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1 Staphylococcal proteases aid in evasion of the human complement system

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22 **Key words:** complement evasion; proteases; *Staphylococcus aureus*

26

27 **ABSTRACT**

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

44

45

46 **INTRODUCTION**

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

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

76 through the expression of complement inhibitors. Unfortunately for the host, versatile
77 strategies of complement evasion have been developed by bacteria [4]. *S. aureus* expresses
78 numerous molecules, both secreted and surface-bound, targeting all stages of complement [5].
79 Their functions range from binding immunoglobulins and acquiring host complement
80 regulators, via inhibition of C3/C5 conversion, to attenuating complement effector
81 mechanisms, e.g. chemotaxis. Further to this, *S. aureus* also secretes several proteases that
82 may provide the bacterium with additional complement resistance in a manner akin to that
83 observed for the periodontal pathogens *Porphyromonas gingivalis*, *Prevotella intermedia* and
84 *Tannerella forsythia* [6-8]. *S. aureus* secretes several major proteases, including two cysteine
85 proteases (ScpA, staphopain A and SspB, staphopain B), a serine protease (V8 or SspA), and
86 a metalloproteinase (Aur, aureolysin) [9]. The role of these enzymes in pathogenicity has
87 been well documented [10]. For example, *S. aureus*-derived proteases are able to inactivate α -
88 1-protease inhibitor and α -1-antichymotrypsin, endogenous protease inhibitors essential for
89 controlling neutrophil serine proteases [11,12]. The cysteine proteases of *S. aureus* degrade
90 elastin, fibrinogen and collagen, potentially leading to tissue destruction and ulceration
91 [13,14], while SspB affects the interaction of neutrophils and monocytes with macrophages
92 [15]. Additionally, V8 degrades human immunoglobulins [16], whilst Aur contributes to
93 staphylococcal immune evasion by cleavage of LL-37 [17]. Furthermore, the action of Aur on
94 complement component C3 was recently characterized in detail, showing that Aur cleaves C3
95 to C3b at a site only two amino acids different from complement C3-convertases.
96 Additionally, it was shown that this C3b is then rapidly degraded by factor H and factor I
97 present in serum [18]. As a result, bacteria are poorly opsonized with C3b and this attenuates
98 phagocytosis and killing by neutrophils [18]. These activities of Aur were related to its
99 proteolytic activity, and a major effect on degradation of C3 was lost in an Aur deficient
100 strain [18]. In addition, Aur activates prothrombin [19], and the staphopains and V8 act on

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

104

105 MATERIAL AND METHODS

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

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

110 **Proteins.** *S. aureus* cysteine proteases, ScpA and SspB were purified from strain V8-
111 BC10 or 8325-4 culture supernatants using a modified method, originally described in [14,22].
112 The *S. aureus* serine protease V8 (glutamyl-endopeptidase) and the metalloproteinase Aur
113 were purified from culture medium of strain V8-BC10 as described [23]. *S. aureus* serine
114 proteases D (SplD) and E (SplE) were expressed recombinantly in *E. coli* BL21 (DE3;
115 Invitrogen), and purified as described [24]. The purity of proteins was evaluated by SDS-
116 PAGE and their activity was confirmed using specific substrates. The activity of ScpA and
117 SspB was determined by active site titration with E-64 (L-trans-epoxysuccinyl-leucylamide-
118 (4-guanido)-butane) (Sigma-Aldrich), or α -2-macroglobulin (BioCentrum). Before use in any
119 assay, ScpA and SspB were pre-activated for 20 min by incubation in assay specific buffers
120 supplemented with 1-2 mM DTT. Purified complement proteins C3 and C5, and the C5a
121 peptide, were purchased from Complement Technology.

122 **Antibodies.** The following Abs against human antigens were used throughout this study:
123 rabbit polyclonal antibodies (pAb) anti-C1q, -C4c, and -C3d (all from DakoCytomation), goat
124 anti-MBL (R&D Systems), goat anti-C5 (Quidel), goat anti-factor B (FB) (Complement
125 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 MgCl₂ and 5 mM CaCl₂). Cells were incubated with a
141 complement-fixing antibody (amboceptor; Behringwerke; diluted 1:3000 in DGVB⁺⁺ buffer)
142 at a concentration of 10⁹ cells/ml for 20 min at 37 °C. After two washes with DGVB⁺⁺, 5×10⁸
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 MgCl₂, and 10 mM EGTA). Erythrocytes at a concentration of 5 x 10⁸ cells/ml were then

151 incubated for 1 h at 37°C with 2% NHS diluted in Mg-EGTA buffer (150 µl total volume).
152 The assay was modified for Aur, where 4% C1q-depleted human serum diluted in GVB⁺⁺
153 buffer (5 mM veronal buffer [pH 7.3], 140 mM NaCl, 0.1% gelatin, 1 mM MgCl₂, and 5 mM
154 CaCl₂) was used. In both variants of the alternative pathway assay NHS was pre-incubated
155 with different staphylococcal proteases for 15 min at 37 °C. Samples were then centrifuged
156 and the amount of erythrocyte lysis determined spectrophotometrically (405 nm).

157 **Complement activation assays.** Microtiter plates (Maxisorp; Nunc) were incubated
158 overnight at 4°C with 50 µl of a solution containing 2 µg/ml human aggregated IgG
159 (Immuno), 100 µg/ml mannan (M-7504; Sigma-Aldrich), 20 µg/ml zymosan (Z-4250; Sigma-
160 Aldrich) in 75 mM sodium carbonate (pH 9.6), or 10 µg/ml acetylated BSA (AppliChem;
161 acetylated as described [27]) in PBS. Between each step of the procedure, plates were washed
162 four times with 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5). Wells were
163 blocked with 1% BSA in PBS for 2 h at RT. NHS (classical and lectin pathway) was diluted
164 in GVB⁺⁺ buffer and used at a concentration of 2% for measurement of deposition of C1q, 1%
165 for C3b and C4b in the classical pathway; 2% for C3b, C4b, ficolin-2 and ficolin-3 in the
166 lectin pathway, 4% for MBL. For the alternative pathway, 3% NHS in Mg-EGTA (all
167 proteases except Aur) or 4% C1q-deficient serum in GVB⁺⁺ (Aur) were used for the
168 deposition of C3b, FB and C5. These concentrations were chosen on the basis of initial
169 titrations. The serum used was mixed with various concentrations of different staphylococcal
170 proteases, pre-incubated for 25 min (NHS) or 15 min (C1q-depleted serum) at 37°C and
171 incubated in the wells of microtiter plates for 45 min at 37°C for C1q and MBL, 20 min at
172 37°C for C3b and C4b (classical and lectin pathway), and 35 min for C3b, FB and C5
173 (alternative pathway). Complement activation was assessed by detecting deposited
174 complement factors using specific Abs against C1q, C4b, C3d, FB, C5, MBL, ficolin-2, and
175 ficolin-3, each diluted in blocking buffer. Bound Abs were detected with HRP-labeled anti-

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

179 **Deposition of C1q on bacteria.** *Staphylococcus epidermidis* CCUG 3709 and *S. aureus*
180 8325-4 were grown in TSB overnight, harvested by centrifugation, washed once in PBS,
181 adjusted to an OD₆₀₀ of 1.0, and incubated with 10 μM CFSE (Sigma-Aldrich) for 20 min in
182 the dark. After incubation, bacteria were washed once and adjusted to an OD₆₀₀ of 0.6 in
183 GVB⁺⁺. NHS (6%) was treated with different concentrations of Aur and V8 for 25 min at
184 37°C, after which time 80-μl aliquots of these samples, were mixed with an 80-μl solution of
185 bacteria, and incubated for 45 min at 37°C (3% NHS final concentration). Thereafter, cells
186 were washed twice in FACS buffer (50 mM HEPES, 100 mM NaCl [pH 7.4], 1% BSA, and
187 30 mM NaN₃). C1q deposition was assessed by incubation of cells with rabbit anti-human
188 C1q F(ab)₂ fragments conjugated with DyLight 633 for 45 min. The geometric mean
189 fluorescence intensity (GMFI) of DyLight 633 was calculated for 25000 CFSE-positive cells
190 using FlowJo software (Tree Star).

191 **Degradation assays.** C3 and C5 (0.2 μM each) were incubated with *S. aureus* proteases at
192 concentrations ranging from 0.06 to 2 μM. Incubations were carried out for 2.5 h in 50 mM
193 HEPES (pH 7.4), 150 mM NaCl, and 5 mM CaCl₂ buffer at 37°C. Proteins were separated by
194 SDS-PAGE electrophoresis using standard Laemmli procedures, and 12% gels. Prior to
195 electrophoresis, samples were boiled for 5 min at 95°C in a reducing sample loading buffer
196 containing 25 mM DTT and 4% SDS. Separated proteins were visualized by staining with
197 silver salts.

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

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

212 Chemotactic activity was measured in a disposable 96-well cell migration system with 3-
213 μm polycarbonate membranes (ChemoTx; Neuro-Probe). Serial dilutions of proteases were
214 incubated with 4% heat-inactivated human plasma (the same as for neutrophil suspensions)
215 for 30 min at 37°C, and thereafter applied to the wells of the ChemoTx microplate. Purified
216 human C5a (Complement Technology) at 12.5 nM, diluted in 4% heat-inactivated human
217 plasma, served as a positive control, whereas plasma alone, proteases (at maximal
218 concentration used in the samples with plasma) diluted in PBS, and PBS alone, were used as
219 negative controls. A volume of 50 μl of 1.0×10^7 neutrophils/ml in 4% heat-inactivated human
220 plasma was applied to each well of the filter top. The microplate was incubated for 60 min at
221 37°C in humidified air with 5% CO₂, before the membrane was removed. Samples were
222 transferred to a new flat-bottom 96-well plate (Sterilin) and mixed with 30 μl cell lysis buffer
223 (0.5% hexadecyl trimethyl ammonium bromide [Sigma-Aldrich] in PBS). Similarly, 30 μl of
224 cell lysis buffer was added to all wells of the emptied ChemoTx microplate. Both plates were
225 incubated for 30 min at room temperature, and subsequently the solutions from corresponding

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

229 **Whole blood killing assay.** *S. aureus* strains (Table 1) were grown overnight in 10 ml of
230 tryptic soy broth. Bacteria were harvested for 5 min at 3000 x g, and the culture supernatants
231 were collected for subsequent use to make bacterial suspensions for the assay. Bacteria were
232 re-inoculated to the respective supernatants at an OD₆₀₀ of 0.15 for the 8325-4 wild-type
233 strain (1.0×10^8 CFU/mL) and its mutants, or an OD₆₀₀ of 0.4 (0.5×10^8 CFU/ml) for strain
234 RN6390 and its mutant. Forty μ l of such cultures were mixed with 360 μ l freshly collected
235 human blood anti-coagulated with Refludan (Pharmion), a recombinant hirudin anticoagulant
236 that does not affect complement activation [29], and incubated at 37°C for 20 min. After
237 incubation, aliquots were removed, serially diluted and plated onto tryptic soy agar. Bacterial
238 survival was calculated via colony enumeration.

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

242

243 RESULTS

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

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

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

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

276 For the cysteine protease ScpA (Fig. 2-4, left panels: “Cysteine proteases”), we found that
277 in the classical pathway, the deposition of C1q was decreased by up to 40% in the presence of
278 1 μ M of this enzyme (Fig. 2A). Consequently, deposition of C4b (Fig. 2B) and C3b (Fig. 2C)
279 was also decreased by >50% at 1 μ M. ScpA also attenuated the lectin pathway as it inhibited
280 the deposition of all three collectins: MBL (Fig. 3A), ficolin-2 (Fig. 3B) and ficolin-3 (Fig.
281 3C), as well as all of the ensuing complement factors, such as C4b (Fig. 3D) and C3b (Fig.
282 3E). Surprisingly, in the alternative pathway, ScpA caused a significant increase in the
283 deposition of C3b (Fig. 4A), whereas deposition of FB (Fig. 4B) and C5 (Fig. 4C) were
284 relatively unaffected.

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

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

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

305 The metalloproteinase Aur (Fig. 2-4, right panels: “Metalloprotease”), like SspB and V8,
306 caused enhanced deposition of the classical pathway initiator, C1q (Fig. 2G), and then
307 inhibited deposition of C4b (Fig. 2H) and C3b (Fig. 2I) at a relatively low concentration (350
308 nM). In the lectin pathway, we found that Aur, like ScpA and V8, decreased the deposition of
309 MBL, ficolin-2 and ficolin-3 (Fig. 3K -M), which was followed by a decrease in C4b (Fig.
310 3N) and C3b deposition (Fig. 3O). Surprisingly, however, there was a significant deposition
311 of C3b in the alternative pathway in the presence of Aur (Fig. 4G), while the deposition of FB
312 and C5 was efficiently decreased (Fig. 4H-I). The data regarding C3b deposition via the
313 classical and lectin pathways are in agreement with previously published findings [18].
314 However, we do see the inhibition of these pathways upstream to C3b, already at the level of
315 C4b formation, which is in contrast with the statement in the previous study [18], where the
316 authors did not observe such inhibition.

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

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

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

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

376 **Expression of proteases by *S. aureus* contributes to enhanced survival in whole**
377 **human blood.** In order to verify the effect of proteases on the survival of *S. aureus* in human
378 blood, we studied the survival of strains lacking different proteases compared to the wild type.
379 For this purpose *S. aureus* strains (Table 1) grown in tryptic soy broth overnight (under
380 conditions that yielded the highest detectable proteolytic activity in the medium of wild type
381 strains (data not shown)) were incubated for 20 min at 37°C in fresh human blood, and the
382 survival was assessed by colony counting from serial dilutions (Fig. 7B). Mutant strains of
383 8325-4 lacking different proteases, or combinations of proteases, showed reduced survival
384 compared to the wild type strain; indicating the involvement of proteolytic enzymes in
385 resistance to killing by human blood. This decreased survival was significant for all mutants,
386 with the most significant effect observed for those lacking Aur. Strains lacking ScpA in
387 RN6390 did not show a significantly different survival compared to the wild-type.

388

389

390 **DISCUSSION**

391 The role of *S. aureus* proteases in the virulence of this bacterium has been documented in
392 numerous studies, showing that they are able to interact with host defense mechanisms and
393 tissue components. In the current study we demonstrate that four major proteases of *S. aureus*
394 provide a powerful strategy for defense against complement. Importantly, the protease genes
395 are highly conserved among clinical *S. aureus* strains, although under *in vitro* conditions,
396 down-regulation of their expression has been observed in some clinical isolates [30]. In
397 contrast, *S. aureus* grown in serum significantly increases the production of proteases [31].
398 The major control of expression and activity of extracellular proteases, similarly to other
399 secreted virulence factors of *S. aureus*, is based on the interplay of two global regulators.
400 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

426 specifically shown to block biofilm formation and nasal colonization by *S. aureus* [41].
427 Perhaps *S. aureus* protease-induced deposition of C1q on the surface of *S. epidermidis* could
428 render it more vulnerable to opsonophagocytosis, resulting in its eradication, leaving the
429 niche free for pathogen colonization. This hypothesis is further supported by the fact that the
430 same proteases did not increase C1q deposition on *S. aureus* itself, but rather seemed to limit
431 the opsonisation of the pathogen with C1q (at least Aur ;Fig. 5 C). The role of C1q in the
432 phagocytosis of bacteria, independently of C3b, has been demonstrated for several species
433 [42,43]. Considering that consumption of C3 in the fluid phase due to Aur has previously
434 been shown [18], this may remain the primary mechanism. Taking into account the vital role
435 of C1q in the nonphlogistic clearance of apoptotic cells, an attractive hypothesis emerges,
436 whereby *S. aureus* promotes the uptake of commensal species without boosting the
437 inflammatory response.

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

451 [15]. The detrimental effects mediated by staphopain-induced C5a can be now added to this
452 scheme.

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

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

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

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

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

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

536

537 **ABBREVIATIONS**

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

548

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560

561 **DISCLOSURE STATEMENT**

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

563

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732

733 **FIGURE LEGENDS**

734 **FIG 1 Staphylococcal proteases diminish the hemolytic activity of human serum. A-C)**

735 Classical pathway. NHS (1%) was supplemented with various concentrations of proteases
736 and pre-incubated for 30 min at 37°C, after which time sheep erythrocytes sensitized with
737 antibodies and diluted in GVB⁺⁺ were added. **D-F)** Alternative pathway. 2% NHS in Mg-
738 EGTA (**D, E**) or C1q-depleted human serum (4%) in GVB⁺⁺ (**F**) were pre-incubated with
739 increasing concentrations of proteases for 15 min at 37°C. Serum was then added to sheep
740 (**A-C**) or rabbit (**D-F**) erythrocytes diluted in their respective buffers. **A-F)** After 1-h
741 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-depleted 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

791 neutrophil-associated myeloperoxidase. PBS and proteases alone were used as negative
792 controls, and human C5a (12.5 nM) was the positive control. Absorbance obtained for the
793 highest migration in the assay, observed with the positive control, was set as 100%. An
794 average of three independent experiments is presented with error bars indicating SD.
795 Statistical significance was determined using a one-way ANOVA and a Dunnett post-test (*p
796 < 0.05, **p < 0.01, ***p < 0.001), and calculated compared to untreated plasma (0 μM
797 proteinase). **B)** *S. aureus* strains 8325-4 (WT), 8325-4 *sspABC*⁻, 8325-4 *sspBC*⁻, 8325-4 *aur*⁻,
798 RN6390 (WT) and RN6390 *scpA*⁻ were incubated for 20 min at 37°C with freshly collected
799 human blood. After incubation, aliquots were removed, serially diluted, and plated on tryptic
800 soy agar plates. Survival was calculated as percent survival compared to the inoculum.
801 Statistical significance of the observed differences between wild-types and corresponding
802 mutant strains was determined using a one-way ANOVA and a Dunnett post-test; *p < 0.05,
803 **p < 0.01, ***p < 0.001.

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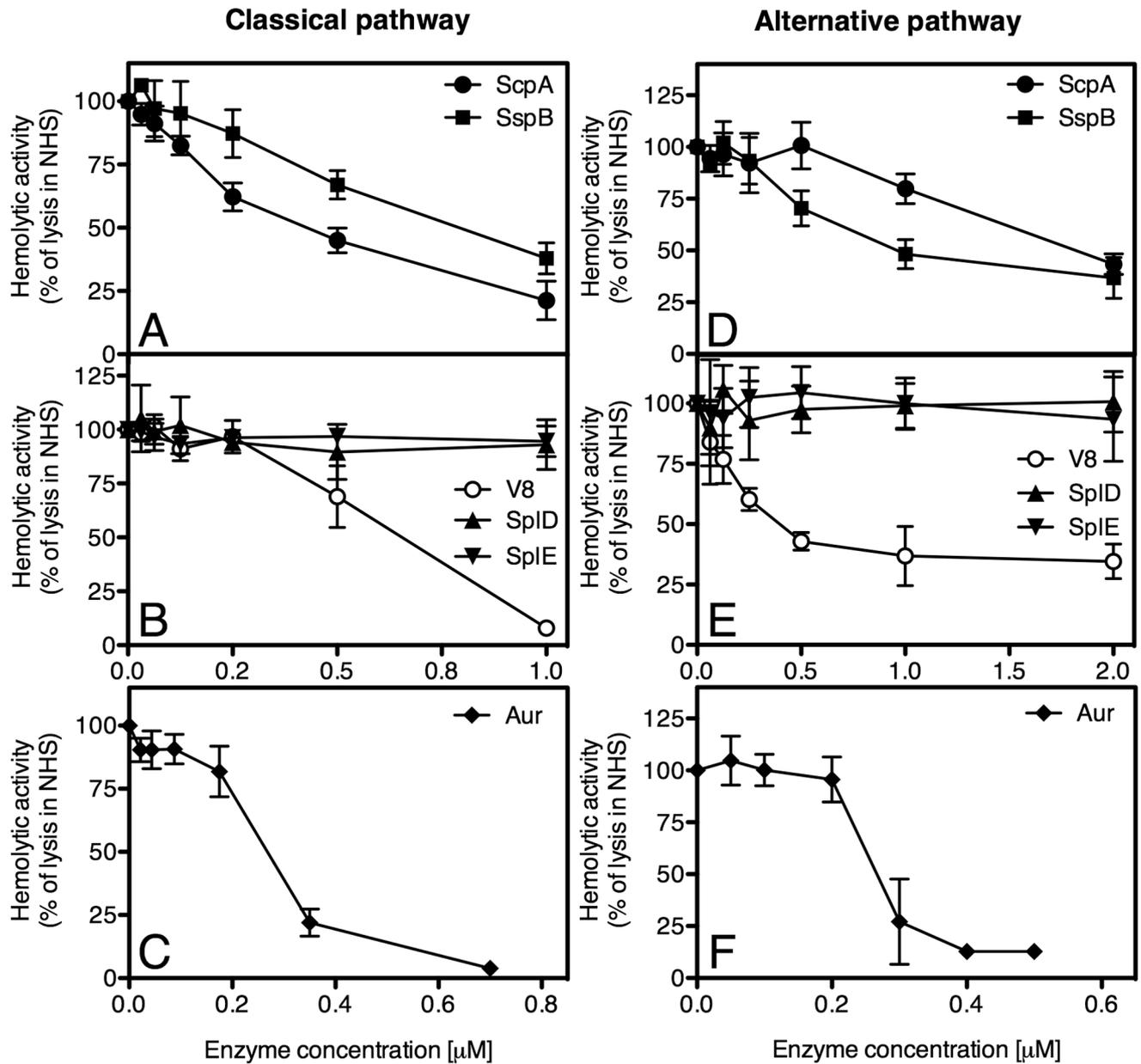
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824 **TABLE 1** Description of bacterial strains used in this study.

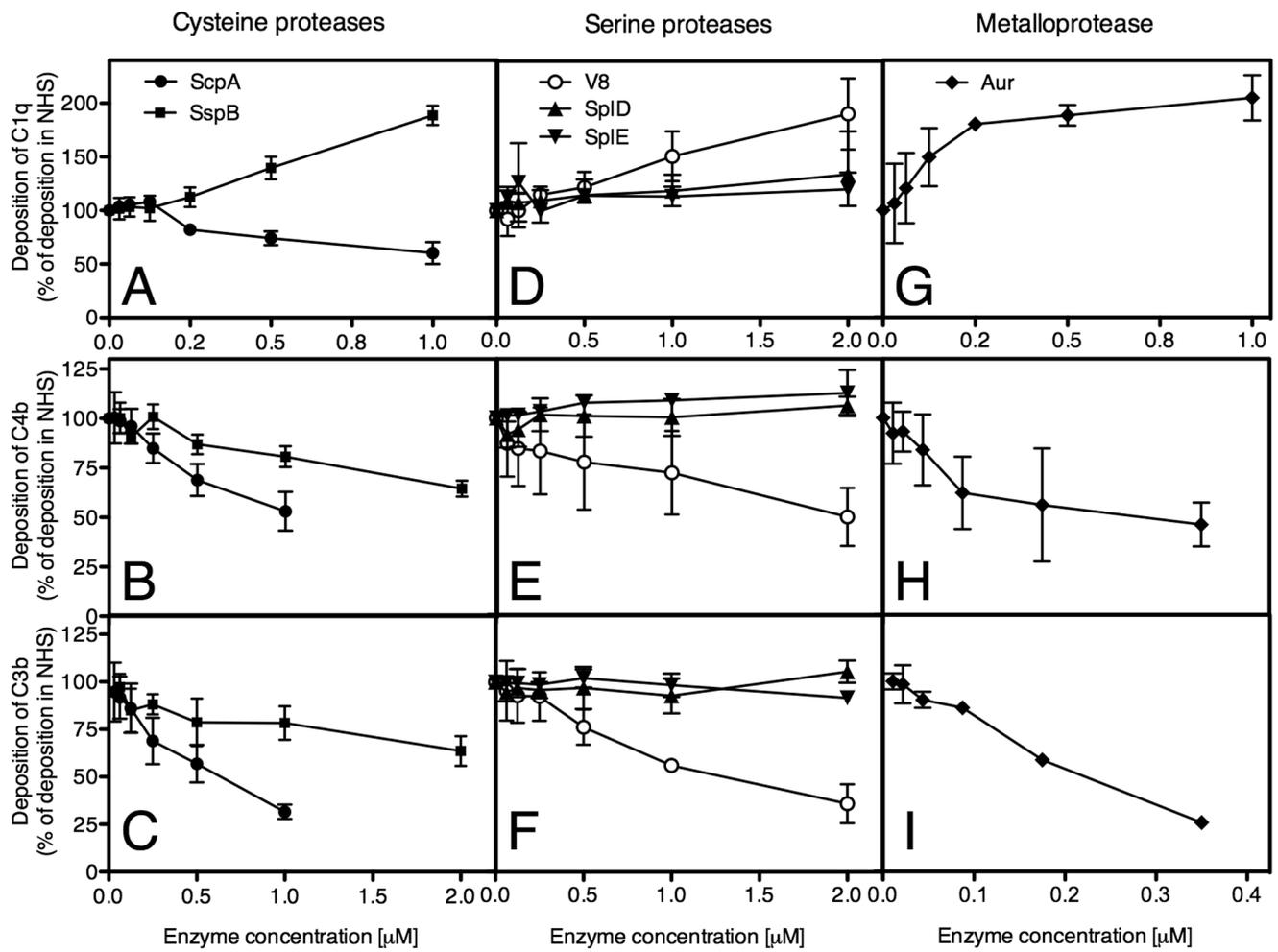
Bacterial strain	Description	References or source
8325-4	<i>S. aureus</i> WT laboratory strain	laboratory stocks
8325-4 <i>aur</i> ⁻	<i>S. aureus aur</i> mutant, no expression of Aur metalloproteinase	[9]
8325-4 <i>sspABC</i> ⁻	<i>S. aureus sspABC</i> mutant, no expression of V8 serine protease, SspB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

RN6390 <i>scpA</i> ⁻	<i>S. aureus scpA</i> mutant, no expression of ScpA cysteine protease	This study
CCUG 3709	<i>S. epidermidis</i> WT laboratory strain	Culture Collection, University of Göteborg, Sweden

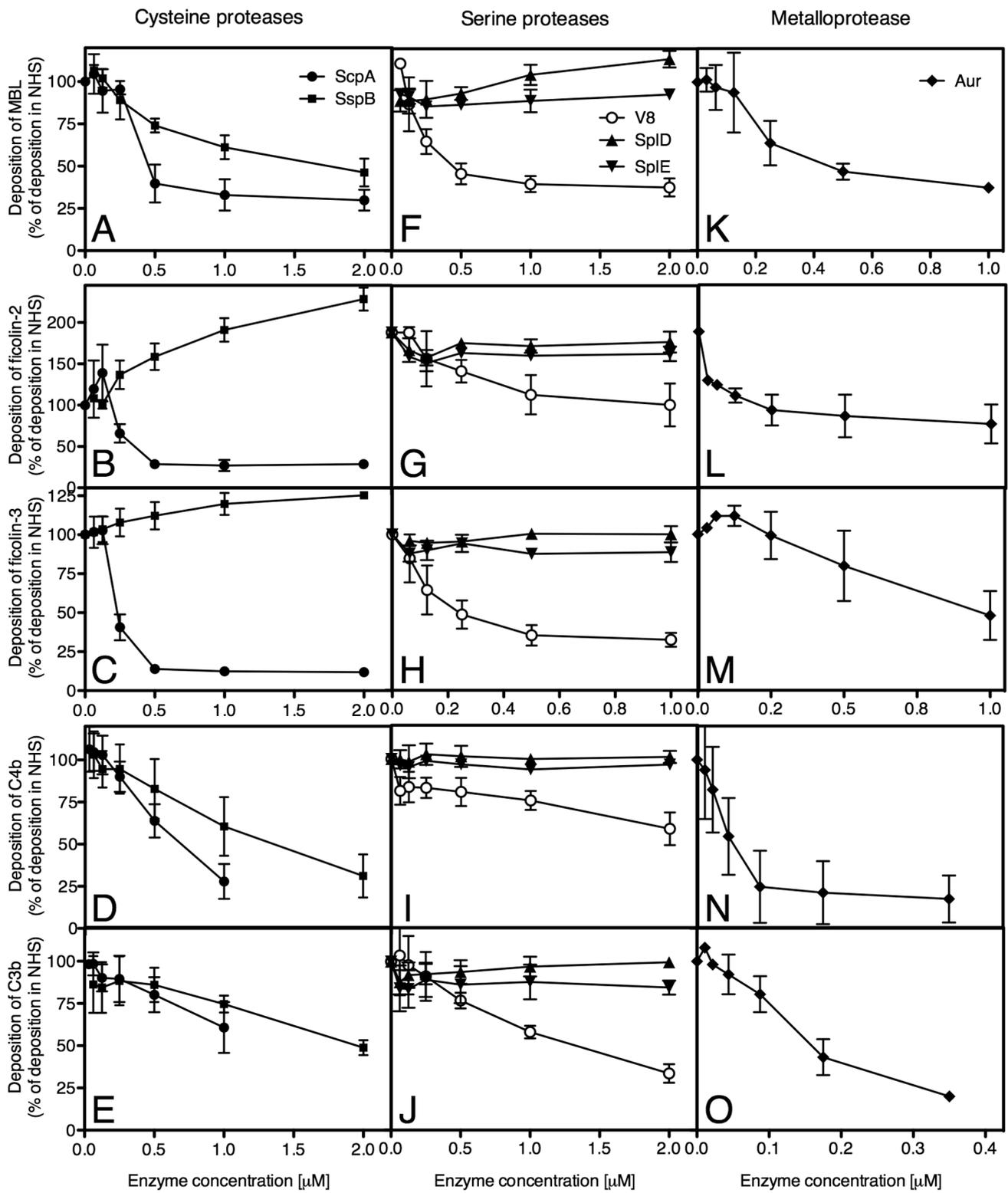
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Classical pathway



Lectin pathway



Alternative pathway

