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Staphylococcal proteases aid in evasion of the human complement system

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Running title: *Staphylococcal proteases inhibit complement*

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ABSTRACT

Staphylococcus aureus is an opportunistic pathogen that presents severe healthcare concerns due to the prevalence of multiple antibiotic resistant strains. New treatment strategies are urgently needed, which requires an understanding of disease causation mechanisms. Complement is one of the first lines of defense against bacterial pathogens, and *S. aureus* expresses several specific complement inhibitors. The effect of extracellular proteases from this bacterium on complement, however, has been the subject of limited investigation, except for a recent report regarding cleavage of the C3 component by aureolysin. We demonstrate here that four major extracellular proteases of *S. aureus* are potent complement inhibitors. Incubation of human serum with the cysteine proteases staphopain A and staphopain B, the serine protease V8, and the metalloproteinase aureolysin resulted in a drastic decrease in the haemolytic activity of serum; whereas two serine-protease like enzymes, SplD and SplE, had no effect. These four proteases were found to inhibit all pathways of complement due to the efficient degradation of several crucial components. Furthermore, *S. aureus* mutants lacking proteolytic enzymes were found to be more efficiently killed in human blood. Taken together, the major proteases of *S. aureus* appear to be important for pathogen-mediated evasion of the human complement system.

INTRODUCTION

Staphylococcus aureus has long been recognized as one of the most threatening opportunistic pathogens. About 20% of the human population are persistent carriers of *S. aureus*, and another 60% are colonized intermittently [1]. The bacterium can remain within the host in a commensal state, but can also cause a wide spectrum of clinical manifestations,

ranging from skin-limited abscesses and wound infections, to life-threatening diseases, including pneumonia, bacteremia, sepsis, endocarditis, or toxic shock syndrome [2]. It has also become a major public health threat due to the increased prevalence of multiple antibiotic resistant strains, such as methicilin-resistant *S. aureus.* The emergence of vancomycin-resistant strains brings back the terrifying spectre of fatal bloodstream infections from the pre-antibiotic era, and emphasizes a need for the development of new treatment strategies, for which a deep comprehension of *S. aureus* pathogenic mechanisms is necessary. In terms of human virulence, *S. aureus* is perhaps the most successful bacterium, as it produces a large arsenal of tightly regulated virulence factors that can be exploited in different host environments [3]. It is clear that complement system evasion by *S. aureus* is an important challenge in the establishment of a successful infection, since the repertoire of staphylococcal molecules targeting this system is extensive. Even though, as a Gram-positive bacterium with a thick layer of peptidoglycan, this pathogen is insensitive to complement-mediated lysis, the three activation pathways of complement ensure that *S. aureus* is quickly recognized and opsonized for efficient phagocytosis. The classical pathway is triggered when the C1 complex binds to invading pathogens, either directly, or via immunoglobulins; whereas the lectin pathway is able to recognize microbial polysaccharides via collectins such as mannose-binding lectin (MBL) or ficolins. Finally, complement can also be activated and amplified through the alternative pathway, which is not so much an activation pathway, but rather a failure to appropriately regulate the constant, low-level spontaneous activation of C3. All three pathways lead to opsonisation of the pathogen with C3b, and fragments thereof. Furthermore, anaphylatoxins C5a and C3a are released to activate and attract phagocytes to the site of infection. The end result of the complement cascade is formation of the membrane attack complex (MAC), and bacterial cell lysis in the case of Gram-negative bacteria. The host manages to protect itself from bystander damage following complement activation

through the expression of complement inhibitors. Unfortunately for the host, versatile strategies of complement evasion have been developed by bacteria [4]. *S. aureus* expresses numerous molecules, both secreted and surface-bound, targeting all stages of complement [5]. Their functions range from binding immunoglobulins and acquiring host complement regulators, via inhibition of C3/C5 conversion, to attenuating complement effector mechanisms, e.g. chemotaxis. Further to this, *S. aureus* also secretes several proteases that may provide the bacterium with additional complement resistance in a manner akin to that observed for the periodontal pathogens *Porphyromonas gingivalis*, *Prevotella intermedia* and *Tannerella forsythia* [6-8]. *S. aureus* secretes several major proteases, including two cysteine proteases (ScpA, staphopain A and SspB, staphopain B), a serine protease (V8 or SspA), and a metalloproteinase (Aur, aureolysin) [9]. The role of these enzymes in pathogenicity has been well documented [10]. For example, *S. aureus*-derived proteases are able to inactivate α-1-protease inhibitor and α-1-antichymotrypsin, endogenous protease inhibitors essential for controlling neutrophil serine proteases [11,12]. The cysteine proteases of *S. aureus* degrade elastin, fibrinogen and collagen, potentially leading to tissue destruction and ulceration [13,14], while SspB affects the interaction of neutrophils and monocytes with macrophages [15]. Additionally, V8 degrades human immunoglobulins [16], whilst Aur contributes to staphylococcal immune evasion by cleavage of LL-37 [17]. Furthermore, the action of Aur on complement component C3 was recently characterized in detail, showing that Aur cleaves C3 to C3b at a site only two amino acids different from complement C3-convertases. Additionally, it was shown that this C3b is then rapidly degraded by factor H and factor I present in serum [18]. As a result, bacteria are poorly opsonized with C3b and this attenuates phagocytosis and killing by neutrophils [18]. These activities of Aur were related to its proteolytic activity, and a major effect on degradation of C3 was lost in an Aur deficient strain [18]. In addition, Aur activates prothrombin [19], and the staphopains and V8 act on

kininogen [20,21], thereby suggesting a possible role of these proteases in septic staphyloccocal infections. In the current study we investigated the impact of the major staphylococcal proteases in complement evasion.

MATERIAL AND METHODS

Ethics statement. The ethics board of Lund University has approved collection of blood from healthy volunteers.

Sera. Normal human serum (NHS) was obtained from ten healthy volunteers, pooled and stored at -80°C. Serum deficient in C1q was obtained from Quidel.

Proteins. *S. aureus* cysteine proteases, ScpA and SspB were purified from strain V8-BC10 or 8325-4 culture supernatants using a modified method, originally described in [14,22]. The *S. aureus* serine protease V8 (glutamyl-endopeptidase) and the metalloproteinase Aur were purified from culture medium of strain V8-BC10 as described [23]. *S. aureus* serine proteases D (SplD) and E (SplE) were expressed recombinantly in *E. coli* BL21 (DE3; Invitrogen), and purified as described [24]. The purity of proteins was evaluated by SDS-PAGE and their activity was confirmed using specific substrates. The activity of ScpA and SspB was determined by active site titration with E-64 (L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane) (Sigma-Aldrich), or α-2-macroglobulin (BioCentrum). Before use in any assay, ScpA and SspB were pre-activated for 20 min by incubation in assay specific buffers supplemented with 1-2 mM DTT. Purified complement proteins C3 and C5, and the C5a peptide, were purchased from Complement Technology.

Antibodies. The following Abs against human antigens were used throughout this study: rabbit polyclonal antibodies (pAb) anti-C1q, -C4c, and -C3d (all from DakoCytomation), goat anti-MBL (R&D Systems), goat anti-C5 (Quidel), goat anti-factor B (FB) (Complement Technology), and mouse monoclonal antibodies (mAbs) anti–ficolin-2 [25] or anti–ficolin-3

126 [26]. Secondary pAb conjugated with horseradish peroxidase (HRP) against rabbit, goat or
127 mouse antibodies, were purchased from DakoCytomation, and goat-anti-rabbit $F(ab')_2$
128 fragments conjugated with Alexa Fluor 647 (AF647) were acquired from Invitrogen. Rabbit
129 $F(ab')_2$ fragments conjugated with DyLight 633 were generated from rabbit pAb anti-human
130 C1q (Dako) using a F(ab')2 Preparation Kit (Pierce) and the DyLight 633 NHS-Ester kit
131 (ThermoScientific).

132 **Bacterial strains and culture conditions.** *S. aureus* strains were cultured in tryptic soy
133 broth (TSB, Difco) or grown on tryptic soy agar plates (TSA) enriched with 1% skimmed
134 milk. For mutant strains, medium was supplemented with erythromycin (5 μg/ml; Sigma-
135 Aldrich). All bacterial strains used in this study are listed in Table 1. The RN6390 *scpA*$^-$
136 mutant was generated from previously published strains [9] using phage transduction
137 techniques described elsewhere [9].

138 **Hemolytic assays.** To assess activity of the classical pathway, sheep erythrocytes were
139 washed three times with $DGVB^{++}$ buffer (2.5 mM veronal buffer pH 7.3, 70 mM NaCl, 140
140 mM glucose, 0.1% gelatin, 1 mM $MgCl_2$ and 5 mM $CaCl_2$). Cells were incubated with a
141 complement-fixing antibody (amboceptor; Behringwerke; diluted 1:3000 in $DGVB^{++}$ buffer)
142 at a concentration of 10^9 cells/ml for 20 min at 37 °C. After two washes with $DGVB^{++}$, 5×10^8
143 cells/ml were incubated for 1 h at 37 °C with 1% NHS diluted in $DGVB^{++}$ buffer (150 μl total
144 volume). Before incubation with erythrocytes, NHS was pre-incubated with various
145 concentrations of different staphylococcal proteases for 30 min at 37 °C. Samples were then
146 centrifuged and the amount of lysed erythrocytes determined by spectrophotometric
147 measurement of released hemoglobin (405 nm). To assess the activity of the alternative
148 pathway, rabbit erythrocytes were washed three times with Mg-EGTA buffer (2.5 mM
149 veronal buffer [pH 7.3] containing 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM
150 $MgCl_2$, and 10 mM EGTA). Erythrocytes at a concentration of 5 x 10^8cells/ml were then

incubated for 1 h at 37°C with 2% NHS diluted in Mg-EGTA buffer (150 µl total volume). The assay was modified for Aur, where 4% C1q-depleted human serum diluted in GVB^{++} buffer (5 mM veronal buffer [pH 7.3], 140 mM NaCl, 0.1% gelatin, 1 mM $MgCl_2$, and 5 mM $CaCl_2$) was used. In both variants of the alternative pathway assay NHS was pre-incubated with different staphylococcal proteases for 15 min at 37 °C. Samples were then centrifuged and the amount of erythrocyte lysis determined spectrophotometrically (405 nm).

Complement activation assays. Microtiter plates (Maxisorp; Nunc) were incubated overnight at 4°C with 50 µl of a solution containing 2 µg/ml human aggregated IgG (Immuno), 100 µg/ml mannan (M-7504; Sigma-Aldrich), 20 µg/ml zymosan (Z-4250; Sigma-Aldrich) in 75 mM sodium carbonate (pH 9.6), or 10 µg/ml acetylated BSA (AppliChem; acetylated as described [27]) in PBS. Between each step of the procedure, plates were washed four times with 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5). Wells were blocked with 1% BSA in PBS for 2 h at RT. NHS (classical and lectin pathway) was diluted in GVB^{++} buffer and used at a concentration of 2% for measurement of deposition of C1q, 1% for C3b and C4b in the classical pathway; 2% for C3b, C4b, ficolin-2 and ficolin-3 in the lectin pathway, 4% for MBL. For the alternative pathway, 3% NHS in Mg-EGTA (all proteases except Aur) or 4% C1q-deficient serum in GVB^{++} (Aur) were used for the deposition of C3b, FB and C5. These concentrations were chosen on the basis of initial titrations. The serum used was mixed with various concentrations of different staphylococcal proteases, pre-incubated for 25 min (NHS) or 15 min (C1q-depleted serum) at 37°C and incubated in the wells of microtiter plates for 45 min at 37°C for C1q and MBL, 20 min at 37°C for C3b and C4b (classical and lectin pathway), and 35 min for C3b, FB and C5 (alternative pathway). Complement activation was assessed by detecting deposited complement factors using specific Abs against C1q, C4b, C3d, FB, C5, MBL, ficolin-2, and ficolin-3, each diluted in blocking buffer. Bound Abs were detected with HRP-labeled anti-

rabbit, anti-goat, or anti-mouse secondary pAbs. Bound HRP-labeled pAbs were detected with 1,2-phenylenediamine dihydrochloride tablets (DakoCytomation), with absorbance measured at 490 nm.

Deposition of C1q on bacteria. *Staphylococcus epidermidis* CCUG 3709 and *S. aureus* 8325-4 were grown in TSB overnight, harvested by centrifugation, washed once in PBS, adjusted to an OD_{600} of 1.0, and incubated with 10 μM CFSE (Sigma-Aldrich) for 20 min in the dark. After incubation, bacteria were washed once and adjusted to an OD_{600} of 0.6 in GVB^{++}. NHS (6%) was treated with different concentrations of Aur and V8 for 25 min at 37°C, after which time 80-μl aliquots of these samples, were mixed with an 80-μl solution of bacteria, and incubated for 45 min at 37°C (3% NHS final concentration). Thereafter, cells were washed twice in FACS buffer (50 mM HEPES, 100 mM NaCl [pH 7.4], 1% BSA, and 30 mM NaN_3). C1q deposition was assessed by incubation of cells with rabbit anti-human C1q $F(ab)_2$ fragments conjugated with DyLight 633 for 45 min. The geometric mean fluorescence intensity (GMFI) of DyLight 633 was calculated for 25000 CFSE-positive cells using FlowJo software (Tree Star).

Degradation assays. C3 and C5 (0.2 μM each) were incubated with *S. aureus* proteases at concentrations ranging from 0.06 to 2 μM. Incubations were carried out for 2.5 h in 50 mM HEPES (pH 7.4), 150 mM NaCl, and 5 mM $CaCl_2$ buffer at 37°C. Proteins were separated by SDS-PAGE electrophoresis using standard Laemmli procedures, and 12% gels. Prior to electrophoresis, samples were boiled for 5 min at 95°C in a reducing sample loading buffer containing 25 mM DTT and 4% SDS. Separated proteins were visualized by staining with silver salts.

Chemotaxis assays. For C5a chemotaxis assays, plasma was used because serum may contain C5a and C5adesArg, which are produced during blood coagulation [28]. Blood was collected with 50 μg/ml Refludan, centrifuged at 2000 rpm for 10 min, with plasma stored in

aliquots at -80°C. To isolate neutrophils, human blood from healthy volunteers was drawn using heparinized blood collection tubes (BD Vacutainer) and left for 15 min at room temperature. Subsequently, blood was layered on an equal volume of Histopaque-1119 (Sigma-Aldrich) and centrifuged for 20 min at 800 x g (room temperature). The polymorphonuclear cell-rich interphase was washed once in 0.5% human albumin (Sigma-Aldrich) in PBS (HyClone), placed onto a 65–85% Percoll gradient (GE Healthcare), and centrifuged for 20 min at 800 x g (room temperature). Cells dispersed in the 70–75% Percoll layers were collected, washed once in 0.5% albumin solution, and adjusted to a concentration of 1.0 x 10^7 cells/ml in a PBS solution of 4% heat-inactivated (30 min, 56°C) Refludan-treated human plasma. The purity of neutrophils (>70%) was determined by flow cytometry using staining with anti–CD16 mAb labeled with allophycocyanin (ImmunoTools).

Chemotactic activity was measured in a disposable 96-well cell migration system with 3-μm polycarbonate membranes (ChemoTx; Neuro-Probe). Serial dilutions of proteases were incubated with 4% heat-inactivated human plasma (the same as for neutrophil suspensions) for 30 min at 37°C, and thereafter applied to the wells of the ChemoTx microplate. Purified human C5a (Complement Technology) at 12.5 nM, diluted in 4% heat-inactivated human plasma, served as a positive control, whereas plasma alone, proteases (at maximal concentration used in the samples with plasma) diluted in PBS, and PBS alone, were used as negative controls. A volume of 50 μl of 1.0 x 10^7neutrophils/ml in 4% heat-inactivated human plasma was applied to each well of the filter top. The microplate was incubated for 60 min at 37°C in humidified air with 5% CO_2, before the membrane was removed. Samples were transferred to a new flat-bottom 96-well plate (Sterilin) and mixed with 30 μl cell lysis buffer (0.5% hexadecyl trimethyl ammonium bromide [Sigma-Aldrich] in PBS). Similarly, 30 μl of cell lysis buffer was added to all wells of the emptied ChemoTx microplate. Both plates were incubated for 30 min at room temperature, and subsequently the solutions from corresponding

wells were pooled together. The activity of neutrophil-associated myeloperoxidase was detected in lysates using 1,2-phenylenediamine dihydrochloride tablets, and the absorbance recorded at 490 nm.

Whole blood killing assay. *S. aureus* strains (Table 1) were grown overnight in 10 ml of tryptic soy broth. Bacteria were harvested for 5 min at 3000 x g, and the culture supernatants were collected for subsequent use to make bacterial suspensions for the assay. Bacteria were re-inoculated to the respective supernatants at an OD_{600} of 0.15 for the 8325-4 wild-type strain (1.0×10^8 CFU/mL) and its mutants, or an OD_{600} of 0.4 (0.5×10^8 CFU/ml) for strain RN6390 and its mutant. Forty μl of such cultures were mixed with 360 μl freshly collected human blood anti-coagulated with Refludan (Pharmion), a recombinant hirudin anticoagulant that does not affect complement activation [29], and incubated at 37°C for 20 min. After incubation, aliquots were removed, serially diluted and plated onto tryptic soy agar. Bacterial survival was calculated via colony enumeration.

Statistical analysis. A one-way ANOVA (InStat) was used to calculate p values to estimate whether the observed differences between experimental results were statistically significant.

RESULTS

Staphylococcal proteases diminish complement activity in human serum. In order to verify if staphylococcal proteases inhibit human complement, purified enzymes were incubated at various concentrations with human serum, and hemolytic assays were used to assess activity of the classical and alternative pathways of complement in pre-treated sera. ScpA, SspB and V8 were found to be efficient inhibitors of the classical pathway, with >70% inhibition observed at a 1-μM concentration (Fig. 1A-B); whereas SplD and SplE were devoid of such activity (Fig. 1B). The metalloproteinase Aur was the most effective,

inhibiting the classical pathway by 98% when present at low micromolar concentrations (0.7 μM) (Fig. 1C).

All proteases that exerted an inhibitory effect on the classical pathway also inhibited the alternative pathway (Fig. 1 D-F). ScpA and SspB (Fig. 1D), as well as V8 protease (Fig. 1E), inhibited the alternative pathway by at least 50% when present at 2 μM. Aur again was the most effective inhibitor, however it has to be considered that to assess its effect on the alternative pathway, a modified hemolytic assay was used since this metalloproteinase requires calcium ions for activity, while the standard buffer used for alternative pathway assays contains EGTA, which will chelate calcium. Therefore, GVB^{++} buffer and C1q-depleted NHS were used for incubation with rabbit erythrocytes. Under such conditions, Aur inhibited the alternative pathway (Fig. 1F) by 90% at 0.5 μM. SplD and SplE did not affect the alternative pathway in any regard (Fig. 1E). In addition, V8 activity was tested in both types of alternative pathway hemolytic assay, with no significant difference found, proving that these two methods are comparable.

Staphylococcal proteases interfere with all three activation pathways by degrading multiple key complement factors. Each complement pathway is a cascade of events activated in a consecutive manner. In order to assess which complement factor(s) were affected by staphylococcal proteases, a microtiter plate-based assay was used. In this assay, depending on the pathway analyzed, complement activation was initiated by various ligands, and the deposition of successive complement factors was detected with specific antibodies. In the case of the classical pathway, complement activation was initiated by aggregated human immunoglobulins. For assessment of the lectin pathway, we used plates coated with mannan (MBL) or acetylated BSA (ficolins). The alternative pathway was activated by immobilized zymosan and the assay was performed using NHS in Mg-EGTA buffer (for all proteases except Aur), or C1q-deficient serum in GVB^{++} buffer (Aur).

For the cysteine protease ScpA (Fig. 2-4, left panels: “Cysteine proteases”), we found that in the classical pathway, the deposition of C1q was decreased by up to 40% in the presence of 1 μM of this enzyme (Fig. 2A). Consequently, deposition of C4b (Fig. 2B) and C3b (Fig. 2C) was also decreased by >50% at 1μM. ScpA also attenuated the lectin pathway as it inhibited the deposition of all three collectins: MBL (Fig. 3A), ficolin-2 (Fig. 3B) and ficolin-3 (Fig. 3C), as well as all of the ensuing complement factors, such as C4b (Fig. 3D) and C3b (Fig. 3E). Surprisingly, in the alternative pathway, ScpA caused a significant increase in the deposition of C3b (Fig. 4A), whereas deposition of FB (Fig. 4B) and C5 (Fig. 4C) were relatively unaffected.

The other cysteine protease of *S. aureus*, SspB (Fig. 2-4, left panels: “Cysteine proteases”), displayed a distinct mode of action towards complement. For the classical pathway, deposition of C1q from human serum was enhanced in the presence of SspB (Fig. 2A). Downstream to C1q, we found a slight inhibition of the pathway, with C4b deposition decreased by 30% at 2 μM protease (Fig. 2B). Accordingly, C3b deposition was also decreased (Fig. 2C). The interference of SspB with the lectin pathway also appeared on the level of collectins, as for ScpA. However, in this case only MBL was sensitive to degradation by SspB (Fig. 3A), whereas deposition of ficolins was greatly enhanced (by up to 120%) for ficolin-2 (Fig. 3B), and slightly (by up to 25%) for ficolin-3 (Fig. 3C). The deposition of consecutive factors, C4b (Fig. 3D) and C3b (Fig. 3E), was consequently inhibited. In the alternative pathway we found that SspB strongly inhibited the deposition of C3b (Fig. 4A) and C5 (Fig. 4C), while there was no effect on FB (Fig. 4B).

The V8 serine protease (Fig. 2-4, middle panels: “Serine proteases”), similarly to SspB, also caused an increase in the deposition of C1q (Fig. 2D). V8 also reduced the deposition of C4b (Fig. 2E) and C3b (Fig. 2F). Like ScpA, V8 was found to inhibit the deposition of all the lectin pathways collectins: MBL (Fig. 3F), ficolin-2 (Fig. 3G) and ficolin-3 (Fig. 3H), and

consequently decreased C4b (Fig. 3I) and C3b (Fig. 3J) deposition. In the alternative pathway, we found that V8 reduced the deposition of C3b (Fig. 4D), as well as FB (Fig. 4E) and C5 (Fig. 4F). The other two serine proteases, SplD and SplE, did not have any effect on any pathway (Fig. 2-4, middle panels: "Serine proteases").

The metalloproteinase Aur (Fig. 2-4, right panels: "Metalloprotease"), like SspB and V8, caused enhanced deposition of the classical pathway initiator, C1q (Fig. 2G), and then inhibited deposition of C4b (Fig. 2H) and C3b (Fig. 2I) at a relatively low concentration (350 nM). In the lectin pathway, we found that Aur, like ScpA and V8, decreased the deposition of MBL, ficolin-2 and ficolin-3 (Fig. 3K -M), which was followed by a decrease in C4b (Fig. 3N) and C3b deposition (Fig. 3O). Surprisingly, however, there was a significant deposition of C3b in the alternative pathway in the presence of Aur (Fig. 4G), while the deposition of FB and C5 was efficiently decreased (Fig. 4H-I). The data regarding C3b deposition via the classical and lectin pathways are in agreement with previously published findings [18]. However, we do see the inhibition of these pathways upstream to C3b, already at the level of C4b formation, which is in contrast with the statement in the previous study [18], where the authors did not observe such inhibition.

Staphylococcal proteases Aur and V8 cause activation and deposition of C1 in the absence of any activator. In classical pathway assays we observed that SspB, V8 and Aur did not inhibit the deposition of C1q, but rather enhanced its deposition on aggregated IgGs over the entire range of enzyme concentrations tested. When human serum was incubated with ScpA, SspB, V8 and Aur in the absence of any immobilized C1 activator, we found that Aur and V8 caused increased deposition of C1q on empty microtiter plates blocked with BSA (Fig. 5A). This effect was not observed for SspB, although elevated deposition of C1q on aggregated IgGs was found previously (Fig. 2A). In addition, Aur and V8 were also found to cause deposition of C1q on bacterial surfaces. To this end, *Staphylococcus epidermidis* was

incubated with NHS containing Aur at different concentrations, and the deposition of C1q was measured using flow cytometry. We found that the addition of Aur to NHS caused a large increase in deposition of C1q on the surface of *S. epidermidis* that mimicked results obtained using microtiter plates (Fig. 5B). We observed the same effect using V8, although to a lesser extent (Fig. 5 B). In contrast, when *S. aureus* was tested in the same conditions, we found that Aur caused a slight reduction of C1q deposition on the surface of the pathogen, whereas V8 had no effect (Fig. 5C). Taken together, our results show that Aur and V8 are able to cause deposition of active C1 complexes on normally non-activating surfaces, such as BSA coated plastic; and in addition can cause increased C1q deposition on bacterial surfaces. This increased C1q deposition is more likely to occur on commensal bacteria, such as *S. epidermidis*, rather than *S. aureus* itself, on which its own protease Aur seems to moderately inhibit C1q opsonisation.

Proteases of *S. aureus* degrade complement factors C3 and C5 and generate biologically active anaphylatoxins. To assess the cleavage pattern of different proteases, purified C3 and C5 were incubated with proteases at various molar ratios. Proteins were then separated by SDS-PAGE and visualized using silver staining. Both, C3 and C5 are composed of covalently linked α- and β-chains. Different cleavage patterns were observed for all of the proteases tested. Specifically, ScpA degraded both C3 and C5, but only at the highest concentrations, and apparently acted on both chains of the molecules, with some preference toward the α-chain (Fig. 6A). Interestingly, SspB specificity did not cause any degradation of purified C3 (Fig. 6B), but efficiently cleaved C3b deposited on the surface of plates coated with mannan (data not shown). In addition, it caused an efficient degradation of C3met (C3 treated with methylamine, resembling C3b) (data not shown), which further proves SspB specificity for the activated form of C3, C3b. Importantly, SspB showed limited degradation of the C5 α-chain (Fig. 6B). Under the same conditions, V8 caused almost complete

degradation of C3 and C5, even at the lowest concentration tested, implicating multiple cleavage sites in both chains of the molecules (Fig. 6C). Aur, as reported previously [18], specifically degraded the α-chain of C3, and released a band corresponding to C3b (Fig. 6 D). Surprisingly, we also found that Aur acted on the α-chain of C5, which was cleaved in a dose-dependent manner (Fig. 6D). SplD and SplE, as expected, did not show any degradation of either of the complement proteins (data not shown). An analysis of C5 cleavage patterns by SspB (Fig. 6B) and Aur (Fig. 6D) indicated that perhaps they were able to release a band with molecular mass corresponding to C5b. Therefore, we assessed if incubation of these proteases with heat-inactivated human plasma would result in generation of the chemotactic peptide C5a, which would subsequently attract purified human neutrophils. ScpA and V8 were also tested in this assay, with purified C5a serving as a positive control. Surprisingly, both staphopain, ScpA and SspB, as well as Aur, were able to stimulate the migration of neutrophils toward heat-inactivated plasma, indicating the release of anaphylatoxins (Fig. 7A). The peak chemotactic activity (comparable to the C5a positive control) produced by ScpA was at 1.5 μM, and at higher concentrations of the enzyme, migration began to decline marginally. The C5a release by ScpA was apparently not accompanied by the generation of intact C5b (most probably once released, the C5b was degraded further to smaller peptides) (Fig. 6A). For SspB, peak migration was achieved at 5 μM; with higher concentrations not tested since at 5 μM some background migration occurred towards SspB alone. To our surprise, Aur was the most active in releasing biologically active C5a, as already 120 nM protease produced a peak of chemotactic activity, with pronounced decline in migration at higher metalloprotease concentrations. V8 did not cause any release of chemotactic activity (data not shown), indicating that although a band corresponding to C5b can be seen transiently at very low concentrations, the cleavage products (including potential C5a) are most probably degraded rapidly to smaller fragments.

Expression of proteases by *S. aureus* contributes to enhanced survival in whole human blood. In order to verify the effect of proteases on the survival of *S. aureus* in human blood, we studied the survival of strains lacking different proteases compared to the wild type. For this purpose *S. aureus* strains (Table 1) grown in tryptic soy broth overnight (under conditions that yielded the highest detectable proteolytic activity in the medium of wild type strains (data not shown)) were incubated for 20 min at 37°C in fresh human blood, and the survival was assessed by colony counting from serial dilutions (Fig. 7B). Mutant strains of 8325-4 lacking different proteases, or combinations of proteases, showed reduced survival compared to the wild type strain; indicating the involvement of proteolytic enzymes in resistance to killing by human blood. This decreased survival was significant for all mutants, with the most significant effect observed for those lacking Aur. Strains lacking ScpA in RN6390 did not show a significantly different survival compared to the wild-type.

DISCUSSION

The role of *S. aureus* proteases in the virulence of this bacterium has been documented in numerous studies, showing that they are able to interact with host defense mechanisms and tissue components. In the current study we demonstrate that four major proteases of *S. aureus* provide a powerful strategy for defense against complement. Importantly, the protease genes are highly conserved among clinical *S. aureus* strains, although under *in vitro* conditions, down-regulation of their expression has been observed in some clinical isolates [30]. In contrast, *S. aureus* grown in serum significantly increases the production of proteases [31]. The major control of expression and activity of extracellular proteases, similarly to other secreted virulence factors of *S. aureus*, is based on the interplay of two global regulators. Positive regulation is provided by the accessory gene regulator (*agr*) quorum sensing system

401 [32,33], whereas the pleiotropic virulence determinant regulator, SarA, is responsible for
402 protease repression [34]. According to the generally accepted hypothesis, dissemination of *S.*
403 *aureus* takes place via transition from adhesive (promoted by *sarA*) to migratory/invasive
404 phenotypes (promoted by *agr*), producing various extracellular proteins. This process is
405 dependent on, amongst other things, proteolytic enzymes, which cleave tissue adhesion
406 molecules [35,36]. In the adhesive form, *S. aureus* must deal with complement factors that
407 can be produced locally on the skin/epithelium [37,38]; yet dissemination into the
408 bloodstream exposes the bacterium to far more challenging conditions, especially in terms of
409 complement activation. Herein we show that four of the major proteases of *S. aureus*: the
410 staphopains (ScpA and SspB), V8 and aureolysin, may help to successfully evade
411 complement.
412 In general we found that all pathways of complement activation were attenuated by *S.*
413 *aureus*; although there appears to be more specific effects of these proteases on complement,
414 which are worth underscoring. Specifically, we demonstrate that the proteases of *S. aureus*
415 decrease deposition of the collectins, MBL and ficolins (Fig. 3). MBL has been proposed as a
416 first-line defense mechanism against *S. aureus* [39], whilst ficolin-2 binds lipoteichoic acid
417 produced by this bacterium [40]. Our results show that these recognition and complement
418 activation pathways might be corrupted by staphylococcal proteases. Interestingly, we found
419 that the deposition of classical pathway collectin, C1q, was not decreased (apart from a
420 relatively small effect by ScpA, Fig. 2A), but rather increased by the action of bacterial
421 proteases. Furthermore, Aur and V8 were found to cause deposition of C1 from serum onto
422 inert surfaces without the need for a specific C1 activator. The increased deposition of C1q in
423 the presence of Aur and V8 occurred not only on blocked microtiter plates but also on the
424 surface of *S. epidermidis*. This organism is a commensal bacterium found on the skin and in
425 the nasal cavity of humans, is known to inhibit pathogen colonization, and has been

specifically shown to block biofilm formation and nasal colonization by *S. aureus* [41]. Perhaps *S. aureus* protease-induced deposition of C1q on the surface of *S. epiderimidis* could render it more vulnerable to opsonophagocytosis, resulting in its eradication, leaving the niche free for pathogen colonization. This hypothesis is further supported by the fact that the same proteases did not increase C1q deposition on *S. aureus* itself, but rather seemed to limit the opsonisation of the pathogen with C1q (at least Aur ;Fig. 5 C). The role of C1q in the phagocytosis of bacteria, independently of C3b, has been demonstrated for several species [42,43]. Considering that consumption of C3 in the fluid phase due to Aur has previously been shown [18], this may remain the primary mechanism. Taking into account the vital role of C1q in the nonphlogistic clearance of apoptotic cells, an attractive hypothesis emerges, whereby *S. aureus* promotes the uptake of commensal species without boosting the inflammatory response.

Interestingly, ScpA, SspB and Aur were found to release biologically active C5a from C5 present in heat-inactivated human plasma. This finding is particularly worth noting considering the increasing number of sepsis cases resulting from *S. aureus* infections, and the central role of C5a in the immunopathogenesis of this life-threatening syndrome [44]. It is known that neutrophils can undergo 'immune paralysis' during sepsis, an effect mediated mainly by excessive C5a levels [45,46]. In particular, C5a rapidly induces C5a receptor internalization, correlating with loss of neutrophil immune functions (chemotaxis ability and reactive oxygen species production) [47]. Increased local production of C5a at infection sites could reduce the number of functional neutrophils, and facilitate the dissemination of *S. aureus*. Notably, SspB has been previously shown to affect phagocytes, i.e. induce apoptosis-like death in human neutrophils and monocytes by selective cleavage of CD11b [48]. In addition, SspB induces the engulfment of neutrophils and monocytes by macrophages, by both the degradation of repulsion signals and induction of 'eat-me' signals on their surfaces

[15]. The detrimental effects mediated by staphopain-induced C5a can be now added to this scheme.

To our surprise, the protease with the highest potential to release biologically active C5a and stimulate migration of neutrophils was Aur. These observations seem at first to be in contrast with the previous study [18], where the authors reported inhibition of C5a generation by Aur based on its effect on calcium mobilization response in U937-C5a receptor cells treated with activated serum in the presence of Aur. However, we may have identified an explanation for this discrepancy as we found that low nanomolar concentrations of Aur, incubated with either C5 or heat-inactivated plasma, induced increased calcium levels in U937-C5a receptor cells (not shown). At higher Aur concentrations, there was no increase in calcium levels (not shown), presumably due to C5a degradation.

Another appealing aspect of C5a production by bacterial proteases is the recently described cross-talk between C5a receptors (C5aR) and TLR receptors, which was demonstrated to be exploited by bacteria for immune evasion. *P. gingivalis*, which is known to generate C5a by means of its proteases, was shown to impair nitric oxide-dependent killing by macrophages utilizing subversive cross-talk between C5aR and TLR2 [49]. There is growing evidence demonstrating the prolonged survival of *S. aureus* in phagocytes [50,51], but the exact mechanisms mediating this have not been clearly described. The proteases ScpA, SspB and Aur appear to be attractive candidates to study in this context.

A detailed study has previously demonstrated that Aur acts on complement component C3 and blocks phagocytosis by converting C3 to active C3b, which then becomes vulnerable to degradation by host complement inhibitor factors H and I [18]. Importantly, we were able to confirm these previously published findings (not shown). C3b release due to cleavage of C3 by Aur is accompanied by C3a production, which is then further processed to smaller fragments in the presence of Aur and serum, and therefore does not induce neutrophil

activation [18]. This seems to be a protective strategy of the bacterium since C3a, in contrast to C5a, has direct antibacterial activity [52]. Interestingly, we found that SspB does not cleave intact C3; however it does degrade C3b, both deposited on a plate and in fluid phase (data not shown). One can speculate that there may be a cooperative action between Aur and SspB, whereby Aur converts C3 to C3b, which is then degraded further by SspB.

To address the overall effect of *S. aureus* proteases on survival of this bacterium we used mutants depleted in protease genes. Due to the fact that proteases are mainly expressed during post exponential phase [9], we used overnight bacteria cultures, since under such conditions there was the highest detectable proteolytic activity in the media of laboratory strains. Most of the analyzed mutant strains lacking proteases, cultured in such conditions and suspended in media from overnight cultures, showed significantly reduced survival compared to the wild type. However, caution is required in the interpretation of these results. Staphylococcal proteolytic enzymes (ScpA, SspB, V8 and Aur) are expressed as zymogens, and must be activated in an interdependent, cascade-like manner. Aur is required for proV8 activation, and releases the mature active form of V8, which in turn activates proSspB [9,53]. Aur appears to be activated via autocatalysis [54], whereas it is still unclear how proScpA (located outside of this activation cascade) is cleaved, although it is also thought to be via an autocatalytic process [55]. In light of this data one would expect that *aur* deletion results in a lack of active Aur, V8 and SspB, and therefore more pronounced effects should be expected for this mutant. However, some activation of proV8 and proSspB has been observed in *aur*-negative mutant strains [9], suggesting the existence of back-up activation mechanisms. A whole blood killing assay is perhaps not sensitive enough to clearly show the differences between different mutants, yet slightly larger effects on bacteria survival was observed (with higher significance) for the *aur*-deficient strain, compared to *sspBC*$^{-}$ and *sspABC*$^{-}$ knock-outs. It is worth noting that we prove a crucial role in survival for SspB using the *sspBC*$^{-}$ knock-out,

501 whereas we cannot clearly say, based on these results, if Aur and V8 play a role in bacterial
502 survival, or if the effects observed with *sspABC*$^{-}$ and *aur*$^{-}$ knock-outs are due to a lack of
503 mature SspB. In contrast, we did not observe an effect on survival for an ScpA-negative
504 mutant.
505 The increased survival of the wild-type strain expressing all *S. aureus* proteases in whole
506 blood might indicate its diminished clearance by opsonophagocytosis and neutrophil
507 activation; processes linked to, and promoted by, complement activation. In keeping with our
508 results, a protease-null strain lacking all 10 exo-proteases exhibited limited growth in serum
509 and largely reduced survival in human blood [56]. However, due to the numerous actions of
510 proteases on other components of host immunity, such as phagocytes or the coagulation
511 system, we are not able to pinpoint exactly what portion of pathogen survivability can be
512 attributed to the effect of proteases on complement. It is hard to design a conclusive
513 experiment since *S. aureus* cannot be killed by complement without the contribution of
514 cellular components.
515 Since the expression and activation of these different proteases seems to be correlated and
516 inter-dependent, we can suppose that they act in concert, and therefore exert an enhanced or
517 even synergistic effect on complement, implying the requirement of much lower
518 concentrations of individual proteases than those used here in *in vitro* experiments. In terms
519 of relative concentrations of individual enzymes, it seems that staphopains A and B are the
520 most intensively secreted of all the staphylococcal proteases [57], allowing for speculation
521 that their influence will be dominant.
522 The overall effect of the proteases seems to be in shutting down complement. However,
523 not only inhibition but also activation of complement appears to be the purpose of these
524 proteases, suggesting that *S. aureus* can in fact modulate complement depending on the
525 conditions. Similar activating effects on complement, in combination with general inhibition

of its cascades, were previously identified for proteases from other human pathogens, including *P. gingivalis*, *P. intermedia* and *T. forsythia*, all of which are involved in periodontal disease [6-8]. The common intersecting points with these strains is the release of anaphylatoxin C5a, and the increase of C1q deposition on inert surfaces in the absence of specific complement activators. Perhaps these findings indicate the existence of a more general mechanism of complement corruption utilized by human pathogens, although more studies are necessary to confirm this hypothesis. It is clear that proteolytic enzymes play an important role in *S. aureus* immune evasion. Our work presents certain key findings in this regard, but still leaves space for a more detail characterization of the effects of individual proteases, and their specific functions.

ABBREVIATIONS

The abbreviations used are: Abs, antibodies; Aur, aureolysin, *S. aureus* metalloproteinase; C3b, activated complement factor 3; C4b, activated complement factor 4; C5aR, C5a receptor; $DGVB^{++}$, gelatin barbiturate (veronal) buffer with dextrose; FB, factor B; GMFI, geometric mean fluorescence intensity; GVB^{++}, gelatin barbiturate (veronal) buffer; HRP, horseradish peroxidase; IgGs, immunoglobulins G; mAb, monoclonal antibody; MAC, membrane attack complex; MBL, mannose-binding lectin; NHS, normal human serum; pAb, polyclonal antibody; ROS, reactive oxygen species; SplD, staphylococcal serine protease D; SplE, staphylococcal serine protease E; ScpA, staphopain A, staphylococcal cysteine protease A, alternative name: ScpA; SspB, staphopain B, staphylococcal cysteine protease B, alternative name: SspB; V8, staphylococcal serine protease, alternative name: SspA

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DISCLOSURE STATEMENT

All authors report no conflicts of interest related to the study.

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FIGURE LEGENDS

FIG 1 Staphylococcal proteases diminish the hemolytic activity of human serum. A-C) Classical pathway. NHS (1%) was supplemented with various concentrations of proteases and pre-incubated for 30 min at 37°C, after which time sheep erythrocytes sensitized with antibodies and diluted in GVB^{++} were added. **D-F)** Alternative pathway. 2% NHS in Mg-EGTA **(D, E)** or C1q-depleted human serum (4%) in GVB^{++} (**F**) were pre-incubated with increasing concentrations of proteases for 15 min at 37°C. Serum was then added to sheep **(A-C)** or rabbit **(D-F)** erythrocytes diluted in their respective buffers. **A-F)** After 1-h incubation, the degree of lysis was estimated by measurement of released hemoglobin

742 (absorbance at 405 nm). Lysis obtained in the absence of proteases was set as 100%. An
743 average of three independent experiments is presented with bars indicating SD.
744
745 **FIG 2 Staphylococcal proteases inhibit the classical pathway.** Serially diluted proteases
746 were incubated for 25 min with 2% (C1q) or 1% (C3b, C4b) NHS diluted in GVB^{++} and
747 added to microtiter plates coated with IgGs. After 20 min (C3b, C4b) and 45 min (C1q) of
748 incubation, plates were washed and deposited C1q **(A, D, G)**, C4b **(B, E, H)** and C3b **(C, F,**
749 **I)** were detected with specific pAbs. Absorbance obtained in the absence of protease was set
750 as 100%. An average of three independent experiments is presented with bars indicating SD.
751
752 **FIG 3 Staphylococcal proteases inhibit the lectin pathway of complement.** Serial
753 dilutions of proteases were incubated for 25 min with 4% (MBL) or 2% (C3b, C4b, ficolin-2,
754 ficolin-3) NHS diluted in GVB^{++} and added to microtiter plates coated with mannan (MBL,
755 C3b and C4b) or acetylated BSA (ficolins). After 20 min (C3b, C4b) or 45 min (MBL,
756 ficolin-2, ficolin-3) of incubation, plates were washed and deposited MBL **(A, F, K)**, ficolin-
757 2 **(B, G, L)**, ficolin-3 **(C, H, M)**, C4b **(D, I, N)** and C3b **(E, J, O)** were detected with specific
758 antibodies. Absorbance obtained in the absence of protease was set as 100%. An average of
759 three independent experiments is presented with bars indicating SD.
760
761 **FIG 4 Staphylococcal proteases inhibit the alternative pathway of complement.** Serial
762 dilutions of proteases were incubated with 3%NHS in Mg-EGTA for 25 min **(A-F)** or 15 min
763 with 4% C1q-depled serum in GVB^{++} **(G-I)**. Samples were then added to microtiter plates
764 coated with zymosan. After 35 min of incubation, plates were washed, and deposited C3b **(A,**
765 **D, G)**, FB **(B, E, H)** or C5b **(C, F, I)** were detected with specific polyclonal antibodies.

766 Absorbance obtained in the absence of protease was set as 100%. An average of three
767 independent experiments is presented with bars indicating SD.
768
769 **FIG 5 Staphylococcal proteases Aur and V8 cause activation and deposition of C1q on**
770 **microtitre plates as well as commensal bacteria. A)** Microtiter plates were blocked with
771 BSA and incubated for 45 min with 5% NHS containing various concentrations of proteases.
772 Deposited C1q was detected with a specific antibody. Absorbance obtained for NHS in the
773 absence of protease was set as 100%. An average of three independent experiments is
774 presented with bars indicating SD. *S. epidermidis* CCUG 3709 (**B**) and *S. aureus* 8325-4 (**C**)
775 were incubated with NHS (3%) and different concentrations of proteases. Deposition of C1q
776 was quantified using flow cytometry with specific $F(ab)_2$ fragments, and the absorbance
777 obtained in the absence of proteases was set as 100%. An average of three independent
778 experiments is presented with bars indicating SD. Statistical significance of the observed
779 differences was estimated using a one-way ANOVA and a Dunnett posttest (*$p < 0.05$, **$p <$
780 0.01, ***$p < 0.001$). ctrl = control.
781
782 **FIG 6 Staphylococcal proteases degrade C3 and C5**. C3 and C5 (0.2 μM each) were
783 incubated with serial dilutions of ScpA **(A)**, SspB **(B)**, V8 **(C)** and Aur **(D)**. Incubations were
784 carried out for 2.5 h at 37°C, with proteins then separated by SDS-PAGE. All gels were
785 stained with silver salts.
786
787 **FIG 7 Proteases of *S. aureus* generate biologically active C5a and their expression**
788 **contributes to survival in whole human blood. A)** Increasing concentrations of ScpA, SspB
789 and Aur were incubated with 4% heat-inactivated human plasma, and then placed in the wells
790 of ChemoTx microplates. Neutrophil migration was measured after 1 h as activity of

neutrophil-associated myeloperoxidase. PBS and proteases alone were used as negative controls, and human C5a (12.5 nM) was the positive control. Absorbance obtained for the highest migration in the assay, observed with the positive control, was set as 100%. An average of three independent experiments is presented with error bars indicating SD. Statistical significance was determined using a one-way ANOVA and a Dunnett post-test (*p < 0.05, **p < 0.01, ***p < 0.001), and calculated compared to untreated plasma (0 μM proteinase). **B)** *S. aureus* strains 8325-4 (WT), 8325-4 *sspABC*$^{-}$, 8325-4 *sspBC*$^{-}$, 8325-4 *aur*$^{-}$, RN6390 (WT) and RN6390 *scpA*$^{-}$ were incubated for 20 min at 37°C with freshly collected human blood. After incubation, aliquots were removed, serially diluted, and plated on tryptic soy agar plates. Survival was calculated as percent survival compared to the inoculum. Statistical significance of the observed differences between wild-types and corresponding mutant strains was determined using a one-way ANOVA and a Dunnett post-test; *p < 0.05, **p < 0.01, ***p < 0.001.

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824 **TABLE 1** Description of bacterial strains used in this study.

Bacterial strain	**Description**	**References or source**
8325-4	*S. aureus* WT laboratory strain	laboratory stocks
8325-4 *aur*$^-$	*S. aureus aur* mutant, no expression of Aur metalloproteinase	[9]
8325-4 *sspABC*$^-$	*S. aureus sspABC* mutant, no expression of V8 serine protease, SspB cysteine protease and its inhibitor SspC	[9]
8325-4 *sspBC*$^-$	*S. aureus sspBC* mutant, no expression of SspB cysteine protease and its inhibitor SspC	[9]
RN6390	*S. aureus* WT laboratory strain	laboratory stocks

RN6390 *scpA*$^{-}$	*S. aureus scpA* mutant, no expression of ScpA cysteine protease	This study
CCUG 3709	*S. epidermidis* WT laboratory strain	Culture Collection, University of Göteborg, Sweden

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Fig. 1

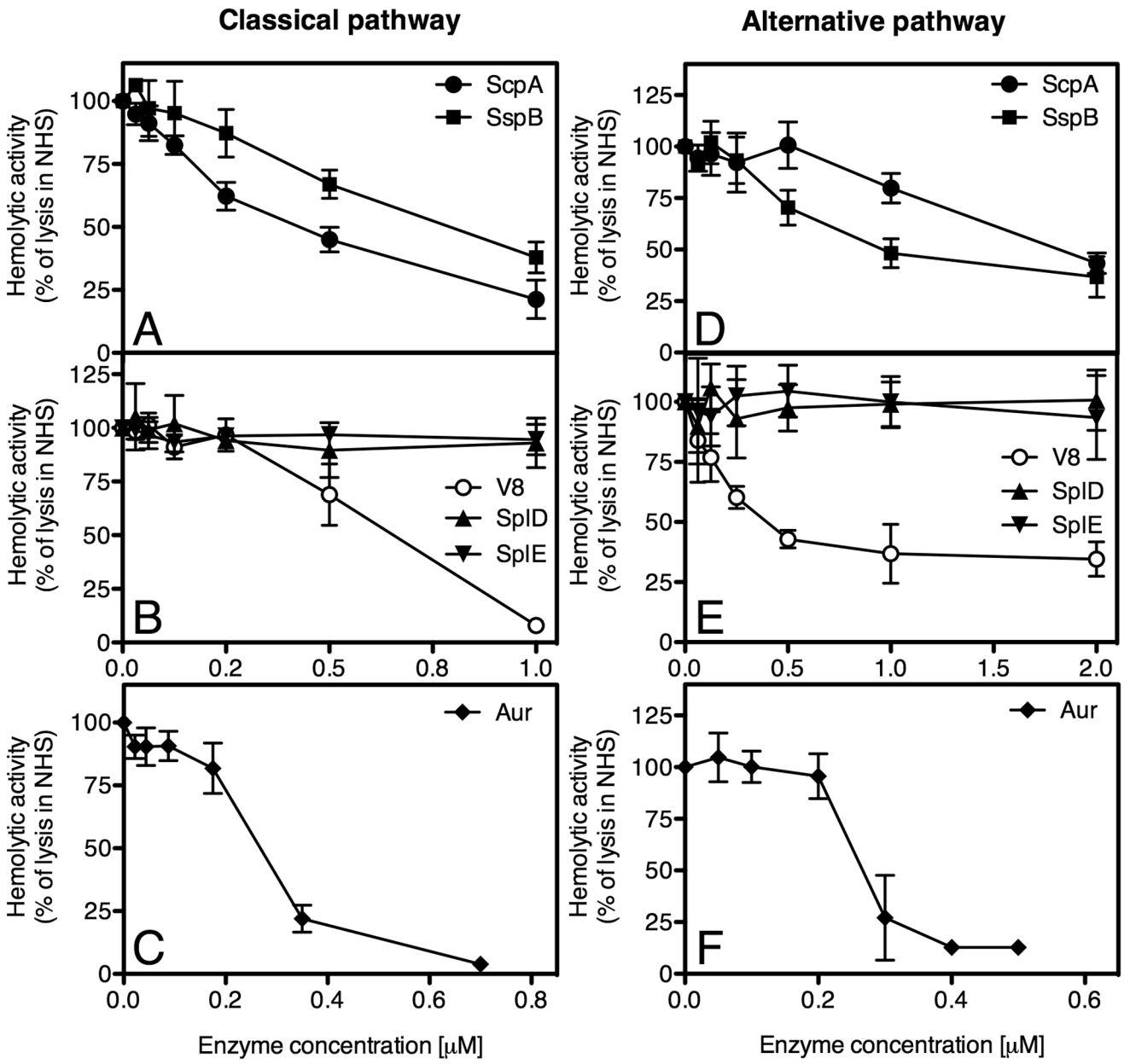
Classical pathway
Alternative pathway
ScpA
SspB
V8
SplD
SplE
Aur
Hemolytic activity (% of lysis in NHS)
A
B
C
D
E
F
Enzyme concentration [μM]

Fig. 2

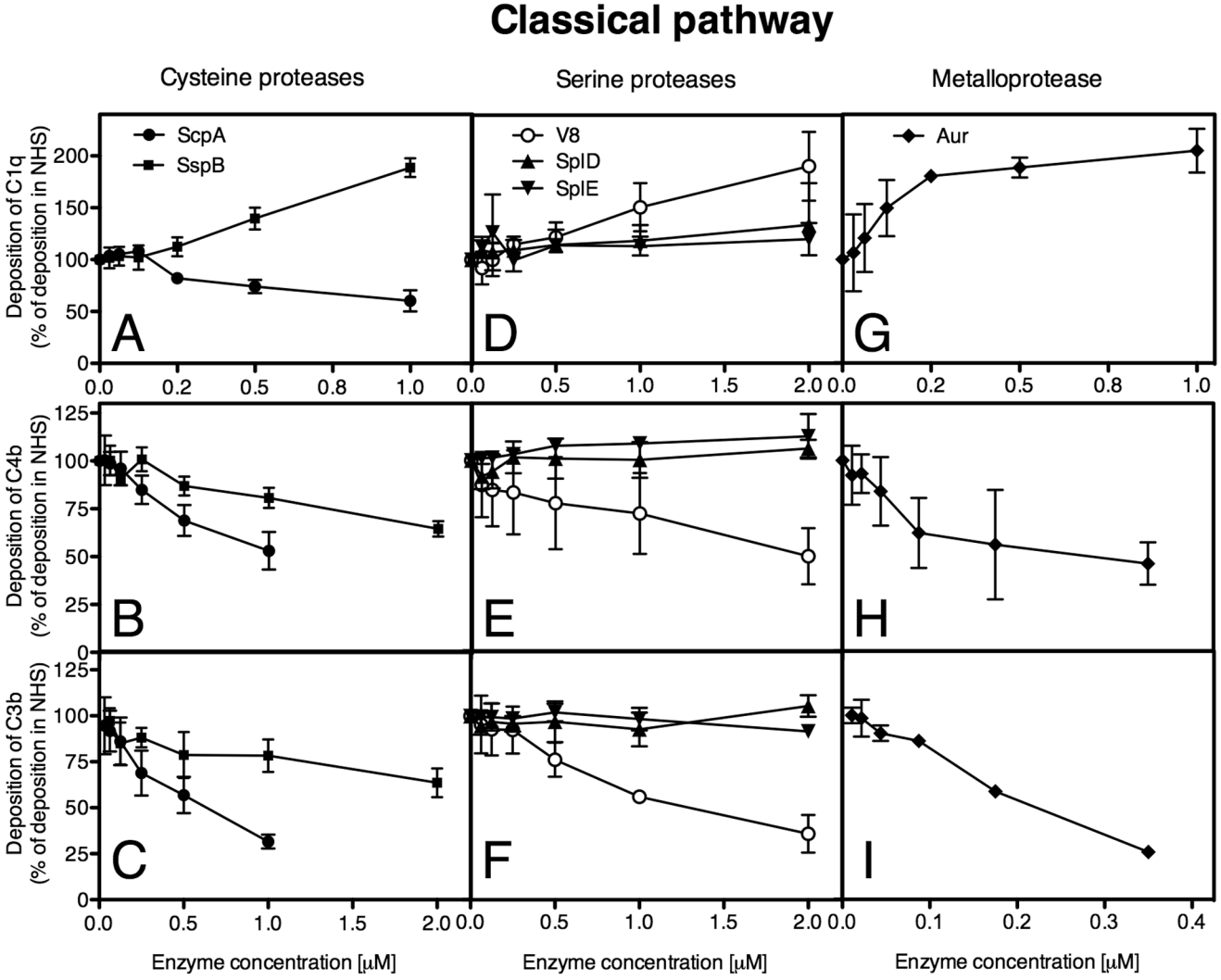
Classical pathway
Cysteine proteases
Serine proteases
Metalloprotease
ScpA
SspB
V8
SplD
SplE
Aur
Deposition of C1q (% of deposition in NHS)
Deposition of C4b (% of deposition in NHS)
Deposition of C3b (% of deposition in NHS)
A
B
C
D
E
F
G
H
I
Enzyme concentration [μM]
Enzyme concentration [μM]
Enzyme concentration [μM]

Fig. 3

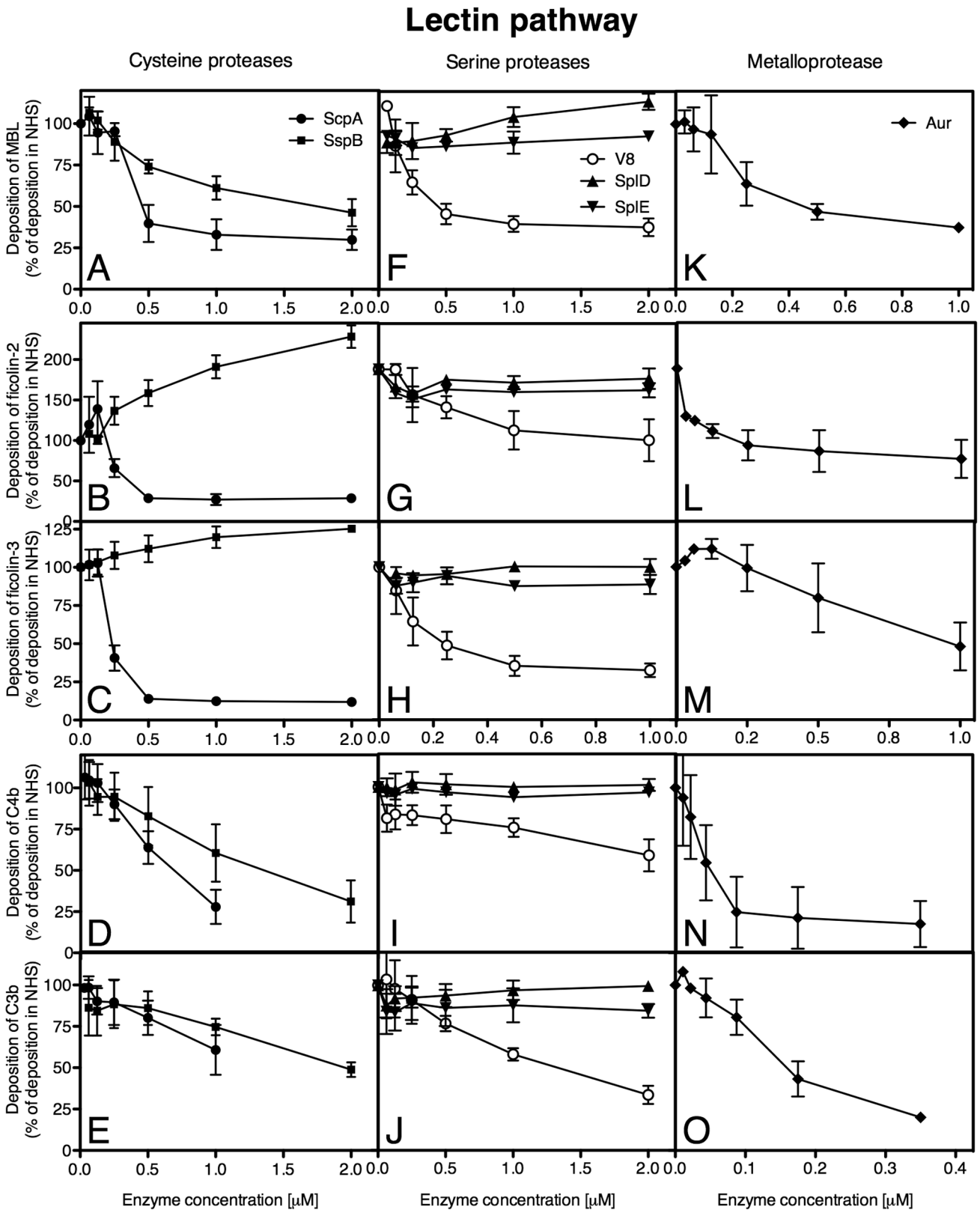
Lectin pathway
Cysteine proteases
Serine proteases
Metalloprotease
ScpA
SspB
V8
SplD
SplE
Aur
Deposition of MBL (% of deposition in NHS)
Deposition of ficolin-2 (% of deposition in NHS)
Deposition of ficolin-3 (% of deposition in NHS)
Deposition of C4b (% of deposition in NHS)
Deposition of C3b (% of deposition in NHS)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Enzyme concentration [μM]
Enzyme concentration [μM]
Enzyme concentration [μM]

Fig. 4

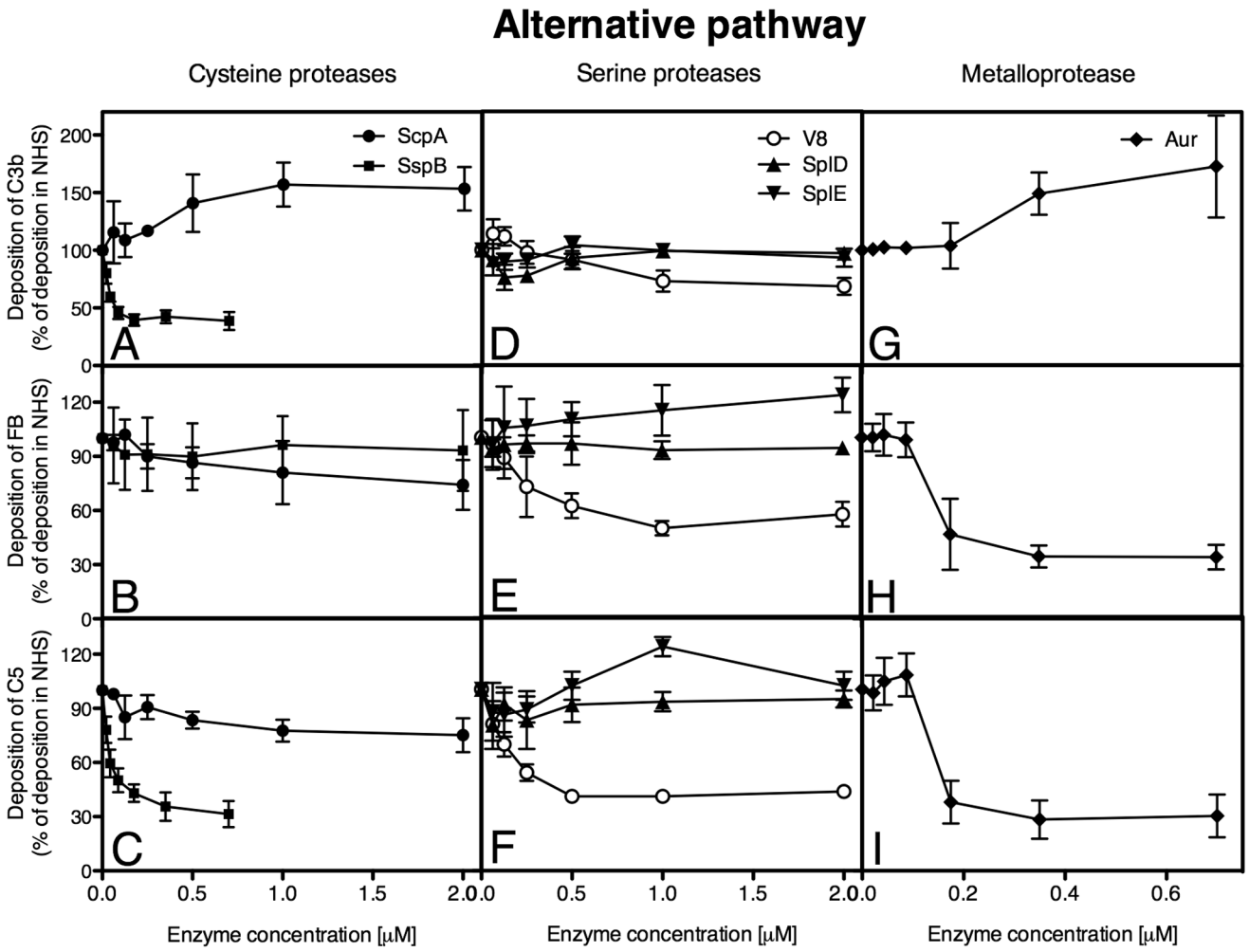
Alternative pathway
Cysteine proteases
Serine proteases
Metalloprotease
ScpA
SspB
V8
SplD
SplE
Aur
Deposition of C3b (% of deposition in NHS)
Deposition of FB (% of deposition in NHS)
Deposition of C5 (% of deposition in NHS)
A
B
C
D
E
F
G
H
I
Enzyme concentration [μM]
Enzyme concentration [μM]
Enzyme concentration [μM]

Fig. 5

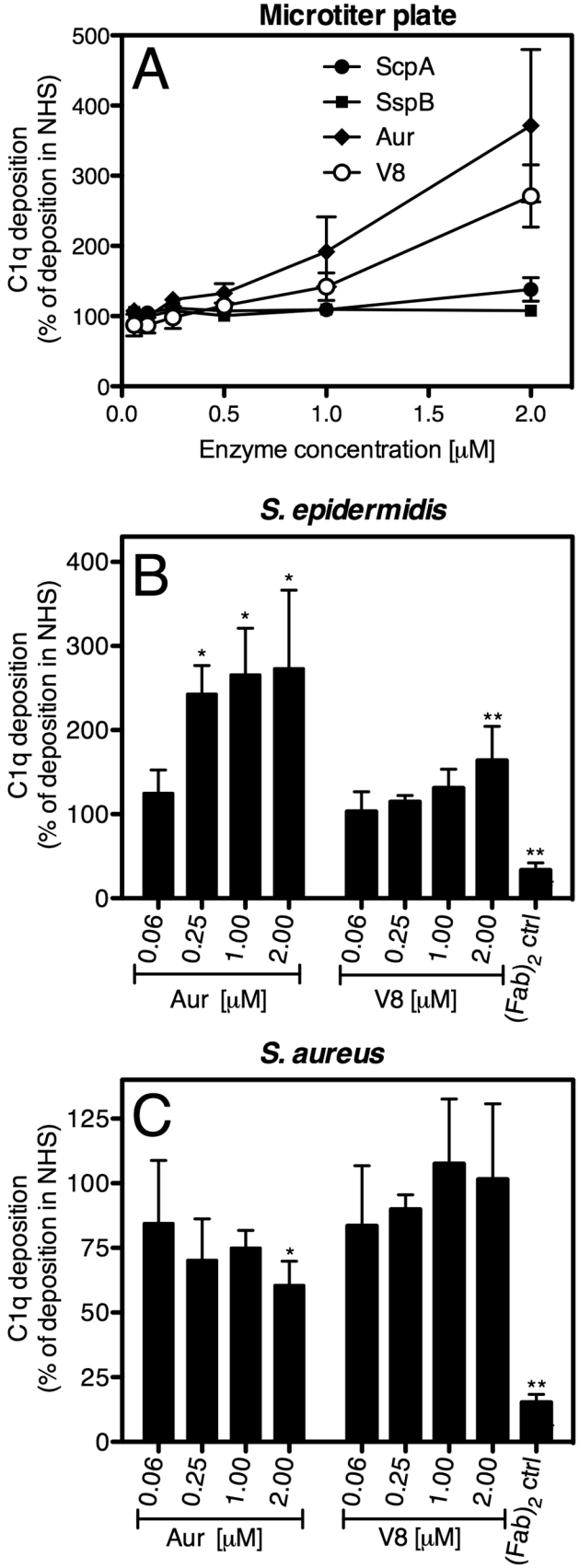
Microtiter plate
A
ScpA
SspB
Aur
V8
C1q deposition
(% of deposition in NHS)
Enzyme concentration [μM]
S. epidermidis
B
Aur [μM]
V8 [μM]
(Fab)2 ctrl
S. aureus
C

Fig. 6

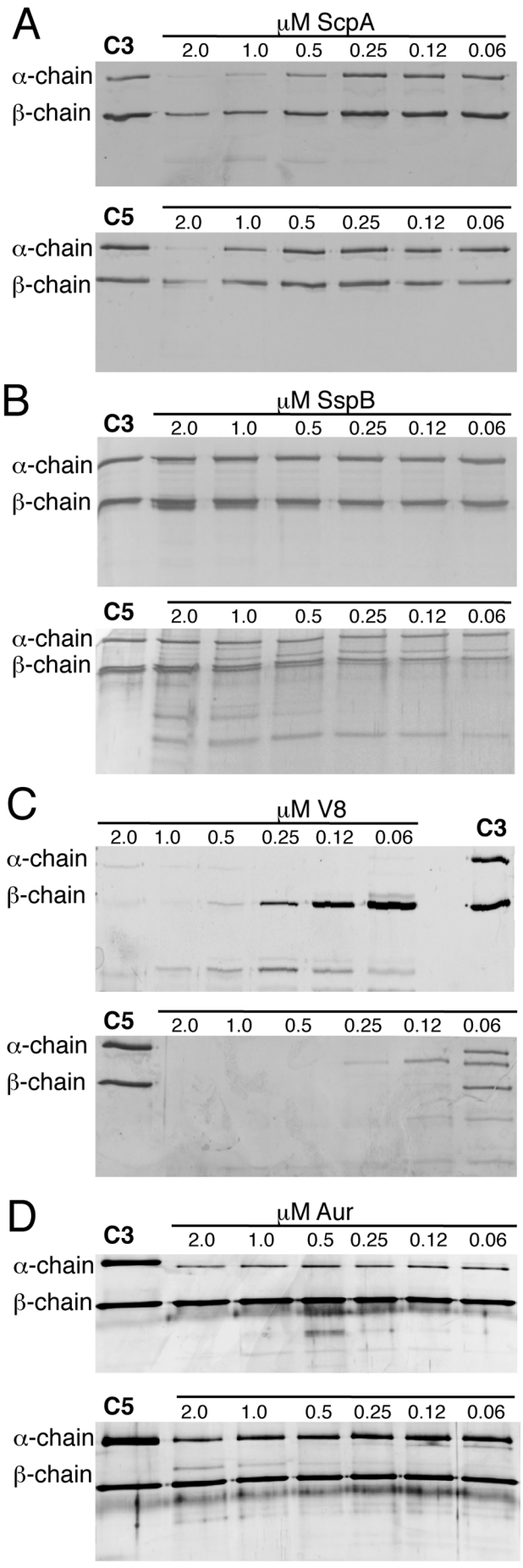
A
μM ScpA
C3
2.0 1.0 0.5 0.25 0.12 0.06
α-chain
β-chain
C5
2.0 1.0 0.5 0.25 0.12 0.06
α-chain
β-chain
B
μM SspB
C3
2.0 1.0 0.5 0.25 0.12 0.06
α-chain
β-chain
C5
2.0 1.0 0.5 0.25 0.12 0.06
α-chain
β-chain
C
μM V8
2.0 1.0 0.5 0.25 0.12 0.06
C3
α-chain
β-chain
C5
2.0 1.0 0.5 0.25 0.12 0.06
α-chain
β-chain
D
μM Aur
C3
2.0 1.0 0.5 0.25 0.12 0.06
α-chain
β-chain
C5
2.0 1.0 0.5 0.25 0.12 0.06
α-chain
β-chain

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Fig. 7