fn, Vn and to endothelial cells. Thus, not only Aas from S. saprophyticus and AtlC from S. caprae (Hell et al., 1998; Allignet et al., 2001), but also Atl from S. aureus bind Fn. AtlE from S. epidermidis also binds Vn and presumably also Fn, although we detected only low binding in ligand affinity blot analysis (Heilmann et al., 1997). However, a discrepancy with false-negative results in ligand affinity blot analysis has been reported before with AtlC (Allignet et al., 2001). From our heterologous expression studies and subsequent adherence and internalization experiments, we concluded that the rAM-R1/R2 domain is the minimal domain allowing substantial adherence to extracellular matrix and plasma proteins as well as to endothelial cells. However, the minimal domain that was significantly sufficient to mediate S. aureus and S. epidermidis internalization by endothelial cells was the rAM-R1/R2/R3 domain. Previously, it was shown that the L. monocytogenes surface protein Ami binds to eukaryotic cells and the adhesive properties are carried out by its C-terminal GW repeats (Milohanic et al., 2001). Moreover, it was demonstrated that the addition of GW repeats (eight GW repeats from Ami versus the three GW repeats of InlB) improved the anchoring of the resulting InlB-Ami hybrid to the cell surface of L. monocytogenes so that it was completely surface-associated in contrast to InlB, which was partially secreted (Braun et al., 1997).

Based on these and our observations, we hypothesized that Atl or Atl-like proteins produced by staphylococcal strains associated with infection and/or persistence may contain a higher number of GW-dipeptide motifs compared with the AtlCT from apathogenic S. carnosus TM300 or more specifically that the presence of the six GW-dipeptide motifs within the repeats R1, R2 and R3 may be required for Atl- or AtlE-mediated uptake. To evaluate this possibility, we analysed the deduced Atl aa sequences from several S. aureus and coagulase-negative staphylococcal strains deposited in databases. Our analyses indicated that Atl and Atl-like proteins are highly prevalent and seem to occur in every staphylococcal isolate. Sequence comparison between the deduced Atl proteins from strains Newman, COL, USA300, MW2, NCTC 8325, MSSA476, N315, MN8 and MRSA252 revealed that they are highly similar sharing between 97% and 99% identical aa. All of these Atl proteins contained six GW-dipeptide motifs. atf-homologous sequences have also been identified from the available genomes of coagulase-negative species that have been associated with infection, such as S. epidermidis, S. saprophyticus,
S. caprae, S. lugdunensis, S. warneri, Staphylococcus haemolyticus and Staphylococcus hominis. Except for Aas from S. saprophyticus, which contains only five GW-dipeptide motifs, each Atl-like homologue of the above mentioned coagulase-negative species contains six GW-dipeptide motifs. Interestingly, sequence analysis of the deduced AtlCT from S. carnosus TM300 (Rosenstein et al., 2009) revealed an imperfect repeat R2 and only three GW-dipeptide motifs with the second, third and fourth motif lacking (Fig. 8), which strongly supports our hypothesis and may explain why S. carnosus TM300 is very poorly internalized. AtlCT consists of 1254 aa with a molecular mass of 136 kDa. In agreement with our assumption, sequence comparison between AtlCT and Atl (or AtlE) revealed a much higher similarity among the enzymatic active domains than within the repeat regions with 77.6% (72.6%) identical aa within the AM domains, 31.8% (32.7%) identical aa within the R1/R2/R3 domains and 54.1% (51.1%) identical aa within the GL domains.

Because Atl bound to and conferred internalization by endothelial cells, we supposed a receptor-mediated uptake mechanism involved in the Atl-dependent internalization. In a search for a potential endothelial cell receptor, we performed pull-down assays and isolated an approximately 70 kDa protein that interacted with Atl. Mass spectrometric analysis identified this protein as human Hsc70. Hsc70 is a constitutively expressed member of the Hsp70 family, which has important intracellular functions including protein folding and trafficking, oligomer assembly, binding to damaged or aberrant proteins, and prevention of toxic aggregate formation (Hightower, 1991). Besides being an intracellularly located molecular chaperone, Hsc70 has been demonstrated to be also present on the surface of human cells, which would be the prerequisite to act as a cellular receptor (Watanabe et al., 2008). Recently, Hsc70 has been demonstrated to contribute to the invasion of the human pathogen Brucella abortus into trophoblast giant cells (Watanabe et al., 2008). To our knowledge, B. abortus that causes brucellosis and infectious abortion has been the only bacterial species reported so far, whose internalization occurs in an Hsc70-dependent manner. However, previous studies showed that Hsc70 is also involved in rotavirus cell entry (Guerreo et al., 2002) and acts as a cellular receptor for syncytium formation by human T-cell lymphotropic virus type 1 (HTLV-1) (Sagara et al., 1998).

The direct interaction between Atl and Hsc70 emphasized a novel receptor-mediated staphylococcal internalization mechanism, which involves the staphylococcal major autolysin/adhesin Atl and Hsc70 as the human host cell receptor. To our knowledge, this is the first report that identified Hsc70 as a receptor for a GW

Fig. 8. Alignment of the deduced amino acid sequences of the Atl, AtlE and AtlCT GW repeats. Deduced amino acid sequences of the repetitive sequences R1, R2 and R3 of the autolysin/adhesins Atl from S. aureus Newman (137 kDa; R1a, R2a and R3a), AtlE from S. epidermidis O-47 (148 kDa; R1e, R2e and R3e) and AtlCT from S. carnosus TM300 (136 kDa; R1c, R2c and R3c) were aligned. While the GW repeats of Atl and AtlE are highly similar and contain six GW-dipeptide motifs, the AtlCT GW repeats are considerably less similar and contain only three GW-dipeptide motifs. The consensus (con) shows the identical amino acids occurring in at least five out of nine repetitive sequences. Gaps (−) were filled in to maximize homologies.
domain-containing surface protein. As a receptor for the GW domains in InlB, gC1q-R/p32 has been identified (Braun et al., 2000). Using flow cytometry, we found that pre-incubation of endothelial cells with monoclonal anti-Hsc70 antibodies significantly inhibited the internalization of the S. aureus strains Cowan 1, SA113 and Newman, and S. epidermidis O-47, but not that of the SA113atl and O-47atlE mutants, strongly further supporting a role for Hsc70 in Atl- or AtlE-dependent staphylococcal internalization. The invasion of B. abortus into trophoblast giant cells was analogously inhibited by a monoclonal antibody against Hsc70 (Watanabe et al., 2008). The strongly increased number of Hsc70 molecules on the surface of endothelial cells upon contact with S. aureus, which is probably due to a general stress response, further suggests an importance of this mechanism during infection.

Taken together, it seems that similar to the adherence to host tissues, the uptake of S. aureus by host cells is rather a multifactorial process. A model of the different mechanisms involved in S. aureus internalization by non-professional phagocytes are summarized in Fig. 9. While in S. aureus, the Atl-mediated uptake by eukaryotic cells appears to represent a ‘back-up’ mechanism, the AtlE-mediated uptake might be the predominant or even sole mechanism involved in the internalization of S. epidermidis or coagulase-negative staphylococci in general.

Hence, the Atl/AtlE-mediated internalization mechanism may represent a universal staphylococcal mechanism that is associated with a basic physiological cell function. Currently, further analyses are on the way to exactly characterize the molecular mechanisms underlying the Atl/AtlE-mediated uptake of staphylococcal cells and subsequent events.

In conclusion, we here propose a novel mechanism of staphylococcal internalization by human host cells, which involves the autolysin/adhesins Atl from S. aureus and AtlE from S. epidermidis and Hsc70 as the human host cell receptor.

**Experimental procedures**

**Bacterial strains, growth conditions, plasmids and cell culture**

Bacterial strains used in this study are listed in Table 1. We used the agr-defective strains S. aureus SA113 and S. epidermidis O-47, because this closely mimics the in vivo situation as the agr-negative geno- and phenotype is widespread among clinical isolates especially from persistent and implant-associated infections. Moreover, agr expression negatively affects atlE expression. Therefore, S. aureus SA113 and S. epidermidis O-47, which produce elevated levels of Atl/AtlE, were especially suitable to analyse the importance of Atl/AtlE in internalization in comparison to their atlE-negative counterparts. Staphylococcal strains were routinely cultivated aerobically at 37°C in Tryptic Soy (TS) broth (TSB, Difco™, BD Bioscience). E. coli strains were grown aerobically at 37°C in Luria–Bertani (LB) medium (Difco™, BD Bioscience). TS and LB agar contained 1.4% agar. TSB was supplemented with 1% xylose to induce atl expression. The production of 6×His-tagged fusion proteins in E. coli was induced by supplementing the culture with 1 mM IPTG. Selection for resistance to antibiotics in staphylococci was performed with 10 μg ml⁻¹ chloramphenicol (Roth, Karsruhe, Germany), 10 μg ml⁻¹ erythromycin (Merck, Darmstadt, Germany), 150 μg ml⁻¹ spectinomycin (Sigma Aldrich, München, Germany) or 10 μg ml⁻¹ tetracycline (Applichem, Darmstadt, Germany) and in E. coli with 100 μg ml⁻¹ ampicillin (Serva, Heidelberg, Germany) and 25 μg ml⁻¹ kanamycin (Serva), when appropriate.

For heterologous expression of the atl gene from S. aureus Newman in S. carnus TM300, the vector pCX19, a derivative of the xylose-inducible expression vector pCX15 (Wieland et al., 1995) and for the production and purification of the 6×His-fusion proteins (rAtl, rAM-R1/R2, rAM, rGL-R3 and rR1–R3), the vector pQE30 (Qiagen, Hilden, Germany), respectively, were used. To produce a 6×His-fusion control protein, the vector pQE40 (Qiagen), which encodes the gene for the mouse DHFR, was used.

For the internalization and adherence assays, the endothelial cell line EA.hy 926 was used (Edgell et al., 1983). EA.hy 926 cells were maintained in Dulbecco’s modified Eagle medium containing 4.5 g l⁻¹ glucose and stable glutamine (DMEM; PAA, Pasching, Austria), supplemented with 10% FBS (PAA), 1× hypoxanthine, aminopterin and thymidine (HAT, Invitrogen, Karlsruhe, Germany), penicillin [Invitrogen, (50 U ml⁻¹)], strepto-
Table 1. Bacterial strains used in this study.

| Strain       | Relevant genotype/plasmid | Relevant properties                     | Source/reference                  |
|--------------|----------------------------|----------------------------------------|-----------------------------------|
| S. carnosus  | Reference isolate          | Non-pathogenic                        | Schleifer and Fischer (1982)      |
| TM300        | (pCX19)                    | Empty vector control                  | This study                        |
| TM300 (pRCatl) | (pRCatl); Cm'               | Expression of atl                      | This study                        |
| TM300 (pRC14) | (pRC14); Cm'               | Expression of atIE                     | Heilmann et al. (1997)            |
| TM300 (pCC15) | (pCC15); Cm'               | Expression of amR1/R1/R2/R3            | Heilmann et al. (1997)            |
| TM300 (pRC18) | (pRC18); Cm'               | Expression of amR1/R1/R2/R3            | Heilmann et al. (1997)            |
| TM300 (pRC20) | (pRC20); Cm'               | Expression of amR1/R2/R3               | Heilmann et al. (1997)            |
| S. aureus    |                            |                                        |                                   |
| Cowan 1      | Reference isolate          | Isolated from septic arthritis         | ATCC 12598; NCTC 8530             |
| Newman       | Clinical isolate           | LACK OF Surface-exposed FNBPs         | NCTC 8178 (Grundmeier et al., 2004)|
| SA113        | ATCC 35556; NCTC 8325 derive | Accepts foreign DNA                  | Iordanescu and Surdeanu (1976)    |
| Col          | Clinical isolate           | Methicillin-resistant strain          | Gill et al. (2005)                |
| SA113 atl    | SA113 atl::spc'            | Deficient in autolysin Atl            | Biswas et al. (2006)              |
| SA113 atl (pCA44) | SA113 atl::spc' (pCA44); Cm' | Control vector                        | Heilmann et al. (1996)            |
| SA113 atl (pCAtl) | SA113 atl::spc' (pCAtl)     | Complemented with atl                 | This study                        |
| SA113 atl (pPCR14) | SA113 atl::spc' (pPCR14)     | Complemented with atIE                | This study                        |
| SA113 atl (pCC15) | SA113 atl::spc' (pCC15)     | Complemented with amR1/R1/R2/R3       | This study                        |
| DU5883       | 8325-4 fnbA::Tc fnbB::Em'   | Deficient in FnbA/FnbB                | Greene et al. (1995)              |
| S. epidermidis|                            |                                        |                                   |
| O-47         | Clinical isolate           | Biofilm-forming clinical isolate       | Heilmann et al. (1996)            |
| O-47atlIE    | O-47 atl::Em'              | Deficient in autolysin Atl             | Heilmann et al. (1997)            |
| O-47atlIE (pTC13) | O-47 atl::Em' (pTC13); Tc' | Control vector                        | Heilmann et al. (1997)            |
| O-47atlIE (pRC14) | O-47 atl::Em' (pRC14)      | Complemented with atl                 | Heilmann et al. (1997)            |
| O-47atlIE (pRC18) | O-47 atl::Em' (pRC18)     | Complemented with amR1/R1/R2/R3       | Heilmann et al. (1997)            |
| M. luteus    | Reference isolate          |                                        | ATCC 4698                         |
| E. coli      |                            |                                        |                                   |
| TG1          | supE hsdR5 thiA(lac-proAB) F' | Cloning and expression host          | Gibson (1984)                     |
| M15          | M15 (pREP4); Kan'          | Cloning and expression host; lac repressor | Qiagen                           |
| UT5600       | K12 strain lacking ompT    | Cloning host; deficient in OmpT protease | New England Biolabs              |

mycin [Invitrogen, (50 μg ml⁻¹)] and amphotericin B [PAA, (250 μg ml⁻¹)]. Cells were split once weekly 1:16 using accutase (PAA) for cell detachment.

DNA manipulations, transformation, polymerase chain reaction (PCR), DNA sequencing, websites and accession numbers

DNA manipulations and transformation of E. coli were performed according to standard procedures (Sambrook et al., 1989). S. carnosus and S. aureus strains were transformed with plasmid DNA by protoplast transformation (Götz and Schumacher, 1987). Because of the defect in cell separation, the S. aureus SA113atl mutant needed a 5x higher concentration of lysostaphin for the generation of protoplasts. Plasmid DNA was isolated using the ‘PrepEase MiniSpin’ Plasmid Kit (USB, Staufen, Germany) and chromosomal DNA was isolated using the ‘PrestoSpin D Bug’ DNA purification kit (Molzym, Bremen, Germany). PCR was carried out with the PCR Extender System (5 Prime; distributed by VWR International, Darmstadt, Germany) in accordance with the protocol of the supplier. The primers were synthesized by Eurofins MWG Operon (Table 2) (Ebersberg, Germany). The DNA sequences of the atl gene and the atl subclones were determined by Eurofins MWG Operon using their standard sequence primers pQefor and pQerev and others (see Table 2) and an ABI 3730XL DNA sequencer.

The DNA and deduced protein sequences were analysed using the program ‘JustBio’ at http://www.justbio.com. The deduced Atl sequences from various sequenced S. aureus strains were compared using the programs BLASTP (Altschul et al., 1997) and FASTA (Pearson and Lipman, 1988) and the alignments were performed using the program CLUSTALW at the European Bioinformatics Institute (EBI, Cambridge, UK). The signals obtained by MALDI-TOF were assigned to peptides of
Heterologous expression of atl in S. carnosus TM300

The atl gene including the ribosomal binding site was amplified by polymerase chain reaction (PCR) from S. aureus Newman genomic DNA using the primers Atl-pCX-F and Atl-KpnI-R, yielding a 3809 bp DNA fragment. The DNA fragment was cloned into the BamHI and Smal sites of the vector pCX19 in S. carnosus, creating the plasmid pCXatl.

Construction and purification of 6×His-rAtl fusion protein and 6×His-rGL-R3 subdomain fusion proteins from E. coli, and production of anti-rAM antibodies

The DNA sequences encoding Atl (rAtl) and different Atl-subdomains, i.e. the amidase (AM) domain (rAM), the AM domain containing the repeats R1 and R2 (rAM-R1/R2), the glucosaminidase domain (GL) containing the repeat R3 (rGL-R3) and the repeat region (rR1–R3) were PCR-amplified from S. aureus Newman (rAtl) or S. aureus COL (Atl-subdomains) genomic DNA using the following primers (see Table 2): rAtl: Atl-BamHI-F/Atl-KpnI-R, rAM: Atl-BamHI-F/Atl-KpnI-R, rAM-R1/R2: Atl-BamHI-F/Atl-KpnI-R, rGL-R3: N-Atl-R, rR1–R3: N-Atl-R.

The resulting DNA fragments (rAtl: 3681 bp; rAM-R1/R2: 2271 bp; rAM: 1173 bp; rGL-R3: 1410 bp; rR1–R3: 1572 bp) were cloned into the BamHI and KpnI sites of the vector pQE30 in E. coli so that the respective gene products are in frame to the codons for 6×His creating the plasmids pQatl, pQAM-R1/R2, pQAM, pQGL-R3 and pQR1–R3. Correct clones were verified by DNA sequencing. Purification of the 6×His-fusion proteins from E. coli cultures containing plasmid pQatl, pQAM-R1/R2, pQAM, pQGL-R3 or pQR1–R3 was performed under denaturing conditions using Ni-NTA superflow columns (Qiagen) according to the manufacturer. rAtl, rAM-R1/R2 and rGL-R3 were additionally purified under native conditions using Ni-TED columns (USB) according to the manufacturer with altered buffer compositions: binding and washing buffer (500 mM NaCl, 50 mM NaH₂PO₄, 5 mM Tris-HCl, 5 mM imidazole pH 8.0), elution buffer (500 mM NaCl, 50 mM NaH₂PO₄, 5 mM Tris-HCl, 250 mM imidazole, pH 8.0). Buffers were supplemented with protease inhibitors (complete EDTA-free protease inhibitor cocktail; Roche, Mannheim, Germany). The purified rAM subdomain was used to immunize rabbits, which was performed by Eurogentec (Seraing, Belgium) according to their standard 87-day immunization programme with subsequent total IgG purification.

SDS-PAGE, detection of bacteriolytic activity (zymogram) and ligand affinity blot analysis

Purified rAtl and rAtl-subdomains (4 μg) or the cell lysates and eluates from the pull-down assays were subjected to SDS-PAGE (10% separation gel and 4.5% stacking gel) and stained with Coomassie Brilliant Blue (R250) (0.1%).

The bacteriolytic activity of rAtl and rAtl-subdomains was determined as described before with minor modifications (Heilmann et al., 1997). Briefly, the 6×His-tag proteins were separated by SDS-PAGE on a polyacrylamide gel containing 0.2% heat-inactivated S. carnosus or M. luteus cells as a substrate in the separation gel (10%) to distinguish between amidase and glucosaminidase activity. After electrophoresis, gels were incubated overnight in 25 mM Tris-HCl (pH 8.0) containing 1% (v/v) Triton X-100 (Sigma Aldrich) at 37°C and then stained with 1% (w/v) methylene blue (Merck) in 0.01% (w/v) KOH and destained with ddH₂O. Bands with lytic activity were observed as clear zones in the blue-stained gels.

To prove the binding of Atl to Hsc70, we performed ligand affinity blot analysis. For this, recombinant human Hsc70 (2 μg; USBio, Swampsott, USA) or BSA (2 μg; Applichem) were separated by SDS-PAGE (10% separation gel) as described above and transferred to a nitrocellulose membrane (Whatman, Dassel, Germany), using a semi-dry blot system (Biorad, München, Germany). The membranes were then blocked in Tris-buffered saline (TBS)-3% BSA for 2 h and washed three times with TBS-0.05% Tween 20 (TBST). Afterwards, the membranes were incubated with 2 μg ml⁻¹ purified rAtl-R1/R2 in 0.3% BSA/TBST for 2 h. In some experiments, 25 μg ml⁻¹ human Fn (Roche) or 10% FBS was added. As a negative control, the membrane was incubated in 0.3% BSA/TBST.
without a ligand for 2 h. Bound rAM-R1/R2 was detected by incubation (1 h) with rabbit anti-rAM IgGs (1.69 μg ml⁻¹). The reaction of proteins with specific antibodies was detected by incubation (1 h) with an anti-rabbit IgG-alkaline phosphatase (AP) conjugate (0.84 μg ml⁻¹; Dako, Glostrup, Denmark) and subsequent colour reaction.

**Preparation of FITC-labelled bacteria, flow cytometric internalization assay and flow cytometric detection of surface abundance of Hsc70**

Overnight cultured staphylococci were washed with PBS, sonicated using an ultrasonic cell disruptor (Branson Sonifier® 250) to separate cell aggregates, and subsequently fixed with 5% formaldehyde for 2 h at room temperature. Special emphasis was put on sonication to disrupt all cell clusters to exclude the possibility that the reduced internalization of the SA113atl and O-47atlE mutants was due to the defect in cell separation resulting in cell clumping and thus would represent a false negative result. Appropriate mechanical cell separation was verified by phase-contrast light microscopy (not shown). Fixed cells were labelled with fluorescein isothiocyanate [FITC isomer I (100 μg ml⁻¹; Invitrogen) in an appropriate staining buffer (1.75% DMSO; 0.5 M Na₂CO₃; 0.5 M NaHCO₃) for 1 h at 37°C. The cells were subsequently washed with PBS (PAA), resuspended in 1% human serum albumin (HSA; Baxter, Vienna, Austria) and the OD₅₄₀ was adjusted to 1.0.

The internalization assay was performed essentially as described before with slight modifications (Sinha et al., 1999). Briefly, EA.hy 926 cells were split into 24-well cell culture plates (Greiner Bio-One) at 4 × 10⁵ cells per well 1 day before the assay. The cells were then washed with DMEM. Internalization medium (DMEM containing 10 mM Heps and 1% HSA) and FITC-labelled bacteria were added at a multiplicity of infection (moi) of 100. For the inhibition studies, the endothelial cells were pre-incubated with either 200 nM of recombinant proteins, monoclonal anti-Hsc70 antibodies (6 μg ml⁻¹; Acris Antibodies, Herford, Germany), or as a control mouse anti-*Aspergillus niger* glucose oxidase antibodies (6 μg ml⁻¹; Dako, Gostrup, Denmark) for 30 min at 37°C and 5% CO₂ prior to infection with staphylococci. Culture dishes were incubated at 4°C for 1 h to allow sedimentation of bacteria and shifted to 37°C and 5% CO₂ for a 3 h internalization period. Finally, lyostaphin (25 μg ml⁻¹; Sigma Aldrich) was added to eliminate adherent staphylococci (20 min, 37°C). After washing thrice with DMEM, the cells were detached with accutase, washed with colourless medium (DMEM without phenol red, PAA Laboratories) and resuspended in 500 μl 1% HSA. After 10 min incubation with the Na⁺/K⁺-ionophore monensin [Sigma Aldrich (70 ng ml⁻¹)] to break down the intra- and extracellular pH gradient, propidium iodide [Sigma Aldrich (5 μg ml⁻¹)] was added, which enters only dead cells and thus enables the exclusion of dead cells from the data analysis. Samples were analysed on a FacsCALIBUR™ (BD Bioscience) as described previously (Sinha et al., 1999), with the exception that experiments were routinely run after addition of propidium iodide. The ratio of internalized staphylococci was calculated in relation to the control strain *S. aureus* Cowan 1, which was set to 100% internalization.

The abundance of Hsc70 molecules on the surface of EA.hy 926 cells infected with *S. aureus* or non-infected was assessed by flow cytometry. EA.hy 926 cells were split into 24-well cell culture plates at a number of 4 × 10⁵ cells per well 1 day before the assay. After washing the cells with PBS, 500 μl of invasion medium was added. The cells were incubated with *S. aureus* (moi: 100) for 60 min. Instantaneous washing was followed by incubation of the cells in 500 μl of invasion medium containing monoclonal anti-Hsc70 antibodies (6 μg ml⁻¹) or control mouse anti-A. *niger* glucose oxidase antibodies (6 μg ml⁻¹) for 1 h at 37°C. The cells were washed twice with PBS, and subsequently incubated with polyclonal FITC-conjugated rabbit anti-mouse IgG (Acris; 4 μg ml⁻¹) for 1 h at 37°C. After cell detachment and subsequent washing with PBS, the samples were analysed on a FacsCALIBUR™.

**ELISA adherence assay**

For the ELISA adherence assays, the wells of 96-well microplates were coated overnight with the human proteins Fg (20 μg ml⁻¹), Fn (10 μg ml⁻¹), Vn (5 μg ml⁻¹), Hsc70 (10 μg ml⁻¹), or 1% BSA in PBS at 4°C and subsequently blocked with 1% BSA or 5% skim milk (Fluka, München, Germany). Vn was previously purified from human serum according to Hayashi (Hayashi, 1994). To assess the adherence to endothelial cells, EA.hy 926 cells were grown confluent in 96-well cell culture plates (Greiner Bio-One), washed with PBS, fixed with ice-cold methanol (Merck) and blocked with 1% BSA. Then, the microplates were washed thrice and each well was incubated with 200 nM of the respective 6 × His-tag fusion protein for 1 h at 37°C. All following incubation steps occurred at room temperature. Unbound proteins were removed by washing thrice with 200 μl of PBS. Bound 6 × His-tag fusion proteins were detected by an anti-5 × His antibody (Qiagen, 0.13 μg ml⁻¹) and AP-conjugated goat anti-mouse IgG (Dako, 0.62 μg ml⁻¹). Detection of the AP-conjugated anti-mouse IgG was performed using the AP-substrate SigmaFast™ p-Nitrophenylphosphate (pNPP) (Sigma Aldrich). The OD₄₀₅ was measured after 30 min of incubation.

**Pull-down assay and MALDI-TOF mass spectrometry**

To identify the proposed endothelial cell receptor for Atl, we performed pull-down assays. For this, confluent EA.hy 926 cells from two cell culture flasks (75 cm², Greiner Bio-One) were detached and resuspended in 10 ml of PBS. The cells were pelleted (1000 g, 3 min), washed twice with PBS, and finally resuspended in 1 ml of lysis buffer (30 mM Heps, 1 mM EDTA, 14 mM NaCl, 2 mM MgCl₂, 1% Triton X-100, 1 mM DTT, protease inhibitors). Complete cell lysis was assured by additional mechanical disruption using a 21 G needle (BD Bioscience). Afterwards, the cell lysate was cleared by centrifugation (10 000 g, 10 min, 4°C). A 10 ml disposable column (Pierce) containing 100 μl of slurry of Ni-NTA agarose (Qiagen) was equilibrated with washing buffer (30 mM Heps, 10 mM imidazole, 500 mM NaCl, 10 mM β-mercaptoethanol). Then, 25 μl of either rAtl or rAM-R1/R2 was bound to the resin and washed once with washing buffer. Next, 500 μl of the EA.hy 926 cell lysate was added and the column was washed thrice. Bound proteins were eluted with elution buffer (30 mM Heps, 250 mM imidazole, 500 mM NaCl, 10 mM β-mercaptoethanol), precipitated with trichloroacetic acid (10%) and consequently analysed by SDS-PAGE. To ensure that proteins from EA.hy 926 cell lysates do not
EA.hy 926 cells were grown to 80% confluency, incubated with S. epidermidis O-47 (moi: 150) for 3 h in internalization medium (see above) and subsequently washed thrice with internalization medium. EA.hy 926 cells were fixed with 2% glutaraldehyde in PBS for 24 h at 4°C and post-fixed with 1% osmium tetroxide. After three washes with PBS, cells were dehydrated through a graded ethanol series and embedded in epoxy resin. Ultrathin sections were prepared, counterstained with uranyl acetate and lead citrate, and examined with a FEI-Tecnai™ 12 electron microscope (FEI, Eindhoven, the Netherlands) at 80 kV. Image conversion was performed using the Ditabis Micron Imaging Scanner (Ditabis Digital Imaging System AG, Pforzheim, Germany).

Statistical analysis

Statistical significance of experimental data was analysed by ANOVA and appropriate post-tests to correct for multiple comparisons: Dunnett’s post-test was applied for Figs 1A and 2B. Bonferroni’s post-test was used to verify the results of Figs 2A, 5, 6B and 7. P-values ≤ 0.05 were considered statistically significant and are indicated with asterisks: * (P ≤ 0.05), ** (P ≤ 0.01) and *** (P ≤ 0.001).

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