Mechanism and consequences of invasion of endothelial cells by Staphylococcus aureus

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Summary
It has become clear that Staphylococcus aureus is a facultative intracellular microorganism. Adherence and invasion are a prerequisite for endovascular infections caused by S. aureus, such as infective endocarditis. These phenomena may also be involved in the pathogenesis of invasive and metastatic infection upon hematogenous dissemination, such as osteomyelitis and abscess formation. The underlying molecular mechanisms have been elucidated in detail, including their likely relevance in vivo. However, the mode of action of recently identified modulators of invasion, such as plS/Pls have not yet been clarified. The potential outcome for host cells and S. aureus following invasion are diverse. Surprisingly, induction of apoptosis in human endothelial cells is more complex than previously thought, since it appears to involve multiple virulence factors. In the light of increasing resistance to antimicrobial therapy, understanding the multifaceted pathogenesis of S. aureus infection in detail is needed for a better prevention and therapy.

Keywords
Bacterial invasion, host cells, Staphylococcus aureus, infective endocarditis, pathogenesis

Background
Staphylococcus aureus and S. aureus-mediated disease
Staphylococcus aureus can be found as a part of the normal human resident flora. Up to two thirds of the healthy population are permanently or transiently colonized by S. aureus with pronounced variations, depending on geographic, ethnic, social, and medical factors (1). The major reservoir is colonization of the anterior nares, but it is also recovered from other moist regions of the body (e.g., the inguinal and perineal area, axilla, forehead, vagina, colon). Colonization may serve as an endogenous source of bacteremia (2, 3) and infection (3, 4). A major risk factor of intravascular and systemic infections by S. aureus is a breach in the epithelial barrier, e.g., by intravascular catheters, implanted foreign body material, or mucosal damage induced by cytostatic chemotherapy (5).

S. aureus is a most versatile microbial pathogen and among the most frequently isolated from community-acquired and nosocomial infections (1, 6). Besides toxigenic disease, S. aureus can cause uncomplicated skin infections (boils), but also soft tissue and wound infections, with or without abscess formation, severe pneumonia, osteomyelitis, and intravascular infections, such as infective endocarditis, sepsis, and septic shock (7, 8).

Endovascular S. aureus infections are among the most difficult to treat. This is probably due to the complex pathogenesis involving a number of host and bacterial factors. Even previously healthy persons may rapidly be vitally endangered by a systemic S. aureus infection. This may be due to a fast destruction of hemodynamically essential valve structures (endocarditis), thrombus formation (septic thrombophlebitis) and hematogenous systemic dissemination with sepsis and abscess formation (central nervous system, visceral organs). The mortality rate due to these complications of systemic infections is high, even if patients are treated early and appropriately. Patients with severe S. aureus infections, such as endocarditis (7) and osteomyelitis (9), often need to be treated for weeks or months, even if the antimicrobial substances are effective in vitro.

Experimental data show that adhesins (10) (bacterial surface components), platelets (11), plasma proteins (12), endothelial cells (13), and sub-endothelial tissue components (14) are involved in the infection of the vessel wall. The initial adhesion process, as well as the course of infection are modulated by networks of different staphylococcal global regulatory systems, and activation processes of host cells and platelets. A detailed overview on the pathogenesis of S. aureus endocarditis can be found in reference 15. Besides being involved in these complex inter-
actions taking place at the vessel wall in vivo, *S. aureus* has been shown to be able to directly adhere to and invade endothelial cells in vitro (16–18).

**Virulence determinants of *S. aureus***

*S. aureus* has a substantially smaller genome of ~2.5–2.9 Mbp (1, 19-21), as compared to several gram-negative bacteria, such as *Escherichia coli* (~4.1–5.6 Mbp), and *Pseudomonas aeruginosa* (~6.2–6.4 Mbp). Nevertheless, there is virtually no organ and tissue which may not be colonized and infected by *S. aureus* as a facultative pathogenic bacterium. This extremely high versatility is, in part, due to the pronounced genomic plasticity of *S. aureus*. Among other mechanisms, the genomic plasticity is based on the insertion of phages and other mobile genetic elements (20), and additional plasmids are found in some strains.

As is the case of enteropathogenic gram-negative bacteria, several pathogenicity islands (SaPI-1, SaPI-2, SaPI-3, etc.; *S. aureus* pathogenicity island) have been identified in *S. aureus* (22, 23). These are chromosomal regions which have been acquired horizontally from other species. Together with these chromosomal regions the respective genes for virulence factors have been passed on. Additionally, SCCmec (staphylococcal cassette chromosome with methicillin-resistant) of MRSA (methicillin-resistant *S. aureus*) can be classified as pathogenicity islands (19).

*S. aureus* can express a large number of surface and secreted proteins (such as exotoxins (24, 25) and exoenzymes (26)), as well as other surface components (e.g. capsular polysaccharides), most of which have been attributed a role as virulence factors, contributing to the establishment or maintenance of an infection (1).

The regulated expression of many virulence factors appears to be growth phase-dependent (1). As has become clear from recent work by several groups, the initial model for regulation of virulence factors is oversimplified and thus cannot explain several data published. At present, several components of a global regulatory network for gene expression in *S. aureus* have been identified, comprising numerous loci such as *agr*, *sar*, *sigB*, *rot*, *arrRS*, *svrA*, and *saeRS* (27, 28). Many of the involved regulatory loci form “two component systems”, a regulatory paradigm found in many bacteria (29, 30). Part of this regulatory network is an intercellular communication system (a quorum sensing system using short circular peptides, AIP/auto-inducing peptide, as a message (29)), which helps to translate the bacterial density into subsequent global gene regulation, in order to adapt to differing environmental conditions (27, 31, 32). In addition, little, if any, data exist regarding the regulation and gene expression in the host in vivo during different stages of infection: e.g. a novel fibrinogen- and fibrinectin-binding cell wall-anchored adhesin, *IsdA*, is expressed only under iron-limited conditions, controlled by Fur (33).

**S. aureus adhesins and invasins**

*S. aureus* has developed a number of mechanisms for adherence to host components, which is an early essential step during microbial pathogenesis. Adhesion of *S. aureus* to host components, such as matrix and plasma proteins (e.g. fibrinectin, fibrinogen, vitronectin, thrombospondin, and collagens), as well as host cells, is mediated by a number of specific staphylococcal surface proteins (adhesins). So far, four different classes of adhesins, including the non-proteinaceous (wall and lipo-) teichoic acids are known in *S. aureus* (Fig. 1). Of note, lack of WTA reduces interaction with endothelial cells and virulence in a rabbit endocarditis model (34).

Recently, 21 cell wall-anchored adhesins, 10 of which were previously unknown, have been identified in silico, based upon the data from six different complete *S. aureus* genomes publicly available (35). In addition, a still growing number of members of other adhesin classes are being identified. Consequently, *S. aureus* has been considered as an extracellular pathogen (36). This still holds true, especially for foreign body infections (e.g. catheters and implants), and the colonization of virtually any host tissue. However, the FnBP-dependent mechanism of adherence to fibronectin also leads to invasion of host cells (37–41). At least for certain conditions, such as infective endocarditis and osteomyelitis, this appears to play an important pathogenic role in vivo, as will be discussed below. An adhesin for thrombospondin has not been clearly identified, despite data arguing for a specific interaction with a putative staphylococcal receptor. Similarly, a vitronectin-binding protein has been described, but not yet further characterized (42).

The importance of the cell wall-anchored adhesins FnBPA and FnBPB (43–47), of the fibrinogen-binding ClfA (46), of

![Staphylococcus aureus adhesins](image-url)

**Figure 1:** Known classes of *S. aureus* adhesins. The four known classes of *S. aureus* adhesins, including the non-proteinaceous (lipo-) teichoic acids, are summarized. Please note that mostly only examples are mentioned. Most recently published in vitro and in vivo data show that wall teichoic acids (WTA) are required for adherence of *S. aureus* strain SA113 to primary human nasal epithelial cells and a human alveolar epithelial cell line (A549), as well as nasal colonization of cotton rats (*Sigmodon hispidus*) (169). In addition, lack of WTA in *S. aureus* strain SA113 reduces interaction with endothelial cells and virulence in a rabbit endocarditis model (34). Lipoteichoic acids are shown as well, since they may serve as binding structures for anchorless adhesins. However, no direct adhesive function has been demonstrated so far in *S. aureus*. Ebh/GSSP is currently classified as anchorless adhesin but contains a putative transmembrane domain. Please refer to the text for more details. CoNS denotes coagulase-negative staphylococci; IE denotes infective endocarditis.
SpA (48), and of the secreted adhesins Eap (49) and Emp (50) can be deduced from the fact that the respective genes are found in nearly 100 % of clinical S. aureus isolates. Unlike in other gram-positive organisms, e.g. for interalin A (InlA) and Inlb in Listeria monocytogenes, these two classes of S. aureus adhesins, cell wall-anchored and soluble/secreted, are not structurally related.

Further S. aureus adhesins with potential relevance for interaction with endothelial cells, which are not discussed in detail in the text are listed in Table 1.

### Covalently cell wall-anchored adhesins

Cell wall-anchored adhesins (MSCRAMMs, microbial surface components recognizing adhesive matrix molecules (10)) are covalently anchored by sortase to the peptidoglycan side chains of peptidoglycan (51). To this end, this class of adhesins possesses a consensus motif (LPXTG) for sortase (52). Two of the 21 cell wall-anchored adhesins recently identified in silico, SasF and SasD (Staphylococcus aureus surface protein) have a modified sortase consensus motif, namely LPGAX, but appear to be sorted normally (35). Initially, MSCRAMMs have been thought to be mono-specific for a given host protein. Now, a different picture has emerged, since more than one ligand has been identified for many of these adhesins. Classic members of this class are SpA (Staphylococcal protein A), FnBPs (fibronectin-binding proteins), Cna (collagen adhesin), ClfA and ClfB (clumping factors, and other fibrinogen-binding proteins). Also belonging to this class are Pls (plasmin-sensitive protein) (53).

Some adhesins of this class have been obviously duplicated and still exert similar or identical functions. This is the case e. g. for FnBPA and FnBPB, or homologues of the fibrinogen-binding clumping factors (ClfA and ClfB), such as the Sdr proteins.

**FnBPA and FnBPB (fibronectin-binding proteins).** The first observation that S. aureus is agglutinated specifically by plasma fibronectin due to a surface component which is distinct from clumping factor and protein A has been reported in 1981 (54). In 1987, isolation of S. aureus FnBPs with an apparent molecular mass of 210 kDa was reported from strain Newman (55). The respective genes coding for the two known forms of FnBPs, FnBPA and FnBPB, fnbA (56, 57) and fnbB (58) have been cloned. The genes fnbA and fnbB are closely adjacent to each other and have most likely resulted from gene duplication. Most, but not all S. aureus WT isolates appear to harbor both genes, fnbA and fnbB, respectively (44, 45), and nearly all isolates have at least one fnb gene (43–47).

Classically, the D-domains have been identified as fibronectin-binding domains. In the prototype sequences of strain 8325-4, three and a half tandem repeats (D1–D4) have been assigned to the D-domain of both FnBPA and FnBPB. More recently, an upstream repeat (D0), located in the C-domain has
been identified. Subsequently, it has been shown that the B-domain of FnBPA is also involved in fibronectin-binding functions (59, 60). All regions thought to be fibronectin-binding domains share a high degree of nucleotide sequence identity between fnbA and fnbB (Fig. 2).

The S. aureus-binding region on fibronectin has been located to the N-terminal 27 kD proteolytic fragment (61), a heparin-binding region. Based on structural data, it has been proposed recently, that the interaction of fibronectin and FnBPs takes place over a wide span of the FnBP molecules encompassing a much larger region than previously identified for binding of fibronectin and adhesion to or invasion of host cells. In this model a sequence of similar fibronectin-binding modules (“beads on a string”) is postulated. These homologous modules can substitute each other (62).

In addition to their classical role as adhesin for fibronectin, and their new role as the major S. aureus invasins, further functions for FnBPs have been identified. A fibronogen-binding function has been described for the A-domain of FnBPA (63). FnBPA appears to bind to fibronogen as well, as shown by agglutination of fibronogen-coated beads (41), used for diagnostic identification of S. aureus. FnBPA, but not FnBBP, binds to and activates human platelets, leading to platelet aggregation (64), conceivably by a direct interaction with a yet unknown platelet receptor. Interaction with platelets via FnBPA and other mechanisms may indirectly promote S. aureus adherence to endothelial cells. In addition, FnBPA appears to mediate T cell adhesion and coactivation (65).

The fibronogen-binding clumping factor A (ClfA) is one of the first S. aureus adhesins investigated. During the last years, it has been recognized that ClfA belongs to the large family of Sdr proteins (Ser [S]-Asp [D] repeat protein) characterized by SD (serine-aspartate) repeats, comprising ClfA (SdrA) and ClfB (SdrB), as well SdrC, SdrD, and SdrE (10). The fibronogen-binding ClfA has been implicated in the pathogenesis of various conditions, such as infective endocarditis (66).

Pls (plasmin-sensitive protein) (53, 67) may be classified as belonging to the Sas (Staphylococcus aureus surface) protein family, since it is structurally related to SasG. So far, Pls has exclusively been identified in MRSA of SCCmec type I (staphylococcal chromosomal cassette with methicillin-resistant type I), a subset of MRSA isolates. The pls gene probably originates, like SCCmec type I itself, from Staphylococcus scuri. Aap (accumulation-associated protein) is a surface protein from Staphylococcus epidermidis structurally related to the Sas family and Pls.

Even though Pls belongs to the class of cell-wall-anchored adhesins, it has a pronounced anti-adhesive (67) and anti-invasive effect, as determined by genetic data in a model using a human embryonic kidney cell line (293 cells) (68). On the other hand, parts of Pls (and potentially Aap) appear to mediate adherence to isolated keratinocytes, much like SasG (69).

Anchorless (secreted or soluble) adhesins

Anchorless (secreted or soluble) adhesins are bound non-covalently to the staphylococcal cell wall after or during secretion. This class of adhesins is also termed “SERAMS” (secreted expanded repertoire adhesive molecules), and is discussed in detail in a separate article (70). They form a kind of putty on the cell wall binding with a broad specificity to different host proteins. Consequently, anchorless adhesins can act in a similar way as cell-wall-bound adhesins. It has been proposed that a surface-bound neutral phosphatase serves as a receptor for sequestration of Eap to the staphylococcal cell wall (71), but generally, immobilization on the staphylococcal cell wall is thought to occur via ionic interactions, as summarized in reference 72.

Some anchorless adhesins, such as Eap and Emp, display a broad binding specificity for a number of matrix (e.g. fibronogen, fibronectin, vitronectin, collagens) (49, 50, 73) and plasma proteins, including thrombospondin. Binding of S. aureus to isolated components of the extracellular matrix (ECM) and of plasma has been extensively studied in vitro, especially for the cell wall-anchored adhesins. However, in vivo, matrix macromolecules form complex networks and alloy-like structures with specific suprastructural organization (74). Consequently, additional or different interactions with adhesins might take place. Furthermore, conformational changes upon binding of matrix proteins may have an impact on binding properties of S. aureus adhesins.

Eap/Map (extracellular adherence protein) (49, 75) /Map, MHC class II-antagonist protein (76)) binds to a number of matrix (e.g. fibronogen, fibronectin, vitronectin, collagens) and plasma proteins, including thrombospondin (49, 73, 75). Eap has an additional immuno-modulatory function: Eap has anti-inflammatory properties due to a reduced recruitment of leukocytes, as detected in a murine peritonitis model (77). This is mediated by an impaired interaction of β2-integrins and ICAM-1, as well as of uPAR and vitronectin, leading to a reduced adhesion and transmigration of leukocytes in vivo.

Emp (extracellular matrix protein-binding protein) has been identified and cloned from strain Newman (50). Like Eap, Emp displays a broad binding specificity for a number of matrix and plasma proteins (50). The affinities of rEmp found in a biochemical interaction analysis (BIA) by SPR (surface plasmon resonance) differ for various plasma and matrix proteins (50): vitronectin binds with high affinity (K_D 122 pM), suggesting an important, albeit still unknown pathogenic role. This high affinity binding of vitronectin might be able to locally recruit a number of factors involved in biologic processes, such as complement activation, hemostasis, and tissue remodeling to the staphylococcal surface.

S. aureus cellular invasion

Cellular model systems for endovascular infection

Mostly, primary endothelial cells (e.g. bovine aortic, and HUVEC) have been used as models to study S. aureus infection in vitro (18, 78–80). Attempts to identify S. aureus adhesins interacting with endothelial cells (81) and host membrane proteins binding to S. aureus (82) have been reported earlier. However, the involved components had not been fully identified at that time.

Since the data obtained and published so far do not support a species or cell type specificity of the adherence and internalization step, we have also included results for other cell types. A substantial proportion of mechanistic data have been acquired using the human embryonic kidney cell line 293 (37, 41, 68, 83).
Since the development of kidney abscesses is a frequent complication upon *S. aureus* bacteremia (84–86), this is nevertheless relevant for the *in vivo* situation, since this probably requires a preceding transcytotic event in endothelial cells, as is discussed below for invasion of osteoblasts *in vivo*.

The major concern for the *in vivo* relevance is a rather general one: simple (subconfluent) monolayers of host cells on solid surfaces do not necessarily reflect the situation of a highly differentiated, polarized endothelial (or epithelial) surface *in vivo*.

**Molecular mechanism of *S. aureus* invasion**

The molecular mechanism for invasion of host cells by *S. aureus* bears a high degree of resemblance to complement-enhanced phagocytosis mediated by β2-integrins in professional phagocytes (macrophages and neutrophil granulocytes). Currently it is unclear, whether it represents a microbial pathogenicity mechanism or rather a mechanism of host defence (87). Most likely, it may have both aspects, depending on the exact setting in a given situation. Invasion of host cells by *S. aureus* proceeds via a modified “zipper-type” mechanism since it does not require live bacteria: live and killed (formaldehyde-fixed) *S. aureus* is equally effective for invasion (37). *S. aureus* is completely internalized in a time-, dose-, and temperature-dependent manner (37), driven by F-actin rearrangement in the host cell (37). Intracellular signaling involves, among others, tyrosine phosphorylation (38), MAPK activation (Erk-1 and Erk-2 phosphorylation) (88) and src kinase activation (89, 90).

The current state of the art regarding the invasion mechanism of *S. aureus* for host cells is depicted in a simplified schematic model (Fig. 4). Data of different groups are congruent, even though they were obtained in different systems (37–41, 59, 60). The identified mechanism is found in a number of cell types including human (37) and bovine epithelial cells (38, 40), human endothelial cells (39, 91) (and Fig. 3), and fibroblasts (37, 59). In addition, chick osteoblasts (92) and human corneal epithelial cells (93) have been examined. Cellular invasiveness is a highly conserved property of *S. aureus* isolates (37, 68), so far with only a few exceptions found in some MRSA isolates.

**Surface interaction between *S. aureus* and host cells**

FnBPs act as *S. aureus* invasins for a number of cell types (37). In the models studied so far, FnBPs are both required and sufficient for efficient invasion, and can replace each other (41) (Fig. 3).

Fibronectin serves as a bridging molecule, binding to integrin αvβ1 (CD49e/CD29; an adhesion receptor, which is the major fibronectin receptor of host cells) and other β2-integrins which are fibronectin receptors (37, 59). FnBPs bind to the N-terminal heparin-binding site of the fibronectin monomer, while the centrally located integrin-binding site, which contains the RGD (arginine-glycin-aspartate) consensus motif, makes contact to the host cell surface. The major FnBP domains involved in this process are the known fibronectin-binding D-domains (37), but it has become clear that additional FnBP domains, such as the B-domain, are also involved (59, 60). These observations have laid the basis to propose a novel domain organization of FnBPs (62, 94).

Some laboratory strains are markedly less invasive for host cells *in vitro*, namely Wood 46, 8325–4 and Newman D2C (37).

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**Figure 3: FnBP-dependent invasion of HUVEC.** Confluent HUVEC were tested for staphylococcal invasion using *S. carnosus* transformants heterologously expressing *S. aureus* FnBP A and FnBP B of strain 8325–4 (41). Invasiveness was as determined using a flow cytometric invasion assay described previously (41), with correction for fluorescence labeling (68). Data shown are means ± SEM of three independent experiments, performed in duplicates. Similar data have been obtained with a lactococcal expression system (91). DU5883: FnBP-negative (fnbA, fnbB) isogenic mutant of 8325–4; TM300: wild type.

**Figure 4: Current model for the mechanism of host cell invasion by *S. aureus*.** The current state of the art model of the invasion mechanism of *S. aureus* is shown. This model summarizes the data from different groups published so far. The mechanism of the anti-invasive effect of Pls has not yet been elucidated. Similarly, it is not clear whether ClpC has a direct impact on invasiveness. WTA are not shown here, since their role for invasion has not yet been formally addressed. Please refer to the text for more details and references.
Now, these exceptions can be explained to a large extent. Strains 8325–4 (11 bp deletion in rabU(95)) and Wood 46 are regulatory mutants, while strain Newman D2C harbors a stop codon in fnbA and fnbB, leading to the production of truncated FnBPs (83). Since both FnBPs are truncated at the end of the C-domains, the LPETG motif is lost. Thus, FnBPs are quantitatively secreted into the culture supernatants and cannot act as adhesins and invasins (83).

The anchorless adhesin Eap does not appear to substantially contribute to adherence to immobilized fibronectin and fibrinogen, but increases adherence to fibroblasts (96, 97). For invasion of host cells, Eap may have a partially compensatory effect for the loss of functional FnBPs (98, 73) in strains Newman and Newman D2C, which produce only truncated FnBPs (83).

Modulation of S. aureus invasiveness
Cellular invasiveness is highly conserved in clinical S. aureus isolates (37, 68) (and Cornelia Werbick and B. Sinha, unpublished data). Apart from the few altered laboratory strains where structural (83), and regulatory (95) mutations are responsible for reduced invasiveness, we could identify only one exception among clinical S. aureus isolates so far. PIs (plasmin-sensitive protein), found in a subset of MRSA isolates (SCCmec I), exerts an anti-adhesive and anti-invasive effect (68). Adhesion to immobilized human fibronectin, human fibrinogen, rabbit IgG and murine laminin is strongly reduced (68). This effect is least pronounced for human fibronectin, corresponding to the fact that invasiveness is only reduced, but not completely lost, in PIs-/pls-positive isolates (68). For reduced invasiveness, pls expression appears to be required (68). At present, the mechanism of the anti-adhesive and anti-invasive effect of PIs/pls is unclear.

Invasiveness appears to be partially modulated by ClpC (casino lytic protein C)(99) (and Petra Becker and B. Sinha, unpublished data), a chaperone of the Hsp100 family. The mechanism for this is still unidentified, but may involve modulation of FnBP expression by ClpC.

Post-invasion events in the host cell
The fates of the host cells and S. aureus following invasion depend on the respective (genetic) tools of the host cells and the S. aureus isolate, respectively. Strain Cowan I can survive within 293 cells for days, without apparent damage to the host cells (37). Strain RN6390 is able to replicate in CFT-1 cells (100) (bronchial epithelial cells of a patient with cystic fibrosis, carrying a AF508 deletion in CFTR, cystic fibrosis transmembrane conductance regulator; the most common mutation in these patients). Most recently, S. aureus intracellular replication has been monitored by real time imaging, using RN6390 transformed with a plasmid carrying a luciferase (lux) and GFP reporter construct (101). This suggests that S. aureus has defence mechanisms, since, much like in professional phagocytes, acidification (37) and (S. Couziniet, S. Zimmerli, W.L. Kelley, B. Sinha; N. Demaurex, D. Lew, J. Schrenzel, manuscript in preparation; and B. Sinha, unpublished data), and phago-lysosomal fusion, as determined by colocalization with LAMP-1 (S. Couziniet, S. Zimmerli, W.L. Kelley, B. Sinha; N. Demaurex, D. Lew, J. Schrenzel, manuscript in preparation) occur after invasion of non-professional phagocytes.

S. aureus defence mechanisms include resistance to low pH, which is partly dependent on ClpC (102) (and Petra Becker and B. Sinha, unpublished data), and “phagosomal escape”; the destruction of the phagosomal membrane (103) (S. Couziniet, S. Zimmerli, W.L. Kelley, B. Sinha; N. Demaurex, D. Lew, J. Schrenzel, manuscript in preparation; and Michelangelo Foti and B. Sinha, unpublished data), which would yield access to the cytoplasm with conditions close to pH 7. However, ultrastructural data alone are not sufficient to definitely prove a phagosomal escape.

Some non-professional phagocytes (e. g. intestinal epithelial cells, such as CaCo-2 and IEC-18), appear to be able to efficiently kill internalized microorganisms (S. aureus and Pseudomonas aeruginosa) in an apparently oxidative-dependent manner (104). CaCo-2 express NOX-1 (NADPH oxidase homolog I), a homolog of the phagocyte NADPH oxidase, originally termed NOH-1 (105). However, these results are not easy to interpret since in CaCo-2 one subunit is missing, thus probably NOX-1 cannot be active (Karl-Heinz Krause, personal communication). The latter finding is consistent with survival of S. aureus strains RN6390 and 502A in enterocytes (CaCo-2 and HT29) for up to 22 h (106). On the other hand, some strains rapidly kill non-professional phagocytes upon close contact. In this study, induction of apoptosis was observed in S. aureus-infected CaCo-2 (106). In addition, S. aureus strain RN6390 is able to replicate and to induce apoptosis in CFT-1 cells upon invasion (100). In leukocytes (Jurkat T cells and freshly isolated mononuclear cells, MNC), apoptotic and necrotic cell death can be induced directly by S. aureus α-toxin, a pore-forming toxin, even without invasion (107–109). Most likely, this reflects differences in the mechanism found in different cell types. For induction of apoptosis in human endothelial cells (HUVEC, and two cell lines), a combination of an invasive and hemolytic phenotype is required (110). The respective virulence factors appear to be agr- and sigB-(gB)-dependent, but distinct from proteases (110). However, not all S. aureus isolates with these properties are pro-apoptotic in vitro, and although very high doses of purified α-toxin was not effective it appears to contribute when produced intracellularly (110).

Biological relevance
To determine the exact role of a single S. aureus virulence factor for a given condition in a given species proved to be not trivial. As found e.g. in the cytokine network of eukaryotes, many components have redundant, as well as pleiotropic effects. Furthermore, the prevalence as well as the expression of virulence factors may vary between different S. aureus isolates and laboratory strains. The adhesive and invasive properties of a given strain may be influenced by proteases, such as the V8 serine protease, which degrades S. aureus surface proteins, such as FnBPs (111). This observation may be extended to other proteases and adhesins, as well. Since the prevalence and expression of different proteases may vary, the presence of an adhesin or invasin, even less the respective gene, may not be sufficient to predict the phenotypic properties of a given strain or isolate. Different isolates may carry different variants of a given virulence factor, potentially being of functional relevance, but mostly factors with a prototype sequence are analyzed in detail. By contrast, the se-
Table 2: Prevalence of the fnb genes in clinical S. aureus isolates.
The prevalence of the genes encoding FnBPA (fnbA) and FnBPB (fnbB) was examined in 163 clinical S. aureus isolates by PCR (narial colonization: 44, invasive infection: 119). A substantially higher number of isolates stemming from invasive infections harbors both genes, and all isolates have at least one gene, however with a clear preference for fnbA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Prevalence (%)</th>
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<tr>
<td>fnbA</td>
<td>79</td>
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<tr>
<td>fnbB</td>
<td>64</td>
</tr>
<tr>
<td>fnbA, fnbB</td>
<td>65</td>
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Adapted from Ref. (45) with permission from Elsevier. *Invasive infections: endocarditis, osteomyelitis, foreign body infections.*

Persistence/ Progression Pronounced

S. aureus, Streptococcus spp. (~ 10^5 CFU)

Table 3: Traumatic endocarditis in rats upon heterologous expression of S. aureus adhesins. In an approach of "adoptive pathogenicity", S. aureus surface proteins were expressed heterologously in largely non-pathogenic lactococci (Lactococcus lactis cremoris). Only FnBPA-expressing lactococci acquire a virulence potential comparable to S. aureus and could persist in vegetations and endothelial cells, as detected by histology. FnBPB had not been tested. Data are extracted from Ref. (66) with permission from the American Society for Microbiology.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Infectivity (ID_{50})</th>
<th>Course of infection</th>
<th>Clinical signs</th>
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<tbody>
<tr>
<td>Lactococcus lactis (WT)</td>
<td>Low ((\leq 10^3) CFU)</td>
<td>Fast resolution</td>
<td>Moderate</td>
</tr>
<tr>
<td>Lactococcus lactis (CIIA \text{+++})</td>
<td>High ((\geq 10^3) CFU)</td>
<td>Fast resolution</td>
<td>Moderate</td>
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<tr>
<td>Lactococcus lactis (FnBPA \text{+++})</td>
<td>High ((\geq 10^3) CFU)</td>
<td>Persistence/ Progression</td>
<td>Pronounced</td>
</tr>
<tr>
<td>S. aureus, Streptococcus spp.</td>
<td>High ((\geq 10^4) CFU)</td>
<td>Persistence/ Progression</td>
<td>Pronounced</td>
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</tbody>
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The invasion mechanism of S. aureus, as identified to date, requires classical host cell adhesion receptors (e.g. for fibronectin), \(\beta_1\)-integrins. In intact, confluent, differentiated, polarized monolayers of epithelial and endothelial cells, these receptors are localized mainly at the baso-lateral cell membrane. This means they are not accessible from the luminal side, but are found below the level of tight junctions (119). Nevertheless, one can postulate that the identified mechanism is of relevance in vivo: in the case of (micro-) trauma, wound healing, and tissue remodeling the monolayer is perturbed. As a consequence, components of the extracellular matrix, such as fibronectin, are exposed and accessible for S. aureus, together with adherence receptors on host cells. Indeed, in vivo cell-associated S. aureus is almost exclusively found in areas with cellular remodeling and de-differentiation (120). Furthermore, after initial adherence, S. aureus appears to be able to induce cell death by several mechanisms, thereby creating its own portal of entry. This is the case e.g. in primary skin infections with PVL-producing (Panton-Valentine-Leucocidin) isolates.

Initial adherence to endothelial cells can be promoted by inflammatory activation of the postcapillary endothelium in venules, as demonstrated recently in vivo (121). On the other hand, infection with S. aureus also induces a pro-inflammatory state in endothelial cells, as determined by expression of cytokines (122–125), Fcy-receptors (126), and adhesion molecules (127). This in turn could facilitate initial adhesion of S. aureus, either directly or indirectly.

Since at least one fnb gene is found in nearly 100% of clinical S. aureus isolates (43–47), and the majority carries both fnbA and fnbB (44, 45), an important role of FnBPs for survival of S. aureus in its natural habitat (ecological fitness) can be postulated. As opposed to some other microorganisms, S. aureus appears to depend mainly on FnBPs as invasins. Furthermore, epidemiologic data clearly support the notion of FnBPs as S. aureus virulence factors, since more isolates from invasive infections than colonizing isolates have both genes (45) (Table 2). This observation is in line with the correlation of fibronectin binding (agglutination) and the propensity to cause invasive disease, published previously (128), as well as with high conservation of cellular invasiveness in nearly all clinical S. aureus isolates (37, 68) (and Cornelia Werbick and B. Sinha, unpublished data). The expression of fnbA and fnbB appears to be regulated differently (129), thus FnBPB might be more important in different growth conditions than FnBPA, such as a later stage of the growth curve.

The potential in vivo relevance of host cell invasion by S. aureus is an ongoing debate (36, 87). However, it is easy to conceive that invasion of host cells is a prerequisite for intracellular persistence, which protects S. aureus from bactericidal host defence mechanisms (87). On the other hand, it may reflect a host defence mechanism, in addition to professional phagocytosis (87). Experimental in vivo data in the case of traumatic endocarditis in rats (66, 91), human xeno-transplanted bronchial segments (from patients with CF, cystic fibrosis, a condition where the CFTR protein is defective, and consequently not sorted correctly to the cell membrane) (43), and infection of chicken osteoblasts (130) support the potential clinical relevance of host cell invasion by S. aureus. The data for traumatic endocarditis clearly demonstrate the essential role of FnBP-mediated host cell invasion by S. aureus in the pathogenesis of endocarditis (66) (Table 3), and demonstrate a cooperative role of fibronectin and fibrinogen-binding by FnBPs (91). Furthermore, induction of apoptosis in human endothelial cells by S. aureus requires
Table 4: Synopsis of selected results for FnBP-dependent functions in strain Newman. Please read the table line by line. Fn, fibronectin; *fnb* mutation, point mutation in the C-domain of *fnbA* and *fnbB* of strain Newman, leading to a truncation of both FnBP and subsequent loss of the cell wall anchor function; HACAT, human keratinocyte cell line; HUVEC, human umbilical vein endothelial cells; n.d., not determined. Adapted from Ref. (83) with permission from the American Society for Microbiology. 4FnBP production assayed by ligand overlay assay with biotinylated fibronectin. *ISP479C, 8325–4 derivative.

<table>
<thead>
<tr>
<th>Model</th>
<th>Function tested</th>
<th>Result</th>
<th>FnBP production†</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilized Fn</td>
<td>Adherence</td>
<td>Higher than ISP479C (166)</td>
<td>Strong</td>
<td>Regulation of adhesive mechanism different from FnBPs examined?</td>
</tr>
<tr>
<td>Immobilized Fn</td>
<td>Adherence</td>
<td>Very low (167)</td>
<td>n.d.</td>
<td>Can be explained by <em>fnb</em> mutation</td>
</tr>
<tr>
<td>Immobilized Fn: Confluent HUVEC</td>
<td>Adherence</td>
<td>Weak (39)</td>
<td>n.d.</td>
<td>Can be explained by <em>fnb</em> mutation</td>
</tr>
<tr>
<td>Confluent HUVEC</td>
<td>Adherence; Invasion</td>
<td>Weak; Background (28)</td>
<td>Strong</td>
<td>Can be explained by <em>fnb</em> mutation</td>
</tr>
<tr>
<td>Subconfluent 293 cells</td>
<td>Invasion</td>
<td>Weak (37)</td>
<td>n.d.</td>
<td>Can be explained by <em>fnb</em> mutation</td>
</tr>
<tr>
<td>Human lung fibroblasts: HACAT</td>
<td>Invasion</td>
<td>Newman, loop reduced compared to WT (98)</td>
<td>n.d.</td>
<td>Role of strongly expressed Eap probably detected due to lack of functional FnBPs</td>
</tr>
<tr>
<td>Rabbit osteomyelitis (foreign body)</td>
<td>Virulence</td>
<td>No difference for <em>fnb</em>-negative mutant (168)</td>
<td>n.d.</td>
<td>FnBP-dependence in fact not tested; results in contrast to Ref. (135) using strain 8325–4 and its isogenic <em>fnb</em>-negative mutant</td>
</tr>
</tbody>
</table>

prior invasion, but invasion is not sufficient (110). This may represent a mechanism in order to re-emerge from an intracellular location. It is thought that variants, SCV (small colony variants), contribute to persistent infection, since they are phenotypically resistant to several antimicrobials and have been found after host cell contact in vitro (131–133), and can be found in clinical specimens from relapsing infections, as well (134).

A pathogenic role for FnBPs in osteomyelitis (murine foreign body model) has been shown using an *fnb*-deficient mutant (135). The postulated mechanism in endocarditis can be probably extended to osteomyelitis, taking together in vitro and in vivo data. The fibronectin-binding capacity of *S. aureus* appears to play a crucial role for the pathogenicity in an osteomyelitis model (guinea pig foreign body) (9). Furthermore, osteoblasts have been shown to be invaded by *S. aureus* in vitro (92, 136) and in vivo (130). By extension, this is of relevance with regard to endothelial cells, as well, since invasion of osteoblasts in vivo most likely is preceded by a (transcytotic) invasion event of endothelial cells after an i. v. challenge.

Some laboratory strains are substantially less invasive for host cells in vitro, namely Wood 46, 8325–4 and Newman D2C (37). Strain 8325–4 is a regulatory mutant: it carries an 11 bp deletion in rsbU (95), thus affecting the function of σ0 (alternative sigma factor, a global regulator), which also controls the expression of adhesins. Strain Wood 46 very likely is a regulatory mutant, as well, since it is the strongest known producer of α-toxin, but phenotypically SpA-negative. Strain Newman D2C (and strain Newman) strongly expresses FnBPs, and now, some of the apparent discrepancies with regard to the role of FnBPs in *S. aureus* pathogenesis can be explained by a specific anomaly found in the *fnbA* and *fnbB* genes of *S. aureus* strain Newman D2C (83) (Table 4). This is of importance for intravascular infections, such as endocarditis, since several in vivo (137–140) and in vitro (141) studies have used a strain of the Newman family. Interestingly, in strain Newman, *fnbA* expression and fibronectin binding are supported even in the absence of *sarA* signals, suggesting a key role for *sarA* (140).

Addition of cell-permeating antimicrobial agents, e.g. clindamycin and rifampicin, to β-lactams and aminoglycosides for the therapy of complicated *S. aureus* infections is thought to improve outcome and reduce relapses. The basis for this can be explained by the protection of intracellular *S. aureus* from most effective standard antimicrobials used to treat *S. aureus* infections, such as β-lactams and aminoglycosides (which have been used in the presented studies for protection assays). The prolonged duration of complicated and the frequently relapsing *S. aureus* infections can be explained by a prolonged intracellular persistence, with reemergence, either statistical or induced by unknown factors.

The invasion mechanism elucidated can be potentially targeted in order to interfere with complications upon hemogenous dissemination, e.g. by using adherence- and invasion-blocking recombinant D1–D3 peptides in bacteremic patients at high risk for secondary complications (e.g. endocarditis and osteomyelitis). In addition, these peptides could be used as adjuvant therapy together with standard combination antimicrobial therapy in patients with complicated and severe infections. This approach (using cefazolin and recombinant D1–D3 peptides) has been reported to have a potentiating curative effect in a guinea pig wound infection/abscess model (142).

Another potential application might be vaccination against FnBPs in order to elicit protective antibodies which block adhesion and invasion. Several surface components, including adhesins, exoproteins, toxins, and capsule polysaccharides have been targeted by experimental vaccination strategies (143).

So far no anti-FnBP vaccination studies have been performed successfully in humans, despite numerous experimental in vitro and in vivo data (144–149). Conformational changes upon binding of matrix proteins may have an impact on binding properties of *S. aureus* adhesins. This phenomenon has been shown e.g. for FnBPs and has been called LIBS (ligand-induced binding site) (150). The D-domains in an unbound state form a random coil, but change their conformation to a β-sheet structure upon binding of fibronectin (151). It is conceivable that this conformational change of adhesins may induce a different or broader binding specificity. The amorphous structure of unbound FnBPs D-domains may be the reason for their relatively weak immunogenicity. The resulting antibodies do not block ad-
hesion (152), and antibodies formed against fibronectin-bound D-domains appear to stabilize the interaction (150). This major obstacle, the failure to elicit blocking anti-FnBP antibodies in humans (152) has been overcome by using short synthetic peptides of the D-domains (153, 154). Passive immunization by anti-ClfA antibodies has been shown to reduce the duration of MRSA bacteremia in an endocarditis model (155), but a similar potent protective effect of anti-FnBP antibodies has not been studied so far. A study targeting CIFA have been successful in a model of infective endocarditis with an MRSA strain (156), and potential clinical application in humans will be facilitated by using humanized antibodies. In any case, so far, it has been impossible to distinguish between a specific effect of blocking antibodies against adhesins, and a general pro-opsonic activity which results in enhanced phagocytosis and subsequent killing. Thus, further studies integrating current knowledge (including targeting of host cell signaling, e.g. by using specific src kinase inhibitors) appear to be warranted.

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Abbreviations

CpfC, clpC: caseinolytic protein C (Hsp100 family chaperone with ATPase activity); clpC gene; CIFA: clumping factor A (major fibrinogen-binding adhesin) protein (→ Map), an anchorless adhesin protein (an Eap isoform); no longer used

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