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Staphylococcus aureus Metalloprotease Aureolysin Cleave Complement C3 To Mediate Immune Evasion

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Complement is one of the first host defense barriers against bacteria. Activated complement attracts neutrophils to the site of infection and opsonizes bacteria to facilitate phagocytosis. The human pathogen Staphylococcus aureus has successfully developed ways to evade the complement system, for example by secretion of specific complement inhibitors. However, the influence of S. aureus proteases on the host complement system is still poorly understood. In this study, we identify the metalloprotease aureolysin as a potent complement inhibitor. Aureolysin effectively inhibits phagocytosis and killing of bacteria by neutrophils. Furthermore, we show that aureolysin inhibits the deposition of C3b on bacterial surfaces and the release of the chemotactic C5a. Cleavage analyses show that aureolysin cleaves the central complement protein C3. Strikingly, there was a clear difference between the cleavages of C3 in serum versus purified conditions. Aureolysin cleaves purified C3 specifically in the α-chain, close to the C3 convertase cleavage site, yielding active C3a and C3b. However, in serum we observe that the aureolysin-generated C3b is further degraded by host factors. We pinpointed these factors to be factor H and factor I. Using an aureolysin mutant in S. aureus USA300, we show that aureolysin is essential and sufficient for C3 cleavage by bacterial supernatant. In short, aureolysin acts in synergy with host regulators to inactivate C3 thereby effectively dampening the host immune response. The Journal of Immunology, 2011, 186: 000–000.
dependent neutrophil chemotaxis by binding to the C5a receptor (C5aR) (20). Next to these secreted molecules, \textit{S. aureus} also produces several surface proteins that interact with the complement system: staphylococcal IgG-binder binds both IgG and C3 to prevent classical and alternative complement activation (21, 22), and clumping factor A and the iron-regulated surface determinant protein H promote degradation of opsonic C3b (23, 24).

Even though \textit{S. aureus} produces a number of proteases, their role in bacterial complement evasion is not known. We studied a role for the metalloprotease aureolysin in complement escape by \textit{S. aureus}. Aureolysin is a 301-aa zinc-dependent metalloprotease that belongs to the family of thermolysins (25). Aureolysin was previously shown to have a role in staphylococcal immune escape as it cleaves the antimicrobial peptide LL-37. In this study, we describe an important role for aureolysin in inhibition of the complement system.

Materials and Methods

Proteins and sera

Aureolysin, purified from \textit{S. aureus} supernatant, was purchased from Bio-Centrum and further purified by gel filtration on a Superdex 75 column (GE Healthcare) using the AktaExplorer system (GE Healthcare). Protease activity was checked by gelatin zymography (26). Purified aureolysin was subjected to SDS-PAGE and verified by mass spectrometry. Heat-inactivated aureolysin was made by incubating aureolysin for 20 min at 65°C. SCIN and CHIPS were produced as recombinant proteins as described (17, 27). C3, C3b, and factor B (fB) were purified from human plasma as described (28). The purified components factor D (fD), H, II, C3a, and C5 were purchased from Quidel. Complement-depleted sera (ΔC1q, ΔC4, and ΔC3) were purchased from Quidel. Normal human serum was obtained from healthy volunteers, who gave informed consent. Heat-inactivated serum was made by incubating serum for 20 min at 56°C.

Bacterial strains and supernatants

\textit{Bacterial strains}. \textit{S. aureus} USA300 strain UAMS-1182 (a generous gift from Dr. Nina van Sorge, University of California, San Diego), Group B \textit{Streptococcus} wild-type strain COH1 (29), and the \textit{S. aureus} clinical isolate (KV27) obtained within the University Medical Center Utrecht were used. All bacteria were cultivated on tryptic soy agar.

\textit{S. aureus} aureolysin mutant. The pKOR1-\Deltaaur plasmid (30) was transduced with phage 80x into USA300 strain UAMS-1182. A markerless \textit{aur} deletion was constructed using the pKOR1 knockout protocol as previously described (31). To construct an \textit{aur} complementing clone, plasmid pBS99 (32) was digested with BamHI and EcoRI to remove the P3-YFP previously described (31). To construct an \textit{S. aureus} aureolysin mutant complementing clone, plasmid (30) was transfected into pCM46 digested by the same enzymes to yield an \textit{aur} complementing clone pCM47.

\textit{Supernatants}. \textit{S. aureus} USA300, the isogenic aureolysin mutant, and the isogenic aureolysin mutant complemented with aureolysin were cultured overnight in Todd–Hewitt broth (THB) and subsequently diluted to an \textit{OD}_{600} of 0.05 in fresh THB. Bacteria were grown to an \textit{OD}_{600} of 1.5 while shaken at 37°C, and supernatants were collected by centrifugation and passed through a 0.45-μm filter. Collected supernatants were concentrated 10 times using Amicon Ultra-10 filters (MWCO 10 kDa; Millipore), dialyzed against PBS and stored at −80°C. Upon usage, bacterial supernatants were diluted for functional analysis.

Phagocytosis and killing

Phagocytosis assays were performed as described (17). In short, serum was preincubated with 0.5 μM BSA, SCIN, or aureolysin in RPMI containing 0.1% human serum albumin (HSA) for 15 min at 37°C. Then, 2.5 × 10^6 freshly isolated human neutrophils and 2.5 × 10^5 FITC-labeled, heat-killed \textit{S. aureus} KV27 were added and incubated for 15 min at 37°C while shaking at 600 rpm. The reaction was stopped by adding 1% ice-cold paraformaldehyde in RPMI containing 0.1% HSA. Phagocytosis was analyzed by flow cytometry (FACS Calibur; Becton Dickinson) measuring fluorescence of 10,000 gated neutrophils. The complement-independent phagocytosis assay was performed as described above, but instead of normal human serum, we used heat-inactivated serum. For bacterial killing assays, Group B \textit{Streptococcus} was grown to an \textit{OD}_{600} of 0.5 in THB and subsequently washed in RPMI containing 0.1% HSA. Then, we preincubated 5% serum with 0.5 μM BSA, SCIN, or aureolysin for 20 min at 37°C. Subsequently, 2.5 × 10^7 Group B \textit{Streptococcus} and 8.5 × 10^7 neutrophils were added and incubated at 37°C. At different time points, a sample was taken, and neutrophils were lysed with Milli-Q. Surviving bacteria were enumerated by plating serial dilutions on Todd–Hewitt agar.

C3b deposition on \textit{S. aureus}

\textit{S. aureus} strain KV27 was grown to an \textit{OD}_{600} of 0.5 in THB and washed in HEPES** buffer (20 mM HEPES, 140 mM NaCl, 5 mM CaCl2, 2.5 mM MgCl2) with 0.1% BSA. Serum was preincubated with 0.5 μM BSA or aureolysin for 20 min at 37°C. Then, we incubated 12.5 × 10^5 bacteria with the preincubated serum for 30 min while shaking at 900 rpm. Bacteria were washed with PBS with 0.1% BSA. C3b deposition was detected using mouse anti-human C3d Abs (Quidel) and FITC-conjugated goat anti-mouse IgG (Protos). Fluorescence of 10,000 bacteria was measured by flow cytometry. C3b deposition with purified components was performed as described above, but instead of using serum, we incubated bacteria with 0.6 μM C3, 20 nM fD, 50 nM fB, and 60 μM C3b in the presence or absence of 0.5 μM aureolysin.

C5a analysis

\textit{S. aureus} strain KV27 was grown to an \textit{OD}_{600} of 0.5 in THB. Bacteria were heat-killed for 30 min at 70°C and washed in RPMI with 0.1% HSA. Then, 10% serum and 0.5 μM BSA or aureolysin were preincubated for 15 min at 37°C and subsequently incubated with 2.5 × 10^5 bacteria for 30 min while shaking at 600 rpm. Bacteria were centrifuged, and C5a was detected in collected supernatants by calcium mobilization: 10-fold diluted supernatants were added to 7.5 × 10^5 fluo-4-AM–labeled U937-C5aR cells (U937-C5aR; a generous gift from Prof. Eric Prossnitz, University of New Mexico), and the increase of intracellular calcium was measured by flow cytometry. To study specificity for C5a, activation was performed as described above but using 10% C5-depleted serum, supplemented with or without 10 μg/ml C5. To block C5aR on cells, cells were pretreated with 10 μg/ml CHIPS for 5 min at room temperature before use in the calcium mobilization assay.

Complement assays

Complement ELISAs were performed as described (33) with modifications. ELISA plates (Maxisorb; Nunc) were coated overnight with 20 μg/ml LPS (Salmonella enteriditis; Sigma), 3 μg/ml IgM (Quidel), or 10 μg/ml mannan (Saccharomyces cerevisiae; Sigma) in 0.1 M sodium carbonate buffer, pH 9.6. Plates were blocked with 4% BSA in PBS with 0.05% Tween for 1 h at room temperature. Diluted complement was added to 96-well plates coated with 10 μg/ml HEPES** buffer (20 mM HEPES, 140 mM NaCl, 0.1% gelatin, 5 mM CaCl2, 2.5 mM MgCl2, 0.05% Tween). Serum or C2-depleted serum was preincubated with 0.5 μM BSA or aureolysin for 20 min at 37°C and subsequently added to the plates for 1 h at 37°C. Deposited C3b and C3b-9 were detected using Abs against C3d and C6, respectively (Quidel), followed by peroxidase-conjugated goat anti-mouse IgG (Southern Biotechnology).

C3 analysis

\textit{C3 cleavage analysis by Western blotting}. \textit{C3 cleavage} was analyzed by incubation of 1 μM C3 with aureolysin or bacterial supernatants for 1 h at 37°C in HEPES** buffer. The reaction was stopped at different time points by adding sample buffer containing DTT. Samples were subjected to SDS-PAGE and visualized by instant blue (Gentaur).

Western blotting. \textit{C3 cleavage analysis by Western blotting} was performed by incubation of 5% serum or 0.6 μM C3 with 0.5 μM aureolysin at 37°C in HEPES** buffer. The reaction was stopped at different time points by adding sample buffer containing DT. All samples were subjected to SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. Cleavage products were visualized with 0.1% Coomassie blue in 40% methanol, excised, and analyzed by N-terminal sequencing (Alphalyse).

\textit{Instant blue staining}. \textit{C3 cleavage} was analyzed by incubation of 1 μM C3 with aureolysin or bacterial supernatants for 1 h at 37°C in HEPES** buffer. The reaction was stopped at different time points by adding sample buffer containing DT. Samples were subjected to SDS-PAGE and visualized by instant blue (Gentaur).

\textit{Western blotting}. \textit{C3 cleavage analysis by Western blotting} was performed by incubation of 5% serum or 0.6 μM C3 with 0.5 μM aureolysin at 37°C in HEPES** buffer. The reaction was stopped at different time points by adding sample buffer containing DT. All samples were subjected to SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. After blocking with 4% skimmed milk in PBS containing 0.1% Tween, C3 was detected by a goat anti-human C3 Ab (Protos), followed by a donkey anti-goat IgG Ab (Jackson) diluted in PBS with 0.1% Tween and 1% skimmed milk. C3b–8s degradation was analyzed by incubating 0.6 μM C3 with 0.5 μM...
aureolysin for 30 min at 37°C. The reaction was stopped by adding 50 nM EDTA. Generated C3b-SN and 0.6 μM C3b were incubated with 5% C3-depleted serum, and samples were immunoblotted as described above. To identify the serum component, various combinations of 0.6 μM purified C3, 45 nM fI, 27 nM fH, and 0.5 μM aureolysin were incubated for 30 min at 37°C in HEPES++ buffer and immunoblotted as described above.

C3a analysis

S. aureus strain KV27 was washed in RPMI with 0.1% HSA and incubated with 10% C5-depleted serum, buffer, and/or 0.5 μM aureolysin for 20 min at 37°C. Bacteria were centrifuged, and the supernatants were analyzed by Western blotting as described earlier but using rabbit anti-C3a serum (Calbiochem) and goat anti-rabbit IgG Ab (Southern Biotech). C3a-mediated neutrophil activation was performed by incubating 0.6 μM C3 or C3a with 0.5 μM aureolysin in RPMI containing 0.1% HSA for 30 min at 37°C. Calcium mobilization was measured as described earlier by adding 10-fold diluted sample to 1 × 10⁶ freshly isolated human neutrophils labeled with fluo-4-AM.

Results

Aureolysin blocks phagocytosis and killing of bacteria

To test whether aureolysin is involved in complement evasion by S. aureus, we first studied its activity in a phagocytosis assay. Therefore, we incubated fluorescently labeled S. aureus with isolated human neutrophils in the presence of human serum and aureolysin. BSA and SCIN served as a negative and positive control, respectively. Fig. 1A shows that aureolysin potently inhibits phagocytosis and that its activity is comparable with that of the complement inhibitor SCIN. Aureolysin blocks phagocytosis in a dose-dependent fashion (Fig. 1B). In 10% serum (containing ~0.5 μM C3), the IC₅₀ of aureolysin is ~0.1 μM implying a molar ratio of aureolysin/C3 of 1:5 at the IC₅₀. To test whether the antiphagocytic effect was a result of complement inhibition, we performed the phagocytosis in complement-inactivated serum and found no inhibition by aureolysin; indicating that aureolysin specifically inhibits complement-mediated phagocytosis (Fig. 1C).

To determine whether aureolysin also blocks bacterial killing, we performed killing assays using isolated neutrophils, human serum, and Group B Streptococcus as a model organism for Gram-positive bacteria (Fig. 1D). Aureolysin strongly prevented bacterial killing indicating that aureolysin helps bacteria to resist complement-mediated killing by neutrophils. In summary, aureolysin blocks complement-dependent phagocytosis and killing of bacteria by human neutrophils.

Aureolysin inhibits C3b deposition and C5a generation

Complement activation is crucial to phagocytosis because it results in the labeling of bacteria with opsonins (C3b molecules) that are recognized by phagocyte receptors. To study whether aureolysin blocks complement activation, we first analyzed whether aureolysin affects opsonization of bacteria with C3b molecules. S. aureus was incubated with serum in the presence or absence of aureolysin, and surface-bound C3b was detected on the bacterial surface with specific Abs and flow cytometry. Fig. 2A shows that aureolysin potently blocks the labeling of bacteria with C3b, again indicating that aureolysin is a complement inhibitor. Downstream of C3 cleavage, the complement cascade continues with the cleavage of C5 into C5a and C5b. C5a binds to the G protein-coupled C5a receptor (C5aR) on phagocytes, crucial to phagocyte activation and chemotaxis. Recent studies demonstrated an important role for C5a in host protection against S. aureus infections (34). To study whether aureolysin blocks C5a formation, we analyzed the formation of C5a during incubation of S. aureus with human serum and aureolysin. Because C5a is released into the extracellular milieu, we collected supernatants of serum-opsonized bacteria and analyzed these supernatants for their potency to induce a calcium mobilization in U937 cells transfected with the C5a receptor (U937-C5aR). To prevent formation of other chemotransmitters during the incubation of bacteria with serum, we used heat-killed S. aureus. We observed that supernatants of serum-opsonized S. aureus increased calcium levels in U937-C5aR cells, and this response could be blocked by pretreatment of cells with the C5aR antagonist CHIPS (Supplemental Fig. 1). Also, supernatants of bacteria incubated with C5-deficient serum could not induce a calcium flux, and this could be restored by repletion of serum with purified C5 (Supplemental Fig. 1). Thus,
this calcium mobilization assay specifically detects C5a in bacterial supernatants. When aureolysin was added during incubation of bacteria with serum, we observed a dose-dependent inhibition of C5a generation (Fig. 2B). Altogether these data show that aureolysin is a complement inhibitor that blocks two critical biological effects of the complement cascade: opsonization of bacteria with C3b and release of the chemoattractant C5a.

Aureolysin blocks the CP and LP

To study how aureolysin blocks the complement cascade, we tested its activity in a well-described complement ELISA where the different complement pathways and activation steps can be assessed separately (33). We specifically measured the effect of aureolysin on the CP and LP. The AP could not be investigated because analysis occurs in a buffer with EGTA to chelate calcium ions (35); this interferes with the calcium-dependent proteolytic activity of aureolysin (36). To assess activation of the CP and LP, human serum was incubated with microtiter plates coated with IgM or mannan, respectively. We observed that aureolysin prevents the deposition of C3b and C5b-9 via both pathways (Fig. 3), which is in line with our previous results showing inhibition of C3b deposition and C5a generation on bacteria. The C4b deposition was not inhibited by aureolysin (data not shown), indicating specificity for a common molecule in both pathways that functions downstream of C4b formation but upstream of C3b deposition. This suggests that aureolysin targets C3, a common molecule for all pathways.

Aureolysin acts as a C3 convertase

Because aureolysin targets C3, we studied aureolysin-mediated C3 cleavage. Therefore, we incubated purified C3 with aureolysin (in a 1:1 molar ratio) at 37˚C and analyzed C3 cleavage by SDS-PAGE. The C3 protein (187 kDa) consists of an α-chain (112 kDa) and a β-chain (75 kDa) that are linked together via disulfide bonds (37). We observed that aureolysin caused a rapid and specific cleavage of the C3 α-chain (112 kDa) into a smaller fragment of around 100 kDa (Fig. 4A). The β-chain of C3 was not affected by aureolysin. N-terminal sequencing revealed that the aureolysin-cleaved α-chain starts with the amino acid sequence NH2-LDE-DII, indicating that aureolysin cleaves C3 between an asparagine (N751) and a leucine (L752), which is a typical cleavage site for aureolysin (38, 39). This specific cleavage was also observed at lower molar ratios of aureolysin/C3 (Supplemental Fig. 2). At a ratio of 1:20, we still observe 50% cleavage of C3, which is a 4-fold lower ratio than the determined IC50 in the phagocytosis

![FIGURE 2. Aureolysin inhibits complement activation on bacteria. A. Inhibition of C3b deposition on S. aureus by aureolysin. Serum was pre-incubated with 0.5 μM aureolysin and mixed with bacteria. Deposition of C3b on the bacterial surface was measured by flow cytometry. B. Dose-dependent inhibition of C5a release by aureolysin. Serum (10%) was pre-incubated with aureolysin and subsequently incubated with heat-killed bacteria. Release of C5a in bacterial supernatants was measured by a calcium mobilization assay using U937-C5aR cells. All figures represent mean ± SE of three separate experiments. For B, the relative calcium mobilization was calculated by dividing the fluorescence after stimulation by the baseline fluorescence.](http://www.jimmunol.org/)

![FIGURE 3. Aureolysin blocks the CP and LP. Serum was incubated with aureolysin or BSA (0.5 μM), and complement activation via the CP and LP was determined via ELISA. Aureolysin prevents C3b and C5b-9 deposition via the CP (A, C) and the LP (B, D). All data shown represent mean ± SE of three separate experiments.](http://www.jimmunol.org/)
assay (Fig. 1B). During activation of C3 by its natural protease, the C3 convertase, the C3 α-chain is cleaved at position R748-S749; this cleavage mediates release of the anaphylatoxin C3a (9 kDa) and formation of C3b, which can deposit on surfaces via its thioester domain. The α'-chain of C3b starts with NH₂-SNLDE-DII (Fig. 4B). Surprisingly, the cleavage sites of aureolysin and the C3 convertase are only 2 aa apart; therefore, we named the aureolysin cleavage products C3a²SN and C3b⁴SN. To investigate whether the released C3a²SN is active, we incubated purified C3 and aureolysin for 30 min at 37°C and mixed this with neutrophils to study calcium mobilization. Fig. 4C shows that aureolysin indeed releases an active C3a molecule by cleavage of C3. Furthermore, we tested whether C3b⁴SN can be deposited on bacterial surfaces by incubating purified C3, aureolysin, and bacteria for 30 min at 37°C and detecting surface-bound C3b (Fig. 4D). As a control, we cleaved C3 with a C3 convertase by mixing purified β, δ, and C3b. Like the C3 convertase, aureolysin increased the C3b deposition on bacteria compared with control. In summary, although aureolysin is a complement inhibitor, it specifically cleaves C3 close to the convertase cleavage site generating active C3a²SN and C3b⁴SN.

**Complement inhibition by aureolysin depends on its proteolytic activity**

To verify that the complement inhibitory effect of aureolysin is due to its proteolytic capacity, we incubated aureolysin for 20 min at different temperatures and subsequently analyzed its potency to cleave purified C3 (Fig. 5A). We observed that aureolysin can no longer cleave C3 when preincubated at 65°C. Subsequently, we tested whether this heat-inactivated form of aureolysin can still inhibit C3b deposition and C5a generation (Fig. 5B, 5C, respectively). In contrast to untreated aureolysin, heat-inactivated aureolysin (at 65°C) does not inhibit C3b deposition on bacteria and release of C5a. This shows that the complement-inhibitory function of aureolysin is a result of its proteolytic activity.

**Aureolysin collaborates with host factors to inactivate C3b**

Our finding that aureolysin closely mimics C3 convertases is in sharp contrast with our initial results in serum where aureolysin functions as a complement inhibitor. To study whether C3 cleavage by aureolysin in serum might be different from purified conditions, we incubated aureolysin with serum or purified C3 and detected C3 cleavage products by Western blotting (Fig. 6A). As shown in Fig. 4A, incubation of aureolysin with purified C3 results in cleavage of the C3 α-chain into C3b α⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥ FileReader
allelic exchange mutagenesis to generate an aureolysin mutant in *S. aureus* strain USA300, a predominant isolate of community-acquired methicillin-resistant *S. aureus* (42). Supernatants of wild-type and mutant (Δaur) strains were tested for their ability to cleave C3. Whereas supernatants of wild-type USA300 cleaved the α-chain of C3, no cleavage of C3 was observed with supernatants of the aureolysin mutant (Fig. 8). Additionally, heterologous expression of aureolysin on an extrachromosomal plasmid (Δaur+aur) restored the C3 cleaving ability of the mutant, indicating that aureolysin is required for C3 cleavage by staphylococcal supernatants. Strikingly, we observed that the cleavage of C3 by aureolysin in the bacterial supernatant was different from purified aureolysin. Aureolysin present in supernatants could fully degrade C3, especially when expressed on a plasmid. Also, the C3 β-chain was cleaved. The remarkable difference in C3 cleavage by supernatants versus purified protease cannot be explained by the protein source, as purified protease was isolated from staphylococcal supernatants. The fact that aureolysin mutant did not show any cleavage of C3 strongly suggests that aureolysin collaborates with other proteases in the supernatant to fully degrade C3.

**FIGURE 5.** Proteolytic activity of aureolysin is important for complement inhibition. A, Aureolysin (0.5 μM) was preincubated for 20 min at different temperatures and subsequently tested for C3 cleavage by incubation with 1 μM purified C3 for 30 min at 37°C. Cleavage was analyzed by SDS-PAGE under reducing conditions and Coomassie staining. B, No inhibition of C3b deposition on bacteria by heat-inactivated aureolysin. Serum (5%) was preincubated with 0.5 μM aureolysin, heat-inactivated (65°C) aureolysin (aureolysin65), or buffer and mixed with bacteria. Deposition of C3b on the bacterial surface was measured by flow cytometry. C, No inhibition of C5a generation by heat-inactivated aureolysin. Serum (10%) was preincubated with 0.5 μM aureolysin or heat-inactivated aureolysin and subsequently added to bacteria. C5a release in the supernatants was quantified by flow cytometry by measuring the calcium mobilization in U937-C5αR cells. Right bar, Cells pretreated with the C5αR antagonist (CHIPS, 10 μg/ml). Data shown in B and C represent the mean ± SE of three separate experiments. In B, the relative C3b deposition is the deposition relative to buffer. For C, the relative calcium mobilization was calculated by dividing the fluorescence after stimulation by the baseline fluorescence.

**FIGURE 6.** Aureolysin inactivates C3 in collaboration with serum components fI and fH. A, Serum-dependent degradation of the C3 α-chain by aureolysin. Human serum (5%) or purified C3 (0.6 μM) was incubated with 0.5 μM aureolysin at 37°C, and C3 cleavage was analyzed at different time points by Western blotting. B, C3b-SN and C3b degradation by human serum. C3b-SN and C3b (0.6 μM) were incubated with 5% C3-depleted serum at 37°C, and C3 cleavage products were detected by Western blotting. C, fI and fH mediate C3b-SN degradation. Purified C3 (0.6 μM), fI (45 nM), and fH (27 nM) were incubated with 0.5 μM aureolysin for 30 min at 37°C, and C3 cleavage products were detected by Western blotting.

**FIGURE 7.** Aureolysin inactivates C3a. Heat-killed *S. aureus* was incubated with C5-depleted serum for 30 min at 37°C. Supernatants were analyzed for their potency to induce calcium mobilization in neutrophils (A) or C3a was detected by Western blotting (B). Data shown represent the mean ± SE of three separate experiments. In A, the relative calcium mobilization was calculated by dividing the fluorescence after stimulation by the baseline fluorescence.

**Discussion**

In recent years, our insights into the way bacterial pathogens escape the complement system have grown enormously. *S. aureus* appears...
to be the “master complement evasion bug” because it produces a large array of small proteins that target different parts of the complement system. These staphylococcal complement inhibitors all have highly specific inactivation mechanisms to block the complement cascade. Whereas some inhibitors specifically bind and inhibit crucial complement enzymes, others sterically hinder important protein–protein interactions. In contrast to S. aureus, other Gram-positive bacteria like Group A streptococci rather use proteases to protect themselves from complement attack (43, 44).

For example, streptococcal cysteine protease SpeB degrades C3 to inhibit bacterial clearance (45), whereas the streptococcal cell-associated peptidase ScpA cleaves C5a to inhibit neutrophil chemotaxis (46). In this study, we describe that S. aureus also uses its proteases to dampen the complement response. Specifically, aureolysin inactivates the central complement protein C3 and thereby blocks important complement-dependent responses such as phagocytosis and neutrophil activation. The molecular mechanism by which aureolysin inactivates C3 is surprising (see Fig. 9 for a schematic overview). In contrast to the streptococcal cysteine protease SpeB that fully degrades C3 (45), aureolysin exerts its function by cleaving C3 at one specific site. Because this site is only 2 aa apart from the C3 convertase cleavage site, aureolysin generates active C3b and C3a and thus functions as a complement activator under purified conditions. Intriguingly, by opening the molecule in a C3 convertase manner, the C3b molecule becomes vulnerable to proteolytic degradation by host regulators in the serum. This inactivation depends on the protease fl and its cofactor fH, which forms a binding platform for fl on C3b (40, 41). In order for aureolysin to function as a complement inhibitor, it is crucial that aureolysin is a secreted protease so it can cleave C3 far away from the bacterial surface. In contrast, the C3 convertases of the complement system are generated on the bacterial surface and therefore they mediate C3 cleavage close to the bacterial surface. This is essential for the covalent attachment of the C3b thiolester domain.

Aureolysin is required and sufficient for cleavage of C3 by S. aureus supernatant. C3 cleavage by supernatants of S. aureus strain USA300 (Wt), its isogenic aureolysin mutant (Δaur), and the complemented mutant (Δaur+ aur). Undiluted (−), 3-fold concentrated (3×), and 10-fold concentrated (10×) supernatants of a stationary culture were incubated with 1 μM purified C3 for 1 h at 37˚C in HEPES++. Cleavage was analyzed by SDS-PAGE under reducing conditions and Coomassie staining.

Future studies will be needed to address the importance of aureolysin in the pathogenesis of S. aureus infections. Next to its role in complement escape, aureolysin has other functions by which it can interact with the host and contribute to bacterial virulence. Aureolysin has been shown to contribute to 1) bacterial spreading and invasion by activating the fibrinolytic system (49), 2) resistance to antimicrobial peptides (26), and 3) inhibition of Ig production by lymphocytes (50). Recent studies suggested that aureolysin can be expressed within the phagocytic vacuole after phagocytosis of S. aureus (51). Furthermore it was shown that the isogenic aureolysin mutant was more efficiently killed by macrophages upon phagocytosis (52). These studies suggest that aureolysin is not only important to prevent phagocytes from taking up bacteria, but that it also protects bacteria inside the phagocytes likely through resistance against antimicrobial peptide killing. We find that the proteolytic strategy of aureolysin toward complement is very efficient, as aureolysin cleaves all C3 in serum into C3b within minutes, thereby inhibiting the phagocytosis in a nanomolar range. Our data with aureolysin mutant bacteria indicate that the expression levels of aureolysin in the supernatant are sufficient for C3 cleavage. However, aureolysin seems to act in synergy with other factors from the bacterial supernatant to degrade C3 fully. Because previous studies indicated that aureolysin is required for activation of the V8 protease (53), this suggests that the V8 protease may act as a cofactor for aureolysin-mediated complement activation in the absence of aureolysin. Fluid-phase C3 is cleaved by C3 convertases (C3bBb) on the bacterial surface. This results in release of C3a and covalent attachment of C3b to the bacterial surface via its thioester domain. C3 activation in the presence of aureolysin. Aureolysin cleaves C3 in fluid phase into C3b and C3aSN. C3b is rapidly degraded by host factors fH and fl preventing its deposition on the bacterial surface; C3aSN is further inactivated by aureolysin.

FIGURE 8. Aureolysin is required and sufficient for cleavage of C3 by S. aureus supernatant. C3 cleavage by supernatants of S. aureus strain USA300 (Wt), its isogenic aureolysin mutant (Δaur), and the complemented mutant (Δaur+ aur). Undiluted (−), 3-fold concentrated (3×), and 10-fold concentrated (10×) supernatants of a stationary culture were incubated with 1 μM purified C3 for 1 h at 37˚C in HEPES++. Cleavage was analyzed by SDS-PAGE under reducing conditions and Coomassie staining.

FIGURE 9. Schematic representation of the complement-inhibitory mechanism of aureolysin. A, C3 activation in the absence of aureolysin. Fluid-phase C3 is cleaved by C3 convertases (C3bBb) on the bacterial surface. This results in release of C3a and covalent attachment of C3b to the bacterial surface via its thioester domain. B, C3 activation in the presence of aureolysin. Aureolysin cleaves C3 in fluid phase into C3bSN and C3aSN. C3bSN is rapidly degraded by host factors fH and fl preventing its deposition on the bacterial surface; C3aSN is further inactivated by aureolysin.
cleavage of C3. Notably, we observed no direct cleavage of C3 by purified V8 (data not shown). Future studies are needed to reveal the exact collaborative action of staphylococcal proteases on C3 in the context of serum. Data from other bacterial pathogens show that bacterial proteases are important virulence factors that affect several different parts of the innate immune system, such as the complement system, intracellular inflammatory signaling pathways, and antimicrobial peptides (9). Next to aureolysin and V8 protease, S. aureus secretes 10 other proteases: the serine protease-like family (SpA–F), the epidermal toxic ETA and ETB, and two cysteine proteases, staphopain A (SpC A) and staphopain B (SpSB). A role in immune evasion has now also been shown for SpSB, which kills neutrophils and monocytes and blocks phagocytosis of S. aureus (54, 55). The fact that all S. aureus proteases are secreted abundantly and regulated by the accessory gene regulator (agr), which controls expression of a large group of secreted virulence factors (56, 57), suggests that staphylococcal proteases are important to bacterial virulence. Also, it seems likely that other functions of these proteases lie in the interactions with the immune system.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental figure 1. C5a specificity of calcium mobilization assay.

Serum (10%) or C5-depleted serum (10%) was incubated with heat-killed bacteria. Release of C5a in bacterial supernatants was measured by a calcium mobilization assay using U937-C5aR cells. C5-depleted serum was repleted with 10 μg/ml C5. Preincubation of cells with the C5aR antagonist CHIPS (10 μg/ml) blocks calcium mobilization. The relative calcium mobilization was calculated by dividing the fluorescence after stimulation by the baseline fluorescence.

Supplemental figure 2. Different aureolysin:C3 ratios.

1 μM purified C3 was incubated with different concentrations of aureolysin for 1 h at 37°C in Hepes++. Cleavage was analyzed by SDS-PAGE under reducing conditions and coomassie staining.