MAJOR ARTICLE

Biofilm Formation Induces C3a Release and Protects *Staphylococcus epidermidis* from IgG and Complement Deposition and from Neutrophil-Dependent Killing

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Background. Biofilm formation is considered to be an important virulence factor of the opportunistic pathogen *Staphylococcus epidermidis*. We hypothesized that biofilm formation could interfere with the deposition of immunoglobulins and complement on the bacterial surface, leading to diminished activation of the complement system and protection from killing by human phagocytes.

Methods. The killing of biofilm-encased and planktonically grown wild-type (wt) *S. epidermidis* and the killing of an isogenic biofilm-negative *ica* mutant (*ica*$^-$) by human polymorphonuclear neutrophils (PMNs) were compared. C3a induction and deposition of C3b and immunoglobulin G (IgG) on the bacteria after opsonization with human serum were assessed by enzyme-linked immunosorbent assay, flow cytometry, and electron microscopy. The virulence of the bacterial strains was compared in a mouse model of catheter-associated infection.

Results. Biofilm-embedded wt *S. epidermidis* was killed less well by human PMNs and induced more C3a than planktonically grown wt and *ica*$^-$ *S. epidermidis*. However, the deposition of C3b and IgG on the bacterial surface was diminished in biofilm-encased staphylococci. wt *S. epidermidis* was more virulent in implant-associated infections and was killed more slowly than *ica*$^-$ in ex vivo assays of killing by PMNs.

Conclusions. The results indicate that prevention of C3b and IgG deposition on the bacterial surface contributes to the biofilm-mediated protection of *S. epidermidis* from killing by PMNs.

Staphylococcus epidermidis has emerged as a frequent cause of hospital-acquired infections, especially in patients with indwelling medical devices [1–3]. Biofilm formation is typically involved in device-associated *S. epidermidis* infections [4] and is considered to be an important virulence factor because bacteria in biofilms are less accessible to antibiotics and immune defenses [5, 6]. *S. epidermidis* biofilms are covered by extracellular material ("slime") [7], protecting them from uptake by polymorphonuclear neutrophils (PMNs) [8]. Moreover, slime-mediated inhibition of phagocytosis also help planktonic (free-floating) *S. epidermidis* evade killing by PMNs [9, 10]. The specific evasion factors and molecular mechanisms of protection remain largely unknown [11].

The *S. epidermidis* slime component polysaccharide intercellular adhesin (PIA) is a β-1,6-linked *N*-acetylglucosamine (GlcNAc) homoglycan [12] encoded by the *icaADBC* operon [13, 14] and is sometimes referred to as "poly-*N*-acetylglucosamine" (PNAG) [15]. The *ica* operon is involved in biofilm formation, a process characterized by the initial attachment of bacteria to an artificial surface, PIA-mediated cell-cell adhesion, and formation of multilayered clusters [16, 17]. Recently, it has been demonstrated that PIA contributes

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to the antiphagocytic property of *S. epidermidis* slime; in the absence of opsonins, a *S. epidermidis ica* mutant was killed faster by human PMNs than its parental strain, and the difference was attributed to an enhanced phagocytosis and correlated with an increased susceptibility of the mutant to host antimicrobial peptides [10]. The underlying resistance mechanisms may include repulsion of antimicrobial peptides, because a portion of the GlcNAc residues are deacetylated by IcaB, resulting in a positive net charge of the polymer [18]. The large amount of PNAG/PIA in *S. epidermidis* biofilms acts as a sink for anti-PNAG antibodies, diminishing opsonization of the bacterial surface and increasing the resistance to PMN killing [19]. Supporting the importance of PIA as a factor in pathogenicity, in animal models of infection *S. epidermidis ica* mutants have been found to be attenuated in virulence [18, 20–23].

Because PMN killing of *S. epidermidis* is known to be complement dependent [24], biofilm-mediated immune evasion may be attributed to diminished opsonization. This may be accomplished by inhibiting the deposition of antibodies on the bacterial surface and/or the activation of the host's complement system. Microbes activate complement via 3 routes. The classic pathway is initiated by antibody-mediated recognition, the alternative pathway is antibody independent and is triggered by microorganisms themselves, and the lectin pathway is activated by the interaction between mannose-binding lectin or serum ficolins and microbial polysaccharides. All complement pathways generate the proinflammatory chemoattractant C3a and lead to deposition of C3b on the bacterial surface, promoting phagocytic uptake [24, 25]. In the present study, we investigated whether biofilm formation helps protect *S. epidermidis* from neutrophil killing in vitro and ex vivo and modulates complement activation and complement and IgG deposition.

METHODS

Bacterial strains. The isogenic biofilm-negative *ica* mutant (*ica*$^-$) was obtained by insertion of Tn*917* into *icaA* of *S. epidermidis* 1457 (wt), leading to a PIA-deficient and thereby to a biofilm-negative phenotype and erythromycin resistance [26].

Serum and isolation of human PMNs. Normal human serum (NHS) was collected from 20 healthy donors, pooled, and stored in aliquots at –70°C. In addition, serum from an asplenic patient with C2 deficiency was used (see the case report [27]). No total hemolytic activity was detectable in this serum, but the alternative pathway hemolytic activity was found to be in the same range as the NHS pool. Blood PMNs were isolated from healthy volunteers by a density gradient technique (Percoll) [28]. Informed consent was obtained from blood donors.

***PMN killing of* S. epidermidis.** For each experiment, staphylococci were grown from frozen stock cultures. Briefly, cryoculture beads (Pro-Lab) were incubated in 1 mL of tryptic soy broth (TSB) with 0.25% glucose for 8 h at 37°C without shaking. Cultures were then diluted 1:100 in TSB with 0.25% glucose and incubated for 16 h at 37°C in either pyrogen-free polypropylene tubes (to obtain planktonically grown bacteria) or in nonpyrogenic, flat-bottomed microtiter plates (to obtain biofilms).

For microtiter plate assays, biofilms were washed 3 times with saline to remove nonadherent bacteria, and wells were filled with 50 μL of Hank's balanced salt solution (HBSS) with 1 mmol/L calcium, 0.05% glucose, and 5 mmol/L HEPES (HBSS-HEPES). Next, the average number of wt cells per well was quantified. Briefly, bacteria were detached by pipetting, wells were washed with saline, and bacteria were transferred to microreaction tubes placed on ice. Residual bacteria were detached with 0.5% trypsin and 0.2% EDTA in saline for 5 min, as described elsewhere [8, 29], and were collected. The complete removal of the bacteria was confirmed by light microscopy. The samples were diluted in 0.9% sodium chloride, 0.15% EDTA, and 0.1% Triton X-100 and were vortexed and sonicated for 1 min at 120 W. Subsequently, the cell numbers were evaluated using a Petroff-Hausser counting chamber for bacteria. In parallel, liquid-grown wt and *ica*$^-$ bacteria were washed, and cells equal in number to those determined for the biofilm-containing wells were centrifuged in the microtiter plates in 50 μL of HBSS-HEPES.

Colony-forming units were counted by plating serial dilutions on Mueller-Hinton broth agar plates. For biofilm-encased wt bacteria, we found a mean $\pm$ SD of $5.8 \times 10^7 \pm 4.6 \times 10^6$ cfu/well and $5.6 \times 10^7 \pm 8.1 \times 10^6$ cells/well, corresponding to a colony-forming unit to cell ratio of 1.08 ± 0.05:1, which indicated that all bacterial cells were alive under this condition. Similar ratios were found for planktonically grown wt and *ica*$^-$ bacteria, ensuring that equal amounts of colony-forming units (and cells) of the staphylococcal strains were used for the experiments. To initiate phagocytic killing, 50 μL of HBSS-HEPES, 20% NHS, and 2.5×10^6 PMNs was added to the bacteria. After 30 min at 37°C, bacteria and PMNs were collected from the wells, vortexed, and sonicated as described above. PMNs were hypotonically lysed, and colony-forming units were quantified.

To compare the killing of preopsonized wt and *ica*$^-$ bacteria by human PMN, wt bacteria were grown as biofilm, and *ica*$^-$ bacteria were grown planktonically, washed, and pelleted into the microtiter plates. Bacteria were opsonized in HBSS-HEPES and 10% NHS for 30 min and then detached by pipetting and washing. Subsequently, 2.5×10^6 cfu/mL were incubated for 30 min with PMNs at a ratio of 1:1 in 200 μL of HBSS-HEPES at 37°C and 200 rpm, and the remaining colony-forming units were counted as described above.

To assess which complement pathways contribute to the effective opsonization of *S. epidermidis,* 2.5×10^6 cfu/mL of planktonically grown wt bacteria were mixed with PMNs at a ratio of 1:1 and shaken at 200 rpm and 37°C in 200 μL of HBSS-HEPES containing 10% NHS, heat-inactivated human serum, or

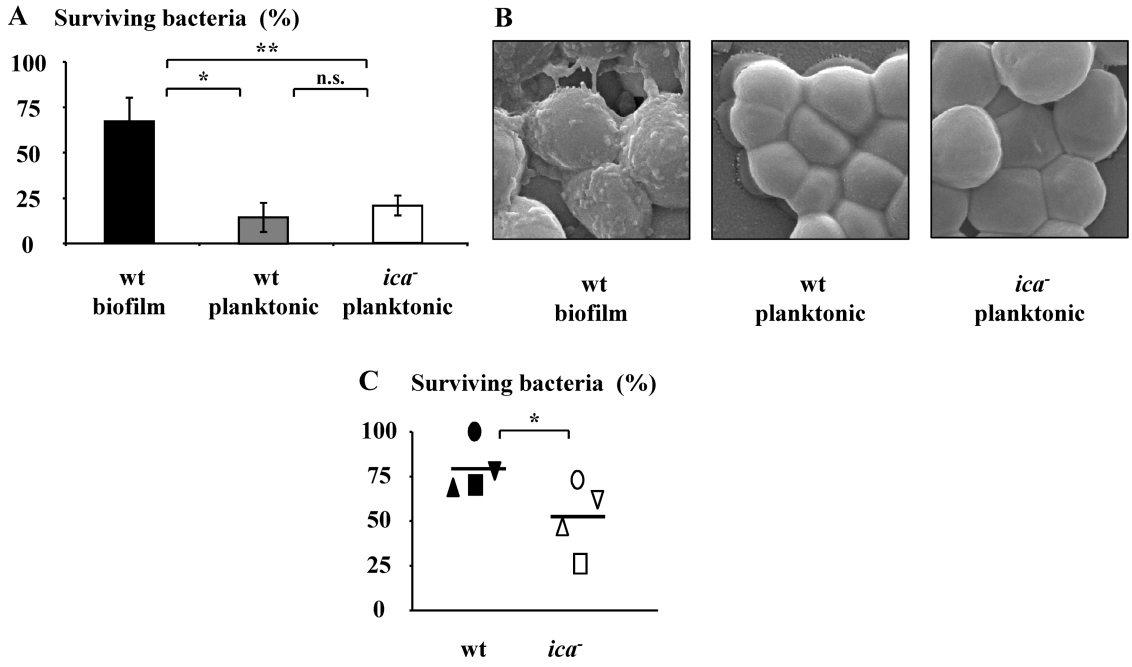

Figure 1. *A,* Protection of *Staphylococcus epidermidis* by biofilm formation from killing by human polymorphonuclear leukocytes (PMNs). *S. epidermidis* wild-type (wt) bacteria were grown as biofilm in microtiter plates *(black bar),* equal numbers of planktonically grown wt *(gray bar)* and biofilm-negative *ica*$^-$ bacteria *(white bar)* were pelleted onto the plates, and bacteria were incubated with human PMNs in the presence of 10% normal human serum (NHS) as the opsonin source. After 30 min, the residual colony-forming units were counted. The mean ± SD percentages of surviving bacteria are shown, based on the initial counts in 3 independent experiments. $^*P < .01$ and $^{**}P < .005$ (analysis of variance [ANOVA]); n.s., not significant. *B,* Scanning electron micrographs of biofilm-encased wt and planktonically grown wt and *ica*$^-$ bacteria. *C,* Killing of preopsonized, biofilm-encased wt *(black symbols)* and pelleted *ica*$^-$ *(white symbols)* bacteria. Bacteria were preopsonized for 30 min with 10% NHS, removed from the microtiter plates, washed, and incubated in suspension with human PMNs at a ratio of 1:1 without any further opsonin source. The percentages of surviving colony-forming units in 4 independent experiments are shown. White and black symbols of the same shape represent data obtained in the same experiment. $^*P < .03$ (ANOVA).

C2-deficient human serum. Samples were drawn after 0, 30, and 60 min, and colony-forming units were counted as described above. In all PMN assays, bacteria were also incubated without neutrophils as a control. Compared with the initial counts, no significant changes in colony-forming unit numbers were observed in these samples.

Complement activation and C3b and IgG deposition. To measure the induction of C3a by and the deposition of human IgG and C3b on biofilm-encased wt and planktonically grown wt and *ica*$^-$ bacteria, staphylococci were incubated in microtiter plates for 30 min with HBSS-HEPES, 10% NHS, or heat-inactivated NHS. Subsequently, bacteria were detached by pipetting and washing, transferred into tubes, and centrifuged at 25,000 *g* for 5 min at 4°C. C3a in the supernatants was measured using a C3a ELISA Kit (Quidel). IgG and C3b deposition was determined by immunoelectron microscopy and flow cytometry. Briefly, preopsonized bacteria were incubated with anti–human C3b (Quidel) or anti–human IgG antibodies and fluorescein-labeled secondary antibodies. The log green fluorescence intensities of 50,000 stained bacterial particles per sample were recorded after excitation with a 488-nm laser, using a Becton Dickinson FACSCalibur flow cytometer.

Electron microscopy. For scanning electron microscopy, staphylococci were fixed in PBS with 2.5% glutaraldehyde for 2 h at room temperature, washed in PBS and double-distilled water, and dehydrated in ethanol. Thin-section scanning electron microscopy was performed as described elsewhere [30]. Briefly, bacteria were prefixed for 20 min on ice in 2% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer, 75 mmol/L ruthenium red, and 75 mmol/L lysine acetate. Samples were washed, fixed without lysine for 3 h on ice, washed again, and postfixed for 1 h with cacodylate buffer and 1% osmium tetroxide. Next, samples were dehydrated and embedded in Epon 812 resin. Thin sections were prepared on an ultramicrotome and collected on carbon/collodium-coated electron microscopic grids. Immunogold labeling was performed with a purified anti–human IgG antibody (Jackson Immunoresearch) or an anti–human C3b antibody (RDI Fitzgerald Industries) and by subsequent treatment with a colloidal gold–labeled secondary antibody.

Infection models. C57BL/6 mice (10–14 weeks old) were obtained from RCC and kept in the animal facility of the Department of Research, University Hospitals Basel; animal experimentation guidelines were followed in accordance with the regulations of Swiss veterinary law. One day before infection, mice

were anesthetized with isofluorane, and 1 flank in each mouse was shaved and depilated. Biofilms were grown on 1-cm segments of Vialon IV catheters (diameter of 2.1 mm; Becton Dickinson). Precultures of wt cells were diluted 1:100 in 1 mL of TSB with 0.25% glucose and placed in microreaction tubes containing 1 catheter segment each. After 16 h, the catheters were washed 4 times with saline, and the average number of biofilm-encased bacteria was determined. Three catheters per experiment were vortexed in saline, 0.15% EDTA, and 0.1% Triton X-100 and sonicated for 2 min at 250 W, and colony-forming units were counted as described above. By this method, *S. epidermidis* can be quantitatively removed from foreign bodies [31]. For all experiments, the average count on the catheters was 1×10^7 cfu.

Mice were infected as described elsewhere [20], with minor modifications. Briefly, mice were anesthetized with 20 mg/kg Ketalar (Pfizer) and 4 mg/kg xylazinum (Graeub), and the shaved flank was cleaned with 70% ethanol. A 3–4-mm incision was made 1–1.5 cm lateral to the spine, and 1 catheter segment, either uncoated or precoated with wt biofilms, was inserted subcutaneously. Next, 20 μL of pyrogen-free saline containing 1×10^7 cfu of *ica*$^-$ bacteria, grown and washed as described above, was injected into the beds of uncoated catheters, and the incisions were closed with wound clips. The diameter of the swelling/edema was measured daily using a caliper. For competition experiments, biofilm-coated catheters were placed subcutaneously, and 1×10^7 cfu of *ica*$^-$ bacteria were added into the catheter beds in saline. Alternatively, mixtures containing 1×10^7 cfu of wt and 1×10^7 cfu of *ica*$^-$ bacteria were injected into uncoated catheters.

On day 7 after infection, mice were anesthetized by use of isofluorane and killed by use of carbon dioxide. For each mouse, the catheter and the surrounding tissue were aseptically removed and separated, transferred into tubes containing 1 mL of saline, and placed on ice. The catheters were washed 4 times with saline. Adherent bacteria were detached and quantified as described above. Tissue samples were homogenized and sonicated, and colony-forming units were counted. For competition experiments, 100 cfu per sample were placed onto Mueller-Hinton broth agar plates with or without 10 μg/mL erythromycin to calculate the percentage of wt and *ica*$^-$ bacteria. Erythromycin-sensitive colonies were considered to be wt bacteria, whereas erythromycin-resistant colonies were considered to be *ica*$^-$ bacteria. Control mice infected with either wt or *ica*$^-$ bacteria alone were included in each competition experiment. In the control mice, all recovered wt and *ica*$^-$ bacteria were still erythromycin sensitive and erythromycin resistant, respectively, showing that the strains maintained their resistance profile in vivo.

For ex vivo opsonophagocytosis assays, wt and *ica*$^-$ bacteria were collected from the catheters on day 7, washed, resuspended in HBSS-HEPES, and incubated (without any further serum source) with human PMNs at a ratio of 1:5 for 30 min at 37°C. Colony-forming units were counted as described above.

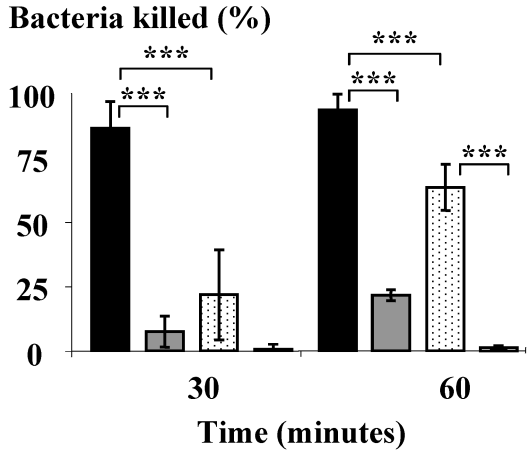

Figure 2. Killing of wild-type *Staphylococcus epidermidis* by human polymorphonuclear leukocytes in suspension at a ratio of 1:1. Pooled normal human serum (NHS; *black bars*), heat-inactivated NHS *(gray bars),* C2-deficient serum *(dotted bars),* or heat-inactivated C2-deficient serum *(hatched bars)* served as the opsonin source. The mean ± SD percentages of killed bacteria are shown for 3 independent experiments. ***$P < .0005$ (analysis of variance).

RESULTS

Biofilm protection of* S. epidermidis *from phagocytic killing. We tested whether *S. epidermidis* evades PMN-mediated killing by biofilm formation. wt *S. epidermidis* was grown as biofilm in microtiter plates, planktonically grown wt and biofilm-negative *ica*$^-$ bacteria were pelleted into the plates, and the bacteria were incubated with human PMNs in the presence of NHS. Biofilm-encased wt bacteria were cleared less efficiently (mean ± SD reduction in colony-forming units after 30 min, 33% ± 13%) than planktonically grown wt bacteria (mean ± SD killing, 86% ± 9%) and *ica*$^-$ bacteria (mean ± SD killing, 79% ± 6%) (figure 1*A*), demonstrating biofilm-mediated protection from neutrophil-dependent killing.

Scanning electron microscopy was used to visualize ultrastructural differences between biofilm-embedded wt bacteria and wt and *ica*$^-$ bacteria grown in suspension. The wt cells in biofilms were embedded by an amorphous extracellular matrix; planktonically grown wt and *ica*$^-$ bacteria lacked this material and adhered to the surface (figure 1*B*). In subsequent experiments, biofilm-encased wt and pelleted *ica*$^-$ bacteria were preopsonized with NHS, removed from the microtiter plates, and incubated with PMNs in suspension. Preopsonized wt bacteria were killed less efficiently than *ica*$^-$ bacteria (figure 1*C*), suggesting that biofilm formation may prevent opsonization by host complement and/or antibodies.

***Requirement of complement for opsonophagocytosis of* S. epidermidis.** We evaluated which complement pathways are

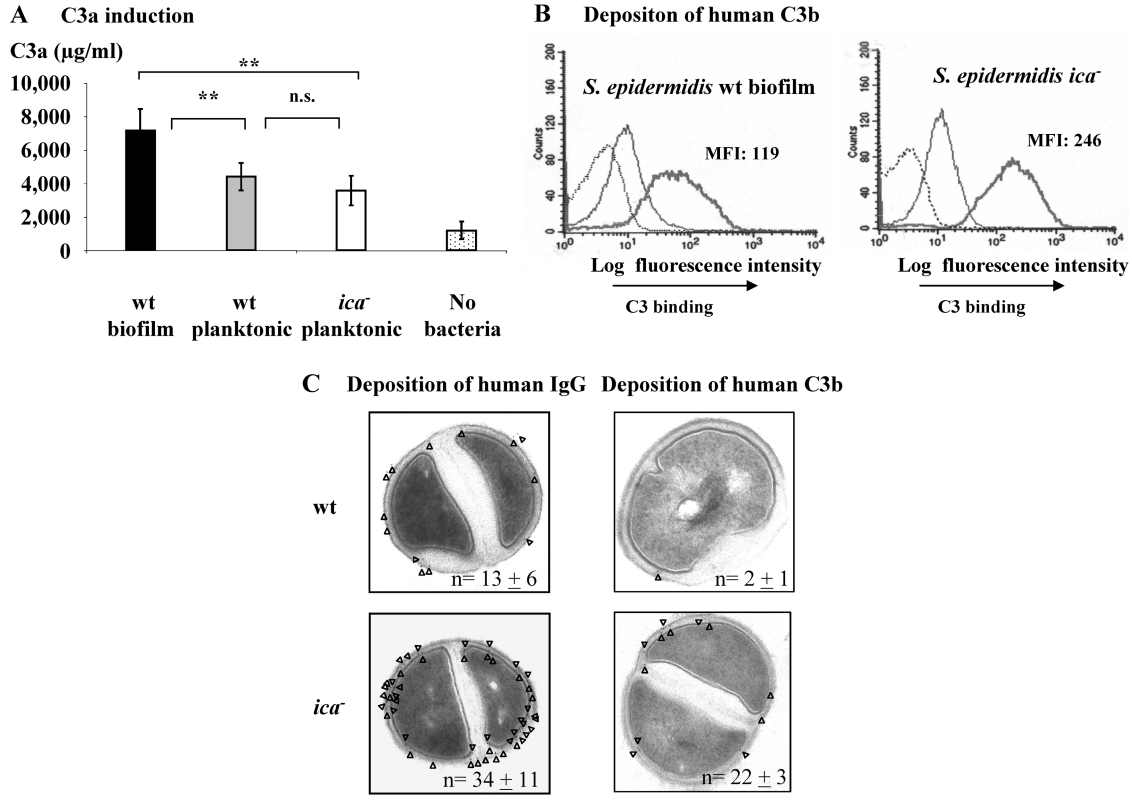

Figure 3. Induction of C3a and deposition of C3b and IgG on the surface of biofilm-encased *Staphylococcus epidermidis* wild-type (wt) and *ica*– bacteria. The bacterial strains were opsonized for 30 min with 10% pooled normal human serum (NHS). *A,* C3a level in supernatants, measured by ELISA. Mean ± SD values for C3a are shown for 3 independent experiments; samples were run in triplicate for biofilm-encased wt bacteria *(black bar),* planktonically grown wt bacteria *(gray bar), ica*– bacteria *(white bar),* and controls without bacteria *(dotted bar).* $^{**}P < .005$ (analysis of variance); NS, not significant). *B,* C3b deposition on opsonized biofilm-encased wt and *ica*– bacteria, as detected by anti–human C3b primary antibody and secondary fluorescein-labeled antibody. Heat-inactivated, and therefore complement-depleted, NHS served as the control. One representative experiment of 5 performed is shown for preopsonization in complete NHS *(bold solid line),* isotype control *(dotted line),* and preopsonization in heat-inactivated NHS *(light solid line).* MFI, mean fluorescence intensity. *C,* Deposition of human IgG *(left)* and human C3b *(right)* on biofilm-encased wt *(top)* and *ica*– *(bottom)* bacteria, as detected by immunogold electron microscopy. One representative experiment of 2 performed is shown; *n* indicates the no. of immunogold particles per square micrometer on the bacterial surface. Arrowheads indicate immunogold particles on bacteria.

required for effective opsonophagocytosis of *S. epidermidis*. *S. epidermidis* was efficiently cleared by human PMNs in the presence of NHS (mean ± SD killing within 60 min, 93% ± 6%). In contrast, no decrease in colony-forming units was observed in the absence of serum, suggesting an absolute requirement of opsonins for the killing of free-floating *S. epidermidis*. The bacteria were killed more slowly if heat-inactivated, and therefore complement-depleted, NHS was used. Still, 22% ± 2% bacteria were inactivated after 60 min, pointing to specific antibodies in NHS (figure 2). C2 deficiency leads to the disruption of the classic and the lectin pathways of complement activation, whereas the alternative complement pathway is intact. Slower killing was observed with C2-deficient serum than with NHS. Heat inactivation of this C2-deficient serum abolished killing, pointing to an absence of specific antibodies. However, the considerable decrease in colony-forming units in C2-deficient serum (mean ± SD killing after 60 min, 63% ± 9%) demonstrated the significance of the alternative complement pathway for inactivation of the bacteria by human PMNs.

Effect of biofilm on activation of complement and deposition of C3b and IgG. We investigated whether biofilm formation leads to reduced activation of the complement cascade and/or to resistance to opsonization with antibodies. wt *S. epidermidis* was grown as biofilm in microtiter plates, planktonically grown wt and *ica*– bacteria were pelleted onto the plates, and the bacteria were opsonized with NHS. Subsequently, the generation of C3a and the deposition of IgG antibodies and C3b on the bacteria were evaluated. Biofilms induced significantly more C3a than planktonically grown wt and *ica*– bacteria (figure 3*A*), indicating that the observed biofilm-mediated evasion of PMN killing was not linked to decreased complement activation. C3b and IgG binding was detected by flow cytometry. The fluorescence intensity of preopsonized wt cells labeled with a primary anti–human C3b antibody and a secondary fluorescein-

labeled antibody was lower than for *ica*⁻ bacteria (figure 3*B*), indicating more C3b on the surface of the latter strain. The percentages of C3b–positive cells were similar and binding was negligible when heat-inactivated serum was used for opsonization (figure 3*B*). The percentage of IgG-positive cells was higher for *ica*⁻ bacteria than for the wt strain (mean ± SD, 91% ± 10% vs. 73% ± 10%; $P < .05$, analysis of variance [ANOVA]); the mean fluorescence intensities were not significantly different. Immunogold electron microscopy was applied to verify these findings. When surface-bound IgG was visualized, we found a markedly decreased number of immungold particles per square micrometer on preopsonized wt vs. *ica*⁻ bacteria (mean ± SD, 13 ± 6 vs. 34 ± 11 particles/μm^2; $P < .05$, ANOVA) (figure 3*C*). Similar results were obtained with surface-bound C3b. The control mouse IgG did not significantly bind to the bacteria, and preopsonization in heat-inactivated NHS did not allow C3b deposition on either strain (data not shown). Taken together, these findings indicate that biofilm-mediated protection from PMN killing correlates with diminished C3b and IgG deposition on the bacterial surface.

Virulence studies and ex vivo opsonophagocytosis. We tested in vivo our hypothesis that inhibition of antibody and complement deposition protects *S. epidermidis* biofilms from clearance. Polyurethane catheters were either precoated with 1×10^7 cfu of wt bacteria or infected with equal numbers of *ica*⁻ bacteria in suspension and implanted subcutaneously into C57BL/6 mice. Both strains led to a local infection with swelling and abscess formation. Mice infected with wt bacteria developed significantly larger-diameter edema than those infected with *ica*⁻ bacteria (figure 4*A*). This may reflect an in vivo correlate of the increased complement activation by biofilm-encased wt bacteria observed in vitro. On day 7, there were significantly more colony-forming units on the catheters and in the surrounding tissue in mice infected with wt bacteria (figure 4*B*).

Two competition assays with different settings were performed to determine further the significance of the ability of biofilm formation on *S. epidermidis* virulence. In the first setting, catheters precoated with wt biofilms were placed subcutaneously before equal numbers of *ica*⁻ bacteria were injected into the catheter beds. Alternatively, mixtures containing equal amounts of wt and *ica*⁻ bacteria were injected into the uncoated catheters. On day 7, the ratio of wt to *ica*⁻ bacteria recovered from the infection site was 3.0 ± 1.9 to 1 for setting 1 ($P < .0001$, ANOVA) (figure 4*C*) and 2.89 ± 1.32 to 1 for setting 2 (figure 4*D*), demonstrating a competitive disadvantage of *ica*⁻ bacteria.

Finally, we compared the susceptibility of wt and *ica*⁻ bacteria in ex vivo assays of opsonophagocytosis. To that aim, catheters from mice infected with wt or *ica*⁻ bacteria were removed 7 days after infection, and bacteria were detached, washed, and subjected to human PMNs without the addition of any opsonin source. Similar to our in vitro findings with preopsonized bio-

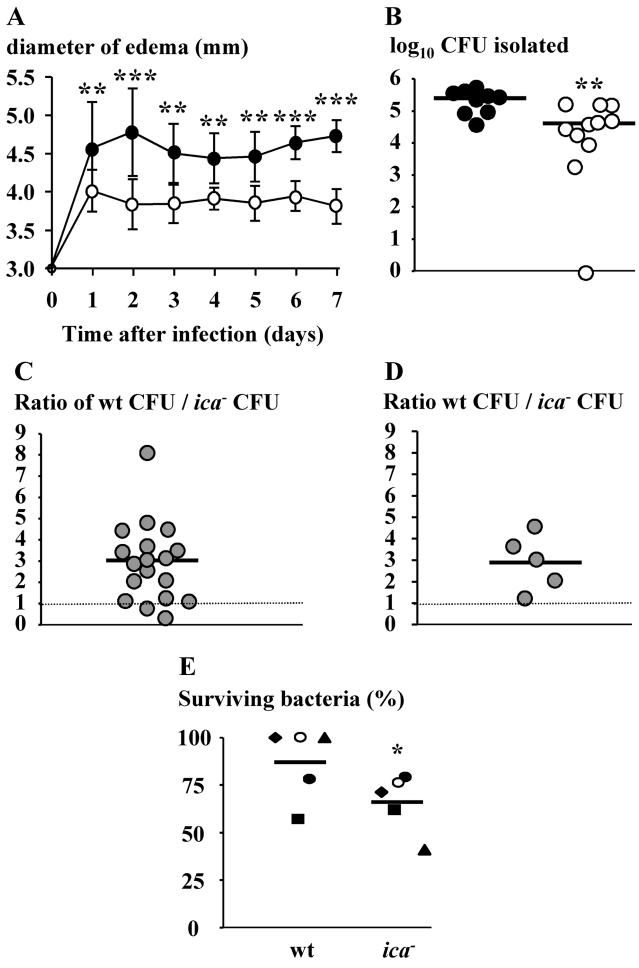

Figure 4. Virulence of wild-type (wt) and *ica*⁻ bacteria in a catheter-related infection model and ex vivo opsonophagocytosis assays. Catheter segments that were precoated with wt bacteria *(black circles)* or infected with equal numbers of *ica*⁻ bacteria in suspension *(white circles)* were implanted into C57BL/6 mice (*A* and *B*). *A,* Mean ± SD edema/swelling sizes in ⩾6 mice per bacterial strain. $^{**}P < .005$ and $^{***}P < .0005$ (Wilcoxon rank-sum test). *B,* Mean total numbers of recovered colony-forming units from the infection sites. $^{**}P < .005$, analysis of variance (ANOVA). *C* and *D,* In vivo competition between wt and *ica*⁻ bacteria. Catheter segments that were precoated with wt bacteria were placed subcutaneously into C57BL/6 mice, and equal numbers of *ica*⁻ bacteria were injected into the catheter beds *(C).* Alternatively, mixtures containing equal amounts of wt and *ica*⁻ bacteria were injected into the catheter beds *(D).* On day 7, colony-forming units of wt and *ica*⁻ bacteria on catheters and in the surrounding tissues were quantified. The colony-forming unit ratios of wt to mutant bacteria at the infection sites are indicated *(gray circles).* Ratios >1 indicate that more wt than mutant bacteria were found; ratios <1 indicate that more *ica*⁻ than wt bacteria were found. Solid bars represent the median values among 18 mice for panel C and among 5 mice for panel D. *E,* Opsonophagocytosis of *Staphylococcus epidermidis* wt and *ica*⁻ bacteria by human polymorphonuclear neutrophils (PMNs) ex vivo. *S. epidermidis* wt and *ica*⁻ harvested 7 days after experimental infection were incubated with human PMNs at a ratio of 1:5, without any additional serum source. Percentages of surviving colony-forming units compared with the initial counts are shown for 5 independent experiments. White and black symbols of the same shape represent data obtained in the same experiment. $^{*}P < .05$ (ANOVA).

film–encased wt and *ica*$^-$ bacteria, the *ica*$^-$ bacteria were inactivated faster ($P < .01$, ANOVA) (figure 4*E*), indicating that biofilms may impair effective opsonization in vivo.

DISCUSSION

In the present study, we have demonstrated that *S. epidermidis* biofilm formation impairs the deposition of IgG and C3b on the bacterium and increases the resistance to phagocyte-mediated killing in vitro and ex vivo. In opsonophagocytosis assays, biofilm-encased wt bacteria were killed more slowly by human PMNs than wt and *ica*$^-$ bacteria grown in suspension. This finding substantiates the earlier observation that *S. epidermidis* cells in disrupted biofilms were protected from PMN killing compared with planktonically grown cells when rabbit polyclonal PNAG-specific antiserum was used as the opsonin source [19].

Using scanning electron microscopy, we demonstrated that bacteria in biofilms were embedded in a fibrous matrix. This phenotype was reminiscent of the extracellular material visualized with scanning electron microscopy in wt *S. epidermidis* by Vuong et al. [10], who also demonstrated that PIA is an integral component of this matrix. The matrix was absent in PIA-negative *ica*$^-$ bacteria and in wt bacteria grown in suspension, which are known to produce PIA in low amounts [32, 33]. However, the presence of the material was correlated with evasion of PMN killing. Along this line, earlier studies demonstrated that slime and PIA protect *S. epidermidis* from uptake by PMNs [8, 10].

Biofilms could contribute in multiple ways to the evasion from phagocyte clearance. Preopsonized *ica*$^-$ bacteria were more susceptible to PMN-mediated killing than the wt strain, indicating that biofilm formation and/or PIA may interfere with complement activation and deposition.

We found that complement is absolutely necessary to mediate PMN-killing of *S. epidermidis* in suspension and that the C2-dependent complement pathways (mannose-binding lectin and classic pathways) are indispensable for rapid phagocytic killing of the bacteria. Vuong et al. [10] showed that unopsonized *S. epidermidis* was readily killed by human PMNs when the bacteria and phagocytes were brought into close contact. This may be explained by extracellular killing mechanisms, such as the formation of neutrophil extracellular traps [34, 35].

S. epidermidis biofilms activated more complement than planktonically grown wt and *ica*$^-$ bacteria, as measured by C3a formation. Nevertheless, IgG and C3b deposition was diminished in biofilm-embedded bacteria, which might contribute to the evasion of PMN killing. To our knowledge, this is the first study to show that biofilm formation interferes with complement deposition on the surface of *S. epidermidis*. The discrepancy between C3a induction and C3b deposition may be explained by the earlier observation that extracellular material in *S. epidermidis* biofilm acts as a sink for specific antibodies [19]. Similarly, complement may be activated by immunoglobulins bound to extracellular material in biofilms, leading to complement activation and C3a generation but to insufficient opsonization of the bacterial surface. In addition, the GlcNAc polymer PIA is strongly produced in *S. epidermidis* biofilms [19] and may promote the lectin pathway of complement activation, because mannose-binding lectin and serum ficolins have lectin activity for GlcNAc. Studies of isolated PIA could clarify whether the exopolymer activates complement.

Confirming the results of earlier studies, we found that biofilm formation contributes to the pathogenicity of *S. epidermidis* [18, 20–23]. Importantly, the inhibitory effect of biofilm formation on complement deposition provides new perspective and context for observations from earlier literature, including the finding that complement significantly contributes to the host defense in *S. epidermidis* infections [36]. In addition, edema formation was more severe in mice infected with wt bacteria than in those infected with *ica*$^-$ bacteria. This may be a consequence of enhanced complement activation and C3a and C5a formation, which are the main mediators of neutrophil infiltration, edema, and inflammation. Biofilm-embedded wt bacteria had a survival advantage over *ica*$^-$ bacteria in competition experiments, suggesting that biofilm-positive cell populations have an advantage over biofilm-negative cells in foreign body–related infections. Along this line, the prevalence of *icaABCD* in isolates from catheter-related bacteremia is >90%, whereas isolates colonizing the skin rarely contain the *ica* operon [37, 38]. Finally, wt bacteria isolated from infected catheters were killed by human PMNs significantly more slowly than *ica*$^-$ bacteria, indicating that biofilm formation may diminish effective opsonization of *S. epidermidis* in vivo.

In summary, biofilm formation impairs IgG and C3b deposition on the bacterial surface and PMN-mediated killing. Therefore, factors involved in biofilm formation may represent promising targets for preventing *S. epidermidis* infections, because inhibition of biofilm formation renders the organism more susceptible to host phagocyte function.

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