Plasmin-Mediated Processing of Clumping Factor A Impacts Bacterial, Aggregation and Abscess Formation during *Staphylococcus aureus* Infection

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Abstract

The interface between pathogenic bacteria and the host plays a critical role during the progression of infection. Bacterial surface components, such as the *Staphylococcus aureus* cell wall-anchored protein clumping factor A (ClfA), interact with host factors to promote pathogenesis. ClfA promotes bacterial aggregation in plasma by binding to fibrinogen. Here, we show that the host serine endopeptidase plasmin cleaves ClfA, disrupting its interaction with fibrinogen and promoting bloodstream survival and virulence in *S. aureus*. We found that plasmin cleaves ClfA between residues Arg214 and Ala215. Cleavage occurs in human serum regardless of whether plasmin becomes activated by host tissue-plasminogen activator or by the activity of the *S. aureus* enzyme staphylokinase. Expressing a plasmin-resistant form of ClfA, and a truncated form that mimics the plasmin-cleaved product on the bacterial cell surface, revealed that following cleavage ClfA cannot mediate aggregation of *S. aureus* in fibrinogen. While truncated ClfA on the cell surface cannot bind to fibrinogen, it promotes the establishment of renal abscesses *in vivo*. Collectively, our findings suggest that activation of plasmin during infection leads to site-specific processing of ClfA, and this not only abrogates fibrinogen binding but also facilitates enhanced *S. aureus* persistence in the host. Thus, we demonstrate that proteolytic processing by plasmin is a mechanism for modulating the host-pathogen interaction during infection.

Introduction

Staphylococcus aureus bacteraemia is among the most common and serious bacterial infections affecting the global population (1). *S. aureus* was the leading bacterial cause of death in 135 countries in 2019 (2), and more than 300,000 people die each year as a direct result of *S. aureus* bacteraemia (3). Complications arise when *S. aureus* evades host immune defences and disseminates to organs and tissues giving rise to metastatic infections such as infective endocarditis, septic arthritis, and infections associated with implanted medical devices. These infections increase morbidity and mortality, often requiring prolonged treatment and, in some cases, surgical intervention.

One of the key virulence factors of *S. aureus* is clumping factor A (ClfA), a cell wall-anchored microbial surface component recognising matrix molecules (MSCRAMM) protein that plays a central role during bacteraemia, septic arthritis and infective endocarditis through fibrinogen-dependent and independent mechanisms (4-10). ClfA binds to fibrinogen, a glycoprotein present in blood plasma, promoting the aggregation of *S. aureus* in plasma. However, fibrinogen also functions in host defense by activating neutrophils and macrophages, as well as trapping bacteria in matrices and inhibiting dissemination (11-15). Fibrinogen-deficient mice have reduced ability to clear *S. aureus* from the peritoneal cavity following infection and disruption of this *S. aureus*-fibrinogen interaction inhibits dissemination from the peritoneum during acute infection (16).

ClfA binds to the extreme C-terminus of the gamma chain of fibrinogen. The C-terminal residues of the gamma chain bind in a trench formed between the separately folded adjacent immunoglobulin G-like domains N2 and N3 domains by the 'dock, lock, and latch' mechanism (17-19). Docking of the peptide induces a conformational change in the flexible extension at the C-terminus of the ClfA N3 domain, which redirects to cover the bound fibrinogen peptide locking it in place. In doing so it forms an β-strand which is complementary to a β-sheet in the N2 domain. The D domain of fibrinogen establishes further interactions with another site on the surface of the ClfA N3 domain (20). The overall stability of the ClfA-fibrinogen complex is enhanced by shear stress (21). ClfA has also been reported to bind to the host protein factor I, facilitating cleavage of complement opsonin C3b into iC3b to inhibit

76 opsonophagocytosis (10, 22). However, no studies have yet detailed the specific
77 mechanism or binding site involved.

78 There is growing evidence that host-derived proteases can alter the *S. aureus*
79 cell surface, potentially impacting its interactions with the host during infection (23-25).
80 The host serine protease plasmin is particularly relevant in the context of infection due
81 to its activation at sites of tissue damage and inflammation (26, 27). Notably, *S. aureus*
82 produces staphylokinase (Sak), a secreted protein that activates host plasminogen to
83 its proteolytically active form, plasmin (28). This interaction suggests a potential
84 mechanism by which *S. aureus* may exploit the host fibrinolytic system to remodel its
85 own cell surface. This study set out to determine if ClfA is susceptible to plasmin and
86 whether plasmin can modulate the biological activity of ClfA.

87 **Materials and Methods**
88
89 **Reagents, Strains, Media, and Growth Conditions.** All strains and plasmids used
90 in this study are listed in Table 1. If not specified otherwise, *S. aureus* strains were
91 cultured on tryptic soy agar (TSA) plates for isolation of individual colonies or for liquid
92 cultures in either tryptic soy broth (TSB) (Difco) at 37°C with shaking (200 rpm).
93 *Escherichia coli* strains were grown at 37°C on lysogeny Agar (LA) or in lysogeny
94 Broth (LB) with shaking (200 rpm) for liquid cultures. Where appropriate, antibiotics
95 were incorporated into media; chloramphenicol (Cm,10 µg/mL), erythromycin (Em, 10
96 µg/mL), ampicillin (Amp, 100 µg/mL); anhydrotetracycline (Atc, 1 ng/mL) (Sigma).
97
98 **Plasmin treatment** Bacteria were grown to stationary phase, then pelleted by
99 centrifugation (7 min, 8000 x *g*), washed with an equal volume of PBS once and
100 pelleted again. Samples were then resuspended in 1 mL PBS and the OD_{600} was read.
101 Bacteria were adjusted to a desired OD_{600} for the assay. Human derived plasmin
102 (Sigma) was then added to the sample to a final concentration of 0.075 U/mL and
103 incubated at 37°C for 1 h in a water bath. Samples were then washed with an equal
104 volume of PBS three times before a final reconstitution to original volume. The
105 protease inhibitor alpha-2-Macroglobulin (α2M, Sigma) was added where indicated at
106 a concentration of 10x that of plasmin used. Recombinant proteins were typically
107 diluted to a concentration of 5 µM in sterile PBS to which plasmin was added to a final
108 concentration of 0.075 U/mL and incubated at 37°C for 1 h in a water bath.
109
110 **Preparation of murine blood and serum.** Whole blood was collected from female
111 C57BL6/JCR mice and was allowed to clot overnight at 4°C. Serum was then
112 harvested from clotted blood by centrifugation at 1100 x g for 11 min. To catalyse the
113 activation of plasminogen to plasmin in human serum, 40% serum was incubated with
114 tissue plasminogen activator (DiaPharma, 25 ug/mL) and *S. aureus* supernatant for
115 20 min at 37°C before being used in the treatment of recombinant proteins as outlined
116 above.
117
118 **SDS-PAGE and Western immunoblotting.** Cell wall extracts were carried out as
119 described by O'Halloran et al., 2015 (29)**.** Briefly, bacteria grown to exponential phase
120 (OD600 = 0.35 – 0.5) or stationary phase (overnight culture) were pelleted and washed

121 with PBS, then adjusted to an OD600 = 10. Bacteria were then incubated with or
122 without plasmin (0.075 U/mL) in a 37°C waterbath for 1 h before being centrifuged and
123 resuspended in 250 µL extraction solution (30% w/v Raffinose, 50 mM Tris-HCL, 20
124 mM MgCl2, pH 7.5, 50 µl/ml complete protease inhibitor [Roche], lysostaphin 10 U/ml)
125 for 8 min at 37°C. Protoplasts were removed by centrifugation at 15000 x *g* for 5
126 minutes.
127 Protein samples were separated by polyacrylamide gel electrophoresis (7.5%,
128 10% or 12.5%). Gels were either stained using Instant Blue (Expedeon) or Gel code
129 (ThermoFisher Scientific) or transferred to nitrocellulose (GE Healthcare) or
130 polyvinylidene difluoride (PVDF, Roche) filters for western immunoblotting.
131 Filters were blocked with 10% (w/v) skimmed milk (Marvel) and then probed
132 with the indicated antibodies**.** Reactive bands were developed using LumiGLO
133 Reagent and peroxide detection solution (Cell signalling Technology) and imaged
134 using the ImageQuant TL (GE Healthcare).
135
136 **DNA manipulation**
137 **Isolation of DNA.** Genomic DNA was isolated using the PureElute Bacterial
138 genomic DNA Purification kit (Edge Biosystems), with the addition of lysostaphin (100
139 µg/ml) to the cell lysis buffer to digest *S. aureus* cell wall peptidoglycan to improve
140 DNA extraction. Plasmid DNA was isolated using Wizard® Plus SVMiniprep kit
141 (Promega) or Qiagen® Plasmid plus Midi-kit
142
143 **Plasmid construction.** Plasmids were constructed by inverse PCR or via sequence
144 and ligase independent cloning (51).Plasmids pQE30::ClfA^{R}40-559* and
145 pRMC2::ClfA^{R} were constructed by sequence and ligase independent cloning (31)
146 where vectors were amplified by Velocity PCR and the insert was amplified from a
147 region of DNA commercially synthesised as a gBlock (IDT). Primers used in this study
148 are listed in Table 2. Plasmids pQE30::ClfAΔ40-214_cSt and pRMC2::ClfAΔ40-214
149 were generated by inverse PCR. Inverse PCR products were treated with DpnI and
150 blunt-end ligation was carried out using the LigaFast® Rapid RNA ligation system
151 (Promega). Chemically competent *E. coli* (32) XL-1 Blue were transformed with the
152 ligation products. Plasmids were then isolated and used to transform *E. coli* Topp3 for
153 protein purification or IM08B (33) for transformation of *S aureus* made
154 electrocompetent as described previously (34). Colony PCRs were carried out to

155 screen for transformants using Phire Hot Start DNA Polymerase (Sigma) as per the
156 manufacturer's instructions.
157
158 **Expression, purification, and modification of recombinant proteins.** Briefly, *E.*
159 *coli* Topp3 carrying plasmids was grown to an OD600 = 0.4 - 0.8, before isopropyl β-
160 D-1-thiogalactopyranoside (IPTG, 1 mM) was added to induce protein expression.
161 Growth was continued for 3 h before cell sedimentation was carried out. The pellet
162 was resuspended in 25 mL Imidazole Buffer (40 mM Tris-HCL, 1 M NaCl, 0.1 M
163 imidazole, pH 7.9) with an EDTA-free protease inhibitor. Bacteria were then lysed
164 using a French Press at 1500 psi before centrifuging the lysates at 18,000 x *g* for 20
165 mins at 4°C. DNase (1 mg/mL) was added to the supernatant before filtration through
166 a 0.45 µm filter (Whatman). Proteins were purified with an N-terminal hexa-histidine
167 (His) affinity tag and a C-terminal Strep-tag II affinity tag for purification via nickel
168 affinity chromatography (5 mL HiTrap affinity column; GE Healthcare) and Strep-
169 Tactin affinity chromatography (Strep-Spin Protein Miniprep kit; Zymo Research)
170 respectively. Protein concentrations were determined by measuring the absorbance
171 at 280 nm using a Nanodrop spectrophotometer and calculations according to Beer-
172 Lambert Law.
173
174 **N-terminal sequencing.** Samples sent for N-terminal sequencing were first incubated
175 with plasmin (0.075 U/mL) as outlined above prior to purification of the digested
176 product by Strep-Tactin affinity chromatography. Purified samples were then
177 subjected to SDS-PAGE, transferred to PVDF membrane and stained with Ponceau
178 S (ThermoScientific). N-terminal sequencing was performed by Edman degradation at
179 Alta Bioscience, Redditch, UK.
180
181 **Synthetic peptide cleavage.** Synthetic peptides used in this study were commercially
182 synthesised by AltaBioscience (Redditch, UK). Peptide size and purity was confirmed
183 by mass spectrometry at AltaBioscience. All peptides were dissolved in molecular
184 grade water to a concentration of 150 µM. As the peptides were 5/6-FAM labelled they
185 were protected from light when in use and stored at -20°C. Synthetic peptides were
186 diluted to a concentration of 37.5 µM in molecular grade water, then incubated with
187 human plasmin (0.075 U/mL) at 37°C for 30 min in a water bath. Reaction products

188 were separated by agarose gel electrophoresis (4% w/v) and gel images were
189 captured under UV light.
190
191 **Bacterial clumping in fibrinogen.** Bacteria were grown to stationary phase, then
192 pelleted by centrifugation (8000 x *g,* 7 min). Cells were washed with PBS and then
193 adjusted to OD600 of 1.5 in 1 ml PBS. Purified human fibrinogen depleted of
194 plasminogen, von Willebrand Factor and fibronectin (Enzyme Research Labs) or
195 murine fibrinogen (Innovative Research) was added to the tube to a final concentration
196 of 18.4 µg/mL and left to stand at 37°C. After incubation for 1 h 45 min, the top 0.5 ml
197 was removed from the tube, diluted 1:2 in PBS for OD600 measurements (Genesys
198 10 UV, Thermo Spectronic) and computed as a percentage of the starting OD600
199 where (100/T0_OD600)*T105_OD600. For every *S. aureus* strain, there was a
200 fibrinogen deficient control. In cases where plasmin treated bacteria were included, on
201 adjustment to an OD600 1.5 the bacteria were incubated with plasmin (0.075 U/mL)
202 for 1 h at 37°C. Following three washes in PBS, the bacteria were resuspended to the
203 original volume in BHI and made up to a final volume of 1 mL with PBS. Fibrinogen
204 (18.4 ug/mL) was then added, and the protocol proceeded as outlined above. In each
205 case for plasmin-treatment, a no plasmin control for each strain was included.
206
207 ***Ex vivo* whole blood survival assay.** The quantification of survival of *S. aureus* in
208 human blood was performed according to the protocol previously described in
209 Zapotoczna et al., 2018 (35). Briefly, *S. aureus* was grown overnight in RPMI then
210 adjusted to 2 x 10^4 cfu/mL. 25 µL was added to 475 µL of freshly drawn EDTA-treated
211 blood obtained from human volunteers. Immediately following inversion, 100 µl of each
212 sample was added to 900 µl ice-cold endotoxin free water. This was the T0 sample.
213 The tubes were then incubated at 37°C on a 360° rotation wheel and after 3 h, serial
214 dilutions were made to quantify CFU/ml. In parallel, bacteria was incubated in serum
215 derived from the same donor. Percentage survival was calculated using the following
216 formula (T3 mean cfu/ml)/(T0 cfu/ml)*100.
217
218 **Mouse model of *S. aureus* bloodstream infection.** Female NMRI, aged 9-10 weeks,
219 were obtained from Envigo (Venray, Netherlands) and housed at the animal facility at
220 the University of Gothenburg. Mice were maintained under standard temperature and
221 light conditions, with unrestricted access to laboratory chow and water. *S. aureus*

222 strains were cultured for 24 hours on horse blood agar plates and then preserved
223 according to established protocols (37). Before each experiment, the bacterial
224 solutions were thawed, washed, and adjusted to the desired concentration. To
225 investigate the clinical relevance of ClfA plasmin cleavage, we used a well-established
226 *S. aureus* sepsis mouse model (38). NMRI mice (10 per group) were intravenously
227 (i.v.) injected via the tail vein with 200 µl of PBS containing 75 ng/ml
228 anhydrotetracycline and the respective *S. aureus* strain Newman *clfA5* expressing
229 pRMC2 variants: ClfA, $ClfA^R$ or $ClfA_{\Delta 40\text{-}214}$ (6×10^6 colony-forming units [CFU]/mouse).
230 Expression of ClfA was induced via an intraperitoneal injection of inducer
231 anhydrotetracycline (5 µg/mouse) on Day 0, 1 h before infection and 1h post-infection.
232 The mice were monitored individually two times daily by an observer (Z.H). The blood
233 samples were collected on both day 3 and day 10 for subsequent Interleukin 6 (IL-6)
234 level analysis. Surviving mice were sacrificed on day 10, the blood, kidneys and livers
235 were aseptically collected.
236 **Quantification of bacterial load in kidneys and livers.** Kidneys and livers
237 were collected aseptically, and kidney abscess scores were assessed by two
238 investigators (T.J. and Z. H.) in a blinded manner (39). The scoring system used
239 ranged from 0 to 3 (0 indicates healthy kidneys; 1, 1–2 small abscesses in the kidneys
240 without structure changes; 2, >2 abscesses, but <75% kidney tissue involved; and 3,
241 large amounts of abscess with >75% kidney tissue involved). The kidneys and livers
242 were homogenized and plated on horse blood agar plates to quantify the CFU counts.
243 **Quantification of Interleukin 6 using enzyme-linked immunosorbent**
244 **assay.** The levels of Interleukin 6 (IL6) in the blood of NMRI mice infected with S.
245 aureus were measured on day 3 and 10 post-infection using a DuoSet ELISA kit (R&D
246 Systems, Abingdon, UK) according to the manufacturer's instructions.
247
248 **Ethical Approval.** Our research complies with all relevant ethical regulations.
249 Informed consent was obtained from all adult participants. Ethical approval for the
250 collection and use of human blood samples from adult volunteers was obtained from
251 the Trinity College Dublin Faculty of Health Sciences Ethics Committee, Ireland
252 (reference 2020111) and through the Human Biomaterials Resource Centre,
253 University of Birmingham, United Kingdom (reference 25/NW/0013). Personal
254 information such as sex and age were not recorded. All participants were over the age
255 of 18 and provided written informed consent. Participants did not receive

256 compensation for their participation. For animal studies conducted in Trinity College
257 Dublin, C57BL/6 mice were bred in-house in the Comparative Medicine Unit, as per
258 College and The Health Products Regulatory Authority (HPRA) welfare guidelines. All
259 animal studies were approved by the TCD Animal Research Ethics Committee (Ethical
260 Approval Number 091210) and HPRA license AE191364/P079. For mouse work, the
261 study was approved by the Gothenburg Ethics Committee of Animal Research (Ethical
262 Approval Number 5.8.18-02443/2021), and all procedures adhered to established
263 guidelines for animal experimentation.
264
265 **Statistical analysis.** Statistical analysis was performed using GraphPad Prism
266 (GraphPad Software, Inc, USA; version 8.0.2.263 and version 10). Unless stated
267 otherwise, the data presented in this study show mean ± standard deviations of at
268 least three independent experiments. Statistically significant differences were
269 calculated by using methods as indicated in the figure legends. P values of less than
270 0.05 were considered significant with *, **, *** and **** representing P values of <0.05,
271 <0.01, <0.001 and <0.0001 respectively. P values of >0.05 represents non-significant
272 values (ns). For experiments with blood or serum, samples from different donors were
273 used for each of the biological replicates.
274
275

276 **Results**

277 **ClfA is cleaved by plasmin.** To determine if cell wall-anchored ClfA was susceptible
278 to plasmin-mediated proteolytic processing in *S. aureus*, bacteria were grown to
279 stationary phase, washed and incubated with plasmin. Cell wall-associated proteins
280 were solubilised from the cell wall using lysostaphin during protoplast formation.
281 Probing cell wall extracts of wild-type *S. aureus* Newman with anti-ClfA IgG in a
282 western blot revealed a ca. 170 kDa immunoreactive band corresponding to full length
283 ClfA (**Fig. 1A**). Incubation with plasmin resulted in the appearance of a ca. 130 kDa
284 band in addition to the ca. 170 kDa band indicating that cell-wall anchored ClfA had
285 been cleaved by plasmin. The broad-spectrum protease inhibitor alpha-2-
286 macroglobulin prevented the activity of plasmin as only the band corresponding to full-
287 length ClfA was detected (**Fig. 1A**). As a control, cell wall extracts were prepared from
288 a mutant deficient in ClfA (Newman *clfA5*). The absence of immunoreactive bands
289 confirmed the specificity of the anti-ClfA IgG (**Fig. 1A**). These results suggested that
290 ClfA on the surface of *S. aureus* Newman can be cleaved by purified plasmin.

291 To explore plasmin cleavage further, three methicillin-resistant *S. aureus*
292 (MRSA) isolates were incubated with plasmin and the integrity of ClfA in cell wall
293 extracts was examined. For each of the isolates, ClfA was cleaved by plasmin as
294 indicated by the disappearance of the ca. 170 kDa band corresponding to full-length
295 ClfA and the appearance of a ca. 130 kDa immunoreactive band following plasmin
296 treatment (**Fig. 1B**). A weak immunoreactive band migrating around 130 kDa was
297 detected in cell wall extracts from LAC that had not been incubated with plasmin. This
298 was likely due to the activity of the *S. aureus* extracellular protease aureolysin that
299 cleaves at the junction of N2 and N3 of ClfA (40). In summary, these findings
300 demonstrate that plasmin cleaves ClfA on the surface of *S. aureus*.

301 Recognition by an antibody specific for ClfA domains N1N2N3 (**Fig. 1**)
302 suggested that ClfA undergoes cleavage towards the N-terminus, leaving a portion of
303 the N1N2N3 domains intact and anchored to the cell wall. To examine this further, a
304 recombinant protein corresponding to the N1N2N3 domains of ClfA with an N-terminal
305 hexahistidine tag and a C-terminal Strep-tagII was purified from *E. coli* ($ClfA_{40\text{-}559*}$).
306 $ClfA_{40\text{-}559*}$ migrates aberrantly with an apparent molecular mass of ca. 95 kDa (**Fig.**
307 **2A**) due to the presence of the elongated unfolded N1 region (41). Incubation of $ClfA_{40\text{-}}$
308 $_{559*}$ with plasmin resulted in the conversion of the ca. 95 kDa band to a single product
309 of ca. 43 kDa. The remainder of the protein could not be detected (**FIG. 2A, B**)

310 suggesting that it was digested into smaller fragments. ClfA$_{40-559*}$ remained mostly
311 intact when the protease activity of plasmin was inhibited by alpha-2-macroglobulin
312 (**FIG. 2A, B**) confirming that cleavage of ClfA$_{40-559*}$ was due to the proteolytic activity
313 of plasmin.

314 To determine if the single band remaining following plasmin treatment retained
315 the N-terminal and/or C-terminal tags, the same samples were probed with anti-His
316 IgG to detect the N-terminal hexa-histidine tag (**FIG. 2C**) or anti-Strep tagII IgG to
317 detect the C-terminal strep tag (**Fig. 2D**). While a band corresponding to full-length
318 ClfA$_{40-559*}$ was detected in samples without plasmin or when plasmin was inhibited with
319 alpha-2-macroglobulin, using anti-His IgG, no band was detected after the addition of
320 plasmin (**FIG. 2C**). These data suggest that the hexa-histidine tag, and therefore the
321 N-terminal region of the recombinant protein, is removed by plasmin cleavage. In
322 contrast, the plasmin-cleaved product reacted with anti-Strep tag II IgG (**FIG. 2D**)
323 indicating that the C-terminus of the protein was intact. Other bands detected in lanes
324 1 and 3 (**FIG. 2B, D**) are likely to be ClfA breakdown product(s) that were already in
325 the sample or formed during the incubation process.

326 To elucidate if ClfA can be cleaved by activated plasmin in human serum,
327 ClfA$_{40-559*}$ was incubated in serum with or without added tissue plasminogen activator
328 (tPA) which catalyses activation of the zymogen plasminogen (**Fig. 2E**) or with
329 concentrated culture supernatants containing the secreted *S. aureus* plasminogen
330 activating-enzyme Sak (**Fig. 2F**). Full length ClfA$_{40-559*}$ was detected following
331 incubation in serum, but only a band corresponding to cleaved ClfA was detected
332 when plasmin was activated (**Fig. 2E**). The addition of alpha-2-macroglobulin
333 protected full-length ClfA from cleavage. The presence of some truncated protein in
334 the absence of tPA or when plasmin activity was inhibited by alpha-2-macroglobulin
335 indicates that this species was present in the protein preparation at the onset of the
336 experiment or developed independently of plasmin activation. No immunoreactive
337 bands were detected in serum only controls confirming the specificity of the polyclonal
338 antibody for ClfA. Full length ClfA$_{40-559*}$ was detected following incubation in serum
339 treated with concentrated supernatants from an *S. aureus* mutant deficient in *sak*
340 (LAC* *sak*::Tn) but not when ClfA was incubated with supernatants from wild-type
341 LAC* (**Fig. 2F)**, indicating that plasmin was activated by Sak and cleaved ClfA (**Fig.
342 2F**). Taken together, these findings indicate that purified human plasmin, or plasmin
343 activated by the host or bacterium during infection is capable of cleaving ClfA.

344

345 **Plasmin cleaves ClfA between residues 214 and 215.** To identify the plasmin
346 cleavage site, $ClfA_{40-559*}$ was incubated with plasmin and the cleaved protein was
347 purified by Strep-Tactin affinity chromatography. The 7 most N-terminal amino-acids
348 of the cleaved product were identified using Edman degradation, revealing that
349 plasmin cleaves the peptide bond after $Arg_{214,}$ towards the end of the N1 domain (**Fig.**
350 **3A**). To verify the plasmin cleavage site experimentally, an 11-mer fluorescein labelled
351 peptide comprising ClfA residues 211-221, was incubated with plasmin and analysed
352 by agarose gel electrophoresis. The 11-mer peptide was chosen based on the
353 evidence from N-terminal sequencing that plasmin would cleave between residues
354 214 and 215 resulting in a clear size shift from a fluorescein-labelled 11-mer peptide
355 to a fluorescein-labelled 4-mer. In agreement with this, the 11-mer peptide (WT)
356 underwent complete conversion to a smaller truncate following plasmin treatment (**Fig.**
357 **3B**). Next a series of peptides where amino-acids 211-216 were individually
358 substituted with serine were tested for susceptibility to plasmin cleavage (**Fig. 3B**).
359 Only the R214S substitution affected cleavage with this peptide undergoing partial
360 truncation following plasmin treatment underscoring the importance of residue Arg214
361 for plasmin recognition and/or cleavage. A scrambled version of the 11-mer peptide
362 (S^c) and peptide where the entire plasmin-cleavage site and surrounding residues
363 were substituted (amino-acids 211-214, WT^R) remained intact post-incubation with
364 plasmin. Similarly, recombinant $ClfA_{40-559*}$ harbouring amino-acid substitutions of
365 residues 211-214 ($ClfA^R{}_{40-559*}$) was resistant to plasmin cleavage (**Fig. 3C**). Overall,
366 these data indicate that substitution of residues 211-214 prevents the cleavage of ClfA
367 by plasmin and that R214 is particularly important for optimal cleavage by plasmin.
368 Failure of plasmin to cleave the scrambled peptide indicates that the order of the
369 residues is critical for plasmin to cleave this sequence.

370

371 **Plasmin cleavage disrupts the fibrinogen binding activity of ClfA.** ClfA binds to
372 fibrinogen in human plasma which facilitates clumping of *S. aureus* by way of the
373 formation of fibrinogen bridges between bacteria (4, 42). The ability of ClfA to interact
374 with fibrinogen was studied by incubating *S. aureus* in a solution of fibrinogen and
375 monitoring the optical density at 600 nm of the suspension as the bacteria clump. A
376 suspension of *S. aureus* expressing full-length ClfA (*S. aureus* $ClfA^+$) treated with
377 plasmin to promote processing of ClfA prior to incubation with fibrinogen, underwent

378 significantly less clumping than untreated bacteria (**Fig. 4A**). Conversely, bacteria
379 expressing ClfA carrying amino-acid substitutions in residues 211-214 that render the
380 protein resistant to plasmin cleavage (*S. aureus* ClfA^{R}) did not change clumping
381 phenotype following plasmin treatment (**Fig. 4B**). Together these results demonstrate
382 that the cleavage of ClfA by plasmin reduces the protein's ability to interact with soluble
383 fibrinogen and promote clumping.
384 Next the ability of *S. aureus* expressing a ClfA truncate that mimics the plasmin-
385 cleaved state, (*S. aureus* ClfA$^{\Delta 40\text{-}214}$), was tested for its ability to clump in a solution of
386 fibrinogen (**Fig. 4C**). *S. aureus* ClfA$^{\Delta 40\text{-}214}$ lost the ability to clump (**Fig. 4C**) while *S.*
387 *aureus* ClfA^{+} (**Fig. 4D**) and *S. aureus* ClfA^{R} (**Fig. 4E**) incubated under the same
388 conditions clumped in a solution of fibrinogen. *S. aureus* ClfA^{-} did not clump
389 demonstrating that clumping activity is mediated solely by ClfA under the conditions
390 used here (**Fig. 4F**). Together these results suggest that removal of residues 40-214
391 either by treatment with plasminogen or genetically, renders ClfA incapable of binding
392 to soluble fibrinogen and promoting clumping of *S. aureus*.
393
394 **Truncation of ClfA increases kidney abscess formation by *S. aureus*.** ClfA
395 promotes renal abscess formation in murine infection models (4, 44-46). Having
396 shown that the plasmin-cleaved form of ClfA interacts poorly with soluble fibrinogen
397 (**Fig. 4**), we sought to determine how this might impact *S. aureus* infection in a mouse
398 model. ClfA was not susceptible to cleavage by mouse plasmin in serum (data not
399 shown) so the effect of plasmin on the activity of ClfA *in vivo* was examined using the
400 *S. aureus* expressing full-length ClfA and its variants. *S. aureus* ClfA$^{\Delta 40\text{-}214}$ resulted in
401 more severe abscess formation (**Fig. 5A**) and a higher bacterial load (**Fig. 5B**) in the
402 infected mouse kidneys compared to *S. aureus* ClfA or *S. aureus* ClfA^{R}, indicating that
403 the ability of *S. aureus* to disseminate to the kidney and establish renal abscesses is
404 enhanced when residues 40-214 are missing from ClfA. There was no difference in
405 bacterial load in the liver following infection with any of the strains (**Fig. 5C**).
406 Comparing the survival of mice challenged with *S. aureus* expressing ClfA, ClfA^{R} and
407 ClfA$^{\Delta 40\text{-}214}$ indicated that there were no significant differences in the survival rates of
408 mice infected with any of the three strains over 10 (**Fig. 5D**). Together these data
409 indicate that removal of residues 40-214 and therefore inactivation of the fibrinogen
410 binding function of ClfA enhances the ability of *S. aureus* to form abscesses in mouse
411 kidneys but does not increase progression to lethal infection.

412

413 **Discussion**

414 This study shows that cleavage of ClfA by plasmin results in a pronounced loss
415 of bacterial clumping in fibrinogen, reduced survival of *S. aureus* in human blood and
416 increased abscess formation in the kidney. We pinpointed the plasmin cleavage site
417 to between residues R_{214} and A_{215} in ClfA and demonstrated that the activity of plasmin
418 leads to the formation of a stable truncate of ClfA on the cell surface lacking residues
419 40-214. Substantial evidence supports fibrinogen binding to bacteria conferring
420 protective benefits to the host while conversely, other studies find it can drive
421 pathogenesis. Such contradictions likely reflect model-dependent factors. In the
422 context of this study, loss of fibrinogen binding resulted in impaired bacterial killing,
423 enhanced dissemination and increased abscess formation. This agrees with recent
424 findings showing that mice expressing a fibrinogen truncate lacking the ClfA binding
425 domain, demonstrate delayed bacterial killing and increased dissemination from the
426 peritoneal cavity (16).

427 The proteolytic cleavage of cell wall-anchored proteins in *S. aureus* has been
428 described previously. In some instances, surface-exposed cell wall-anchored proteins
429 are completely degraded by proteases, such as FnBPA and FnBPB. However, in other
430 cases, cleavage at specific sites by bacterial or host proteases serves as a post-
431 translational modification that modulates protein function. For example, ClfB loses its
432 ability to bind fibrinogen following cleavage by aureolysin and SasG no longer inhibits
433 bacterial clumping following proteolytic processing by trypsin. In this study, we
434 demonstrate that removal of residues 40-214 from the mature ClfA protein abolishes
435 its ability to interact with soluble fibrinogen. This finding was unexpected, as the
436 canonical fibrinogen binding domain of ClfA is thought to reside entirely within the N2
437 and N3 domains (residues 229–545). Interestingly, the loss of fibrinogen binding in the
438 Δ40–214 ClfA variant mirrors the behaviour of ClfB, where cleavage by aureolysin
439 outside of the minimal fibrinogen-binding domain similarly results in a complete loss
440 of function. These observations suggest that the ClfA-fibrinogen interaction is more
441 complex than previously appreciated. While residues 229-545 are sufficient to support
442 recombinant protein binding to a peptide derived from fibrinogen, they are clearly not
443 sufficient to promote bacterial interactions with fibrinogen and fibrin in the
444 bloodstream. Elucidating the structure of the entire ClfA protein in complex with
445 fibrinogen would reveal the full binding interface and uncover how ClfA engages

446 fibrinogen/fibrin. This new finding highlights the drawbacks of studying host-pathogen
447 interactions using recombinant bacterial adhesin domains *in vitro* and indicates that
448 the interaction between ClfA and fibrinogen, and likely other MSCRAMMs and their
449 ligands, is far more complex than the dock, lock and latch mechanism encapsulates
450 and best studied using whole bacteria expressing the protein of interest.
451 Plasmin cleaved ClfA at the carboxyl terminus of arginine, consistent with its
452 known substrate specificity. Proteolytic processing of ClfA results in the release of
453 residues N-terminal of A_{215}, yet the corresponding fragment (the majority of domain
454 N1) is undetectable by SDS-PAGE suggesting it undergoes rapid secondary
455 degradation. Recombinant N1N2N3 domains of MSCRAMMs often resolve into a
456 single proteolytically stable fragment corresponding to N2N3, even in the absence of
457 added protease (65). This pattern of selective degradation of the N1 domain, has been
458 seen for several MSCRAMMs and is likely driven by intrinsic disorder within N1 and
459 trace protease contamination during recombinant protein preparation (65). Our *in vivo*
460 experiments were conducted exclusively with truncated ClfA variants, confirming that
461 the observed phenotypes stem from loss of ClfA function rather than activity of the
462 released fragment. However other future studies should investigate if the released
463 portion, or peptides derived from it, have biological activity.
464
465 **Funding information**
466 M.B.T. was supported by a Postgraduate Ussher Fellowship Award from Trinity
467 College Dublin. J.A.G. is supported by a Royal Society Wolfson Fellowship (Award
468 193002). This work was also supported by the University of Birmingham. Z.H. and T.J.
469 are supported by Swedish Medical Research Council 2024-02667 and the Swedish
470 state under the agreement between the Swedish Government and the county councils
471 ALFGBG-1005076. E.C.L. and R.W.W. received support from Science Foundation
472 Ireland Frontiers for the Future award 19/FFP/6484. R.W.W. received support from
473 the Irish Research Council GOIPG/2019/4236. The funders were not involved with the
474 design of this study, collection or analysis of data, decision to publish or preparation
475 of the manuscript.
476
477 **Acknowledgements**
478 We thank Deirdre Muldowney and Kevin Lyons for technical assistance.
479

480 **Conflict of Interest**

481 The authors declare there are no conflicts of interest of a financial or any other nature.

482

483

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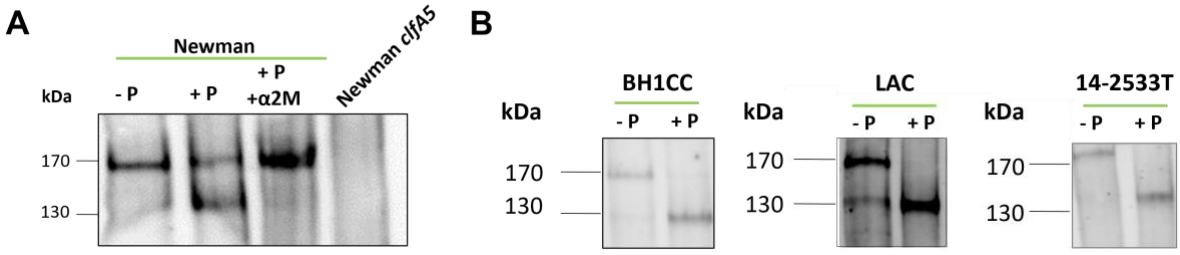

716

717 **FIG. 1. Cell wall-associated ClfA is cleaved by plasmin**. Stationary phase cultures
718 of *S. aureus* strains Newman, Newman clfA5, BH1CC, LAC and 14-2533T were
719 adjusted to an OD_{600} = 10 and incubated at 37°C for 1 h with and without plasmin (±
720 P, 0.075 U/ml) and alpha-2 macroglobulin (+ α2M, 1 mg/ml) before cell wall proteins
721 were extracted and separated by SDS-PAGE and probed with anti-ClfA A domain
722 polyclonal IgG (1:1000) in a western blot. Bound primary antibodies was detected with
723 protein A peroxidase (1:500). Blots presented are representative of two independent
724 experiments.

725

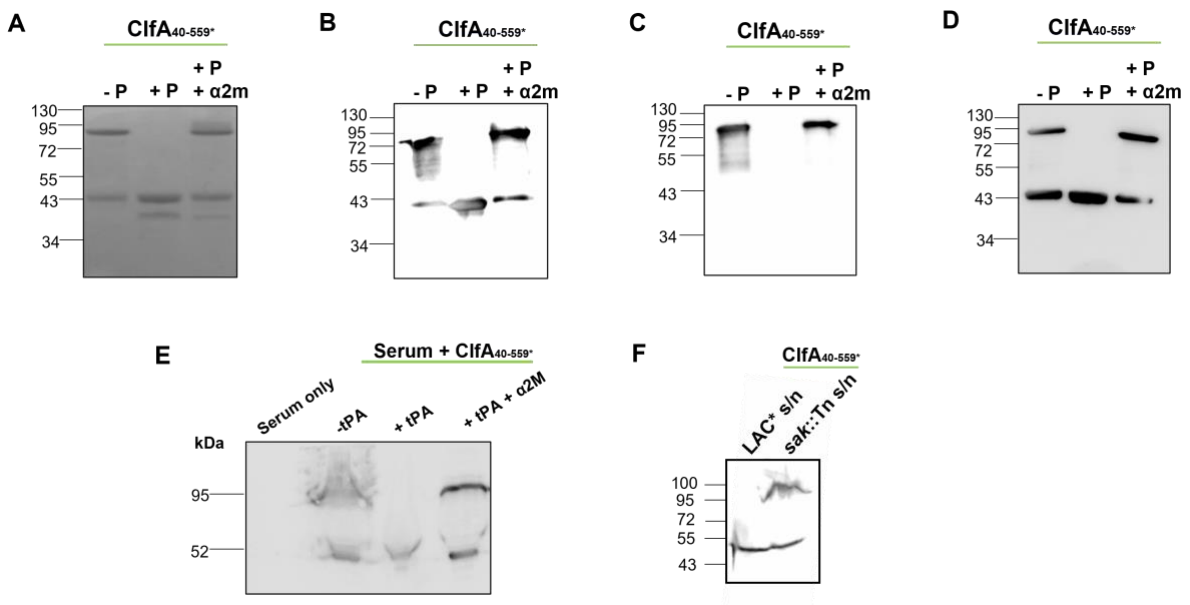

FIG. 2. The ClfA N1N2N3 domains are cleaved by plasmin. ClfA40-559* (5 μM) was incubated in PBS with or without added human plasmin (± P, 0.07 U/mL) and alpha-2 macroglobulin (+ α2M, 1 mg/ml), where indicated, at 37°C for 1 h (A-D). Alternatively, ClfA40-559* (5 μM) was incubated in human serum (40%) that was pre-treated with either E) tissue plasminogen activator (± tPA, 25 μg/mL) or F) concentrated supernatants (s/n) from *S. aureus* strain LAC* or its *sak* deficient mutant (*sak*::Tn) for 20 min at 37°C. Serum only controls did not include ClfA40-559*. Samples were separated by SDS-PAGE and either stained with SimplyBlue Coomassie stain (A) or probed in a western immunoblot with anti-ClfA IgG (1:1000; B, E, F), anti-His 7E8 IgG (1:1000; C) or anti-Strep Tag II IgG-HRP (1:2500; D). Bound anti-ClfA IgG was detected with protein A peroxidase (1:500) and bound anti-His IgG was detected with HRP conjugated rabbit anti-mouse IgG (1:2500). Blots represent two independent experiments.

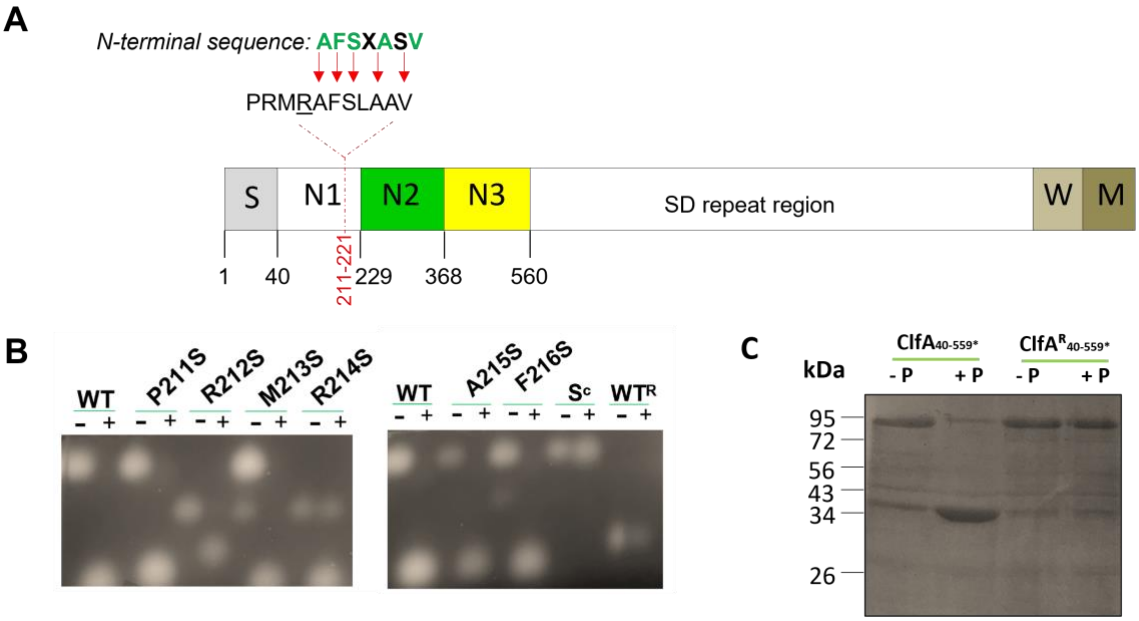

FIG. 3. Plasmin cleaves ClfA between residues 214 and 215. A) Domain organisation of ClfA. The amino acid coordinates of ClfA signal peptide (S), N1N2N3 domains, SD repeat and wall (W) and membranes-spanning (M) regions are shown. The 7 most N-terminal amino-acids of the plasmin-cleaved product identified using Edman degradation are indicated. Sequence identity is indicated in green with red arrows highlighting matches to the ClfA sequence. Arg214 predicted to be cleaved by plasmin is underlined (R). B) A fluorescently-labelled 11-mer peptide (WT) encompassing the region around the proposed cleavage site at Arg214 (residues 211-221) or variants P211S, R212S, M213S, R214S, A215S and R216S harbouring single amino-acid substitutions with serine (37.5 µM) were incubated in the absence (-) or presence (+) of plasmin (0.075 U/mL) for 30 min at 37°C. Peptide S^c was a scrambled version of the WT peptide and WT^R a peptide with four substitutions (P211A, R212S, M213A, R214S). Samples were analysed by agarose (4% w/v) gel electrophoresis. Gels represent three independent experiments. C) Recombinant ClfA40-559* and $ClfA^R$40-559* (5 µM) were incubated with or without plasmin (± P, 0.075 U/mL) at 37°C for 1 h and analysed by SDS-PAGE. Gels were stained with Instant Blue Coomassie stain. The gel image presented is representative of two independent experiments.

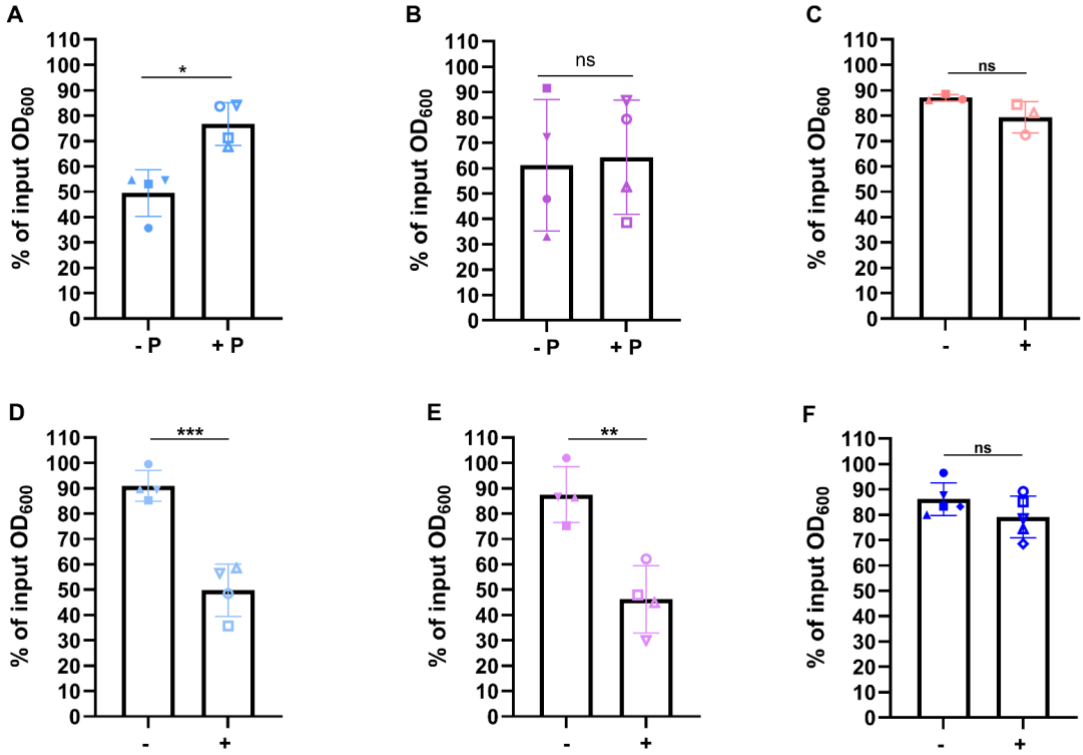

FIG. 4. Plasmin cleavage disrupts ClfA-mediated clumping in fibrinogen. Stationary phase cultures of *S. aureus* ClfA^{+}(A) and ClfA^{R} (B) were adjusted to OD_{600} = 1.5 in PBS and incubated in the absence and presence of plasmin (± P, 0.075 U/mL) for 1 h at 37°C. Bacteria were washed and incubated statically at 37°C for 105 min in the presence of fibrinogen (18.4 µg/mL). *S. aureus* ClfA$^{\Delta 40\text{-}214}$ (C) ClfA^{+} (D) ClfA^{R} (E) and ClfA^{-} (F) were incubated in PBS with (+) and without (-) fibrinogen (18.4 µg/mL) for 105 min at 37°C. The OD_{600} after 105 min is expressed as a percentage of the original input OD_{600} at 0 h. Each symbol represents the % of input OD_{600} from a single matched experiment and the bars represent the mean value for each condition from four independent experiments. Error bars represent standard deviation, and statistical significance was determined by an unpaired student t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, non-significant.

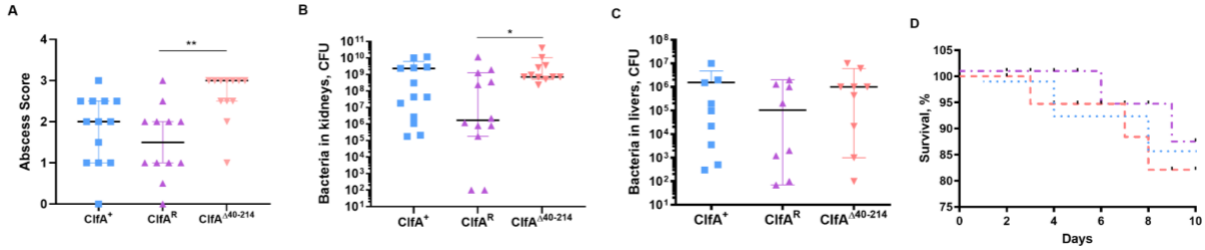

FIG. 5. Truncation of ClfA increases renal/kidney abscess formation by *S. aureus*. *S. aureus* ClfA^{+}, ClfA^{R} and ClfA$^{\Delta 40\text{-}214}$ were intravenously administered to NMRI mice (10 per group) via the tail vein with 200 µl of PBS containing 75 ng/ml anhydrotetracycline. Expression of ClfA was induced via an intraperitoneal injection of inducer anhydrotetracycline (5 µg/mouse) on Day 0, 1 h before infection and 1h post-infection. Kidneys and livers were collected aseptically, and kidney abscess scores (A) were assessed in a blinded manner; 0 represented healthy kidneys; 1 indicated one to two small abscesses without structural changes; 2 denoted more than two abscesses affecting less than 75% of the kidney tissue; and 3 indicated extensive abscess formation involving more than 75% of the tissue. Bacterial load in the kidney (B) and liver (C) were quantified by homogenising the organs and culturing on horse blood agar. (D) Mouse survival after infection. Percent survival of mice (n = 20 per group) after infection with 6 x 10^{6} CFU/mouse. The dashed line colour pairs to symbol colour for strains in panel A-C. Error bars represent 95% confidence intervals and statistical significance was determined by one-way Anova multiple comparisons with the Kruskal-Wallis post test. *P < 0.05; **, P < 0.01. P > 0.05, non-significant.

797 **Table 1.** Strains and plasmids used in this study

Strains	Description	Reference or source
Staphylococcus aureus		
BH1CC	Clinically isolated MRSA; CC8	66
Newman	MSSA; CC8	67
Newman *clfA5*	Derivative of Newman deficient in ClfA, frameshift mutation	68
S. aureus ClfA^{+}	Newman *clfA5* (pRMC2::*clfA*)	This study
S. aureus ClfA^{R}	Newman *clfA5* (pRMC2::*clfA*^{R})	This study
S. aureus ClfA$^{\Delta 40\text{-}214}$	Newman *clfA5* (pRMC2::*clfA*$_{\Delta 40\text{-}214}$)	This study
S. aureus ClfA^{-}	Newman *clfA5* (pRMC2)	This study
LAC*	Clincally isolated MRSA; CC8, Em sensitive	69
LAC* *sak*::tn	Derivative of LAC* defective in *sak*. *sak*::Tn Em^{R}	70
14-2533T	Clinically isolated MRSA; CC22	35
Escherichia coli		
XL-1-Blue	Cloning host. *recA1 endA1 gyr96 thi-1 hsdR17 suppe44 relA1 lac*	Stratagene
Topp3	Deficient in protease or expression of recombinant proteins [F'proAB+ lacqZΔM15 Tn10 (Tet^{R}) (Kan^{R})]	Stratagene
IM30B	Plasmid propagation. SA30BΩPN25-*hsdS* (CC8-1)	33
IM08B	Plasmid propagation. SA30BΩPN25-*hsdS* (CC30-1)	33
Plasmids		
pQE30::*clfA*40-559	PQE30 derivative encoding the full-length A region (domains N123) of ClfA (residues 40-559). Amp^{R}	O'Connell et al., 1998

pQE30::*clfA*40-559*	pQE30::ClfA40-559 derivative with C-terminal Strep tag II and linker DNA. Amp^R	This study
pQE30::*clfA*^{R}40-559*	PQE30:: ClfA40-559* encoding DNA with residues 211-214 substituted with AlaSerAlaSer. Amp^R	This study
pRMC2	Tetracycline-inducible expression vector derived from pALC2073 encoding -10 box of tetR promoter. Cm^R	71
pRMC2::*clfA*	pRMC2 containing full-length *clfA* gene from strain Newman. Cm^R	72
pRMC2::*clfA*^{R}	pRMC2::*clfA* with DNA encoding residues 211-214 of ClfA substituted for AlaSerAlaSer. Cm^R	This study
pRMC2::*clfA*$^{\Delta 40\text{-}214}$	pRMC2::*clfA* lacking DNA encoding residues 40-214 of ClfA. Cm^R	This study

798 CC = Clonal complex; Tn = Transposon; ^{R} = resistant; Em = erythromycin; Cm =
799 chloramphenicol; Amp = Ampicillin
800
801

802 **Table 2.** Primers used in this study

Primer	Description	Sequence
Plasmid construction & mutagenesis		
cSt_F	Inverse PCR for inclusion of C-terminal Strep-Tag II on pQE30::ClfA40-559	*GCTGCTTGGTCTCATCCTCAATTTGAAAAATAAAAGCTTAATTAGCTGAGCTTG
cSt_R	Inverse PCR for inclusion of C-terminal Strep-Tag II on pQE30::ClfA40-559	*CTCTGGAATTGGTTCAATTTC
cSt screen_F	Amplification of 166 bp region within cSt	ATAATTTGGCGCTCTATG
cSt_screen_R	Amplification of 166 bp region within cSt	ATCAACAGGAGTCCAAGCTC
cStSLIC_F	PCR to delete a 547 bp region of ClfA N1N2 from plasmid pCF-40 cSt	ACCCTGAAAATGTTAAAAAGACAGG
cStSLIC_R	PCR to delete a 547 bp region of ClfA N1N2 from plasmid pCF-40 cSt	GTTGAAACATTTTCCGCATTTGTA
ASAS_F	Amplification of a 547 bp region of ClfA N1N2 containing $Ala_{211}SerAlaSer_{214}$ mutation.	CTACAAATGCGGAAAATGTTTCAAC
ASAS_R	Amplification of a 547 bp region of ClfA N1N2 containing $Ala_{211}SerAlaSer_{214}$ mutation.	CCTGTCTTTTTAACATTTTCAGGGT
214_F	Inverse PCR for removal of a 522 bp region of ClfA N1 on pQE30::ClfA40-559*	*GCATTTAGTTTAGCGGCAG
214_R	Inverse PCR for removal of a 522 bp region of ClfA N1 on pQE30::ClfA40-559*	*TGCATCTGCTTCTTTACTGC
pQE30 MCS_F	Amplification of cloned sequence within pQE30 MCS. Screening primer.	GAGCGGATAACAATTATAATAG

Primer	Description	Sequence
pQE30_MCS_R	Amplification of cloned sequence within pQE30 MCS. Screening primer.	CCGAGCGTTCTGAACAAATC
pRMC2 MCS_F	Amplification of cloned sequence within pRMC2 MCS. Screening primer.	ATTCAGGCTGCGCAAC
pRMC2 MCS_R	Amplification of cloned sequence within pRMC2 MCS. Screening primer.	TTGTTGACATATATCATTG

803 * = 5' phosphorylation; F = forward; R = reverse