Fibronectin-Binding Proteins and Fibrinogen-Binding Clumping Factors Play Distinct Roles in Staphylococcal Arthritis and Systemic Inflammation

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Staphylococcus aureus is a commonly encountered pathogen in humans, and it has the potential to cause destructive and life-threatening conditions, including septic arthritis. The pathogenicity of staphylococci depends on the expression of virulence factors. Among these, staphylococcal cell-surface proteins with tissue-adhesive functions have been suggested to mediate the colonization of host tissues, a crucial step in the establishment of infection. We investigated the relative contribution of the fibronectin-binding proteins (FnBPs) and fibrinogen-binding clumping factors (Clfs) to staphylococcal virulence in an experimental model of septic arthritis. The results show that these 2 sets of proteins play distinctly different roles in the development and progression of septic arthritis. Although Clfs significantly contributed to the arthritogenicity of S. aureus, FnBPs had no effect on the development of arthritis. Conversely, FnBPs played an important role in the induction of systemic inflammation characterized by interleukin-6 secretion, severe weight loss, and mortality.

Staphylococcus aureus is an important human pathogen—it most frequently causes superficial infections of the skin, but it also has the potential to cause severe systemic infections, such as endocarditis, osteomyelitis, and septic arthritis [1]. The increasing ability of S. aureus to withstand antibiotic treatment is alarming [2] and calls for the development of alternative treatment strategies. An improved knowledge of the bacterium-host interactions taking place during the establishment and course of infection might provide new targets for antistaphylococcal prophylaxis and intervention in the future.

The pathogenicity of S. aureus depends on the expression of a variety of virulence factors—bacterial products that, by different means, enable the establishment of an infection. Tissue-adhesive functions exerted by cell wall–associated proteins of S. aureus seem to be of crucial importance in this context [3].

Clumping factors (Clfs) A and B are 2 structurally related fibrinogen-binding proteins that are expressed on the surface of S. aureus. Both proteins mediate the fibrinogen-dependent adhesion and clumping of S. aureus cells [4–7]. However, the ligand-binding A regions of ClfA and ClfB interact with different parts of the fibrinogen molecule (the γ-chain for ClfA and the α-chain for ClfB) [6–8].

Fibronectin-binding proteins (FnBPs) A and B [9–11] enable staphylococcal adherence to and invasion of a range of cell types, including epithelial cells, endothelial cells, fibroblasts, and osteoblasts [12–23]. Through the formation of a fibronectin bridge to the fibronectin-binding integrin αvβ3, expressed on the host cell surface, FnBPs trigger bacterial invasion [12, 20, 21]. Such an invasion might provide a means by which the staphylococci evade host defenses and resist antibiotic killing.
Although most studies have focused on the fibronectin-binding activity of the C-terminal D repeats of FnBPs [24], other regions of these proteins also mediate fibronectin binding [20, 25]. Moreover, the A region of FnBPA has recently been shown to interact with the C terminal of the fibrinogen γ-chain with an affinity similar to that of ClfA [26], which suggests that ClFs and FnBPs may have additive functions with respect to fibrinogen/fibrin-bindin during in vivo infection.

The relevance of studying the role that FnBPs and ClFs play in virulence determinants is underscored by previous reports that have shown that genes encoding these proteins are present in virtually all clinical isolates [27, 28]. Interestingly, Proctor et al. [29] observed that S. aureus strains isolated from patients with invasive infection were more readily agglutinated by fibronectin, compared with noninvasive isolates, which possibly indicates that FnBPs contribute to the development of invasive infections.

The objective of the present study was to investigate the relative contributions of FnBPs and ClFs to the development of septic arthritis and systemic inflammatory response during S. aureus infection. For this purpose, we used a well-established murine model of staphylococcal infection [30, 31] to study gene knockout mutants derived from S. aureus LS-1, a strain isolated from a mouse that spontaneously developed septic arthritis [32].

**MATERIALS AND METHODS**

**Mice and bacterial strains.** Female NMRI mice were obtained from B&K Universal and were maintained in the animal facility at Göteborg University, Göteborg, Sweden, under standard conditions of light and temperature, with free access to standard laboratory chow and water. The mice were used for experiments as approved by the local ethical board.

S. aureus strain LS-1, which was originally isolated after a spontaneous outbreak of S. aureus arthritis in a mouse colony [30, 32], was used as the control (wild-type [wt]) strain in the study. The following mutants of LS-1 were also used: a clfA/cfpB double-mutant strain (DU6013), an fnbA/fnbB double-mutant strain (DU6012), and a clfA/cfpB/fnbA/fnbB quadruple-mutant strain (DU6014) (table 1). Briefly, the strains were constructed by the transduction of previously isolated insertion mutations, with the exception of a frameshift mutation clfA5 constructed in LS-1 by allele replacement. Insertion mutations were transduced from 8325-4 or Newman backgrounds into LS-1 by use of bacteriophage 85 [33]. Each mutation was validated by phenotypic analysis and Southern hybridization. Phenotypically, the clfA/cfpB and fnbA/fnbB double-mutant strains were clearly deficient in their abilities to adhere to immobilized fibronectin and fibronectin, respectively. The clfA/cfpB/fnbA/fnbB quadruple-mutant strain was completely deficient with respect to both binding activities. Furthermore, the mutant strains were not affected in their ability to secrete hemolysins, as verified by culturing on blood-agar plates.

**Infection procedure.** Before inoculation, the bacterial strains were cultured on tryptic soy-agar plates for 48 h at 37°C, after which the collected colonies were suspended in PBS supplemented with 10% dimethyl sulfoxide and 5% human albumin (crystallized; ICN Biomedicals). Bacterial suspensions were kept in aliquots at −20°C until they were used. The number of colony-forming units in the suspensions was determined by culturing of several diluted aliquots on horse blood–agar plates. Before inoculation, bacterial suspensions were thawed and washed in PBS. Bacterial pellets were diluted to the desired cell density. Two hundred microliters of PBS that contained ~10^7 cfu of S. aureus was intravenously administered to 8-week-old mice. Viable counts of injection suspensions were assessed in all experiments, and the variations (0.8–1.3 × 10^7 cfu/mouse) were judged to be unlikely to affect the course of infection.

**Clinical examination of infected mice.** The S. aureus–infected mice were examined individually in a blinded manner. To evaluate the severity of arthritis, an arthritic index was constructed for each mouse at each time point. For this purpose each paw was evaluated and given an estimated score based on a 0–3 point scale (0, no swelling or erythema; 1, mild swelling and/or erythema; 2, moderate swelling and erythema; 3, marked swelling and erythema). The scores for the 4 paws were thereafter added to form the arthritic index. The overall condition of each mouse was also examined by assessing any change in weight and signs of systemic inflammation (e.g., reduced alertness and ruffled coat). In cases of severe systemic infection, mice were killed by cervical dislocation and considered to have died from sepsis.

**Histopathological examination.** Histopathological exami-

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**Table 1. Staphylococcus aureus strains used in the present study.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host and genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>DU6004</td>
<td>LS-1 clfA clfB: tetK [Tc]</td>
<td>Fitzgerald et al.*</td>
</tr>
<tr>
<td>DU6012</td>
<td>LS-1 fnbA: tetK[TcR] fnbB: ermC [Emr]</td>
<td>Present study</td>
</tr>
<tr>
<td>DU8883</td>
<td>8325-4 fnbA: tetK[Tc] fnbB: ermC [Emr]</td>
<td>[11]</td>
</tr>
<tr>
<td>LS-1</td>
<td>Wild type</td>
<td>[32]</td>
</tr>
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nation of the joints was performed after routine fixation decalcification and paraffi embedding of specimens. Tissue sections from upper and lower extremities were prepared and stained with hematoxylin-eosin. All slides were examined in a blinded manner and scored with respect to severity of synovitis and cartilage/bone destruction as follows. Synovitis scores were 0, no synovitis; 1, mild synovial hypertrophy (synovial membrane thickness of >2 cell layers); 2, moderate synovial hypertrophy and infiltration of inflammatory cells; and 3, marked synovial hypertrophy and infiltration of inflammatory cells. Cartilage and bone-destruction scores were 0, no destruction; 1, mild destruction; and 2, marked destruction. Histopathological scores for synovitis and erosivity, respectively, were constructed for each mouse by summing the scores for the examined joints (elbow and wrist/carpal joints [front extremities] and knee and ankle/tarsal joints [hind extremities]).

Determination of bacterial load in kidneys. Kidneys were homogenized and mixed with cold PBS. The suspension was serially diluted and thereafter spread onto horse blood-agar plates. The number of colony-forming units formed after 24 h of incubation at 37°C was used to assess the bacterial load.

Interleukin (IL)–6 analysis. For measurement of IL-6 levels in serum samples, a bioassay based on the murine hybridoma cell line B13.29, subclone B9, was used as described elsewhere [34]. This cell line is dependent on exogenously supplied IL-6 for its growth [35, 36].

Statistical analyses. The Mann-Whitney U test or Kruskal-Wallis test (with post hoc analysis) was used to compare severity of arthritis, weight changes, histopathological scores, and serum IL-6 levels between groups. The incidences of arthritis and mortality during the experimental period were analyzed with Fisher’s exact test. \( P \leq .05 \) was considered to be statistically significant.

RESULTS

Contribution to septic arthritis and infection-induced weight loss of FnBP and Clf expression. Septic arthritis was induced with the wt control and with \( clfA^{-} clfB^{-} fnbA^{-} fnbB^{-} \). As shown in figure 1a, the wt strain gave rise to significantly more severe clinical arthritis than did the quadruple-mutant strain (\( P = .005 \) on day 2, \( P = .04 \) on day 6, and \( P = .06 \) on day 14). The incidence of arthritis did not differ significantly between groups (8 and 6 of 10 mice, respectively, developed arthritis in the wt and quadruple-mutant groups). The weight loss induced by the quadruple-mutant strain was strikingly less pronounced (\( P \leq .002 \) for all time points), compared with that induced by the wt strain (figur e 1b). Also, the wt strain caused higher mortality (5/10 mice died; \( P = .03 \)) than did the quadruple-mutant strain (all 10 mice survived). These results indicate that \( >1 \) of the proteins ClfA, ClfB, FnBPB, and FnBPB contribute to \( S. aureus \) virulence and enhance the severity of arthritis and sepsis.

To further investigate the contribution of these 4 proteins to \( S. aureus \) virulence, the impact of \( clfA^{-} clfB^{-} \) or \( fnbA^{-} fnbB^{-} \) was compared with that of wt LS-1 and that of \( clfA^{-} clfB^{-} fnbA^{-} fnbB^{-} \). Interestingly, throughout the experiment, the mice infected with \( clfA^{-} clfB^{-} \) displayed significantly less-severe arthritis than did the mice infected with the wt strain (figur e 2a; \( P < .001 \). This was partly due to a higher incidence of arthritis induced by the wt strain (83% vs. 50% induced by \( clfA^{-} clfB^{-} ; P = .03 \). Notably, the carriage of intact ClfA and ClfB genes by the fnbA fnbB double-mutant strain significantly contributed to arthritogenicity, because it gave rise to more-severe (figur e 2a; \( P < .001 \)-02) and frequent (92% vs. 59%; \( P = .05 \)) arthritis than the quadruple-mutant strain.

In contrast, the \( fnbA^{-} fnbB^{-} \) strain was not attenuated in its ability to cause arthritis, compared with the wt strain (figur e 2a; incidence of arthritis, 83% and 92% for LS-1- and fnbA fnbB-infected groups, respectively). Furthermore, the arthritogenicity of the quadruple-mutant strain was not reduced,

![Figure 1](https://academic.oup.com/jid/article-abstract/191/5/791/1240447)
compared with that of the clfA−clfB− double-mutant strain (which carries the genes for fnbA and fnbB) (fige 2a; incidence of arthritis, 50% and 59%, respectively, for the clfA−clfB− and quadruple-mutant–infected groups). These findings indicate that Clfs are important mediators of arthritis and that FnBPs are less important in this regard.

After inoculation, LS-1–infected mice lost significantly more weight than did mice infected with the clfA−clfB− double-mutant strain (fige 2b; *P < .001–.05, days 2–7). This difference was most pronounced early after inoculation. Notably, the fnbA−fnbB− double-mutant strain (which carries the clfA and clfB genes) gave rise to more weight reduction than did the quadruple-mutant strain only transiently, on day 2 (fige 2b, *3; *P < .05). Furthermore, clfA and clfB gene carriage did not significantly affect mortality (48% and 23% mortality for LS-1– and clfA−clfB−–infected mice, respectively [P not significant] and 8% and 0% mortality for fnbA−fnbB− and quadruple-mutant–infected mice, respectively [P not significant]).

With respect to infection-induced weight loss at all time points, the fnbA−fnbB− strain was significantly less virulent than was the LS-1 (fige 2b; P < .001–.01). Also, the clfA−clfB− double-mutant strain was significantly more virulent than the quadruple-mutant strain in this respect, from days 4 to 10 (fige 2b, *4, *5, and *6; *P < .001). Furthermore, the incidence of mortality was significantly higher after inoculation with strains carrying fnbA and fnbB genes, compared with that after infection with their counterparts (48% and 8% mortality for LS-1– and fnbA−fnbB−–infected mice, respectively [P < .03], and 23% and 0% mortality for clfA−clfB− and quadruple-mutant–infected mice, respectively [P < .05]).

Accordingly, both Clfs and FnBPs affect weight loss after staphylococcal infection, although they act during different stages of the infection. The expression of FnBPs by staphylococci clearly contributes to mortality, whereas our results provide no evidence that Clfs play a role in this respect. Notably, we did not observe any significant differences between any of the staphylococcal strains with respect to the number of bacteria retrieved from the kidneys 9–10 days after inoculation (data not shown).

In summary, our finding indicate that Clfs are important arthritogenic factors that, by mediating the rapid onset of localized joint inflammation also contribute to slight weight loss, whereas FnBPs contribute mainly to the induction of systemic inflammation which is characterized by severe weight loss and mortality, without affecting arthritis development.

**Contribution to synovitis and erosive damage of cartilage and bone by Clf expression.** Histopathological examination of joint sections revealed that inoculation with the wt strain LS-1 caused significantly more synovitis (fige 3; P < .001) and cartilage and bone erosion (fige 3; P < .001) than did inoculation with the clfA−clfB− double-mutant strain. Furthermore, the fnbA−fnbB− double-mutant strain gave rise to significantly more synovitis (fige 3; P < .05) and erosive damage (fige 3; P < .01) than did the quadruple-mutant strain (clfA−clfB− fnbA−fnbB−). These findings indicate that the expression of Clfs contributes to synovitis as well as to persistent damage of cartilage and
more, the tribute to the induction of systemic inflammation further . However, mice infected with the clfA mutant had significantly lower levels of IL-6, a proinflammatory cytokine, after inoculation with Staphylococci. As shown in figure 4, the levels of IL-6, measured in serum samples collected 9–10 days after inoculation, were significantly lower in mice infected with the clfA mutant than in mice infected with the quadruple mutant (clfA clfB fnbA fnbB). The levels of IL-6 in mice infected with the clfA mutant were significantly lower than those in mice infected with the wt strain (P < .01). However, mice infected with the clfA clfB double mutant did not differ significantly from mice infected with the wt strain, which indicates that the Clfs are of less importance in this respect. This finding was further supported by the lack of significant difference between the groups infected with the clfA clfB fnbA fnbB mutant and the clfA clfB double-mutant strains, respectively, because the latter of these strains carries intact clfA and clfB genes. However, fnbA fnbB-infected mice had significantly lower serum IL-6 levels than did the infected mice (P < .001), which clearly indicates that FnBPs contribute to the induction of systemic inflammation. Furthermore, the clfA clfB double mutant induced significantly higher IL-6 levels than did the quadruple mutant, most likely because of the expression of FnBPs (P < .001). The levels of IL-6 in serum were significantly lower after inoculation with Staphylococci. Data are presented as medians (center lines), interquartile ranges (boxes), and 80% central ranges (whiskers).

**Figure 3.** Histopathological findings in mouse joints 9–10 days after inoculation with Staphylococci. Data are presented as medians (center lines), interquartile ranges (boxes), and 80% central ranges (whiskers). Although not indicated in the figure, mice that were inoculated with LS-1 displayed significantly more synovitis (P < .01) and cartilage and bone erosion (P < .01) than did mice infected with the clfA-clfB fnbA fnbB mutant. This finding was further supported by the lack of significant difference between the groups infected with the clfA clfB fnbA fnbB mutant and the clfA clfB double-mutant strains, respectively, because the latter of these strains carries intact clfA and clfB genes. However, fnbA fnbB-infected mice had significantly lower serum IL-6 levels than did the infected mice (P < .001), which clearly indicates that FnBPs contribute to the induction of systemic inflammation. Furthermore, the clfA clfB double mutant induced significantly higher IL-6 levels than did the quadruple mutant, most likely because of the expression of FnBPs (P < .001).

**Figure 4.** Serum interleukin (IL)-6 levels 9–10 days after inoculation with Staphylococci. Data are presented as medians (center lines), interquartile ranges (boxes), and 80% central ranges (whiskers). Although not indicated in the figure, mice that were inoculated with LS-1 displayed significantly more synovitis (P < .01) and cartilage and bone erosion (P < .01) than did mice infected with the clfA-clfB fnbA fnbB mutant. This finding was further supported by the lack of significant difference between the groups infected with the clfA clfB fnbA fnbB mutant and the clfA clfB double-mutant strains, respectively, because the latter of these strains carries intact clfA and clfB genes. However, fnbA fnbB-infected mice had significantly lower serum IL-6 levels than did the infected mice (P < .001), which clearly indicates that FnBPs contribute to the induction of systemic inflammation. Furthermore, the clfA clfB double mutant induced significantly higher IL-6 levels than did the quadruple mutant, most likely because of the expression of FnBPs (P < .001).

**DISCUSSION**

Septic arthritis caused by S. aureus is a severe joint infection that is highly associated with irreversible joint damage and death in humans [37]. The staphylococci are believed to reach the joint area via hematogenous spread from an initial nidus of infection, such as an infected wound [38]. The bacterium–host interactions that take place during the establishment of a joint infection remain largely unknown. However, tissue-adhesive proteins expressed on the staphylococcal cell surface likely contribute to the pathogenicity of staphylococci. Indeed, staphylococcal cell-surface proteins that mediate attachment to collagen, fibronectin, and fibrinogen/fibrin have all been shown to promote the colonization of catheter-damaged heart valves [39–44]. In the present study, we investigated the relative impact of FnBPA, FnBPP, ClfA, and ClfB on the development and progression of murine septic arthritis and systemic inflammation. For this purpose, we used S. aureus LS-1, a murine septic arthritis isolate, and gene knockout mutants derived from this strain.

Our results clearly indicate distinct roles of Clfs and FnBPs in the development and progression of staphylococcal infection. Although the expression of Clfs potentiated the induction of localized joint inflammation and contributed to erosive lesions of cartilage and bone, FnBPs did not affect arthritis development at all. On the other hand, FnBPs significantly contributed to systemic inflammation characterized by weight loss,
IL-6 secretion, and mortality. Notably, Clfs also contributed to weight loss. However, this effect was most pronounced during the early stage of infection, and the Clfs did not significantly affect mortality or serum levels of IL-6. Thus, we believe that the mild weight loss triggered by Clf expression is primarily due to the development of localized joint inflammation whereas the severe FnBP-dependent weight loss seen later on is characteristic of a systemic proinflammatory response. 

The functional overlap between Clfs and FnBPs that has been suggested in the colonization of damaged heart valves [39–43] does not hold true with respect to the induction of arthritis. Our finding indicates that the staphylococcal fib onecin-binding phenotype is insufficient for the induction of septic arthritis. However, staphylococcal interaction with fib onecin is likely involved in the systemic inflammation triggered by FnBP expression.

Interestingly, fib onecin binding mediated by FnBPs is required for staphylococcal invasion of several different cell types, including endothelial cells [12, 19, 20]. The internalization of S. aureus by endothelial cells in vitro has resulted in the production of proinflammatory cytokines and chemokines, such as IL-8, monocyte chemotactic protein–1, IL-6, and IL-1β [45–48]. Furthermore, endothelial adhesiveness for monocytes and granulocytes increased on staphylococcal internalization, because of the up-regulation of adhesion molecule expression [49]. Because the vascular endothelium constitutes a substantial surface structure in close contact with the blood, the invasion of endothelial cells by fib onecin-coated bloodborne staphylococci could be an important trigger for the severe systemic inflammation associated with FnBP expression.

As for the contribution of Clfs to staphylococcal arthritis, we question that their fibrinogen-binding functions play an important role. Indeed, we have recently demonstrated that ClfA-mediated arthritis genesis is retained despite the in vivo depletion of fibrinogen [50]. In addition, FnBPA has recently been shown to interact with the C terminal of the fibrinogen γ-chain with an affinity similar to that of ClfA [26]. Thus, if an interaction between ClfA and the fibrinogen γ-chain C terminal were a prerequisite for ClfA-mediated arthritis genesis, FnBPA would also be expected to contribute to the induction of arthritis, at least to some extent. However, as has been shown in the present article, this is not the case. Rather, we find it likely that one or both of the Clfs promote the establishment of joint infection by mediating interaction(s) with as yet unknown host ligand(s).

Virtually all clinical isolates of S. aureus carry genes that encode the virulence factors identified in the present study [27, 28], which further suggests their importance in the pathogenesis of staphylococcal infections. Because of the frequent occurrence of these proteins, they are strong candidate target structures for antistaphylococcal prophylaxis and intervention in humans. Indeed, finding in animal models of infection have supported this idea [51–58].

To summarize our findings Clfs were identified as important arthritogenic factors, whereas FnBPs contributed to infection-induced weight loss and mortality without affecting the development of arthritis. Thus, these virulence factors play distinct roles in the pathogenesis of staphylococcal infections.

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