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Orla M. Fleury^{*1}, Maeve A. McAleer^{*2,3,4}, Cécile Feuillie⁵, Cécile Formosa-Dague⁵, Emily Sansever⁶, Désirée E. Bennett⁷, Aisling M. Towell¹, WH Irwin McLean⁸, Sanja Kezic⁹, D. Ashley Robinson⁶, Padraic G. Fallon^{*2,3}, Timothy J. Foster¹, Yves F. Dufrêne^{5,10}, Alan D. Irvine^{**2,3,4}, Joan A. Geoghegan^{**1}

¹Department of Microbiology, Moyne Institute of Preventive Medicine, School of Genetics and Microbiology, Trinity College Dublin, Ireland

²Clinical Medicine, Trinity College Dublin, Ireland

³National Children's Research Centre, Our Lady's Children's Hospital Crumlin, Dublin, Ireland

⁴Paediatric Dermatology, Our Lady's Children's Hospital Crumlin, Dublin, Ireland

⁵Institute of Life Sciences, Université catholique de Louvain, Croix du Sud, 4-5, bte
L7.07.06, B-1348 Louvain-la-Neuve, Belgium

⁶Department of Microbiology and Immunology, University of Mississippi Medical Center, Jackson, MS 39216, USA

⁷Epidemiology and Molecular Biology Unit, Temple Street Children's University Hospital,
Dublin, Ireland

⁸Dermatology and Genetic Medicine, University of Dundee, UK

21 ⁹Coronel Institute of Occupational Health, Academic Medical Center, Amsterdam, The
22 Netherlands

23 ¹⁰Walloon Excellence in Life sciences and Biotechnology (WELBIO), Belgium

24

25 *, ** equal contributions

26 #Corresponding author: E-mail: geoghegj@tcd.ie. Telephone: +35318961188. Fax:

27 +35316799294

28 **Running Title:** ClfB-mediated adherence of *S. aureus* to corneocytes from AD

29

30 **Abstract**

31 *Staphylococcus aureus* skin infection is a frequent and recurrent problem in children with the
32 common inflammatory skin disease atopic dermatitis (AD). *S. aureus* colonises the skin of
33 the majority of children with AD and exacerbates the disease. The first step during
34 colonisation and infection is bacterial adhesion to the cornified envelope of corneocytes in
35 the outer layer stratum corneum. Corneocytes from AD skin are structurally different to
36 corneocytes from normal healthy skin. The objective of this study was to identify bacterial
37 proteins that promote the adherence of *S. aureus* to AD corneocytes. *S. aureus* strains from
38 clonal complex 1 and 8 were more frequently isolated from infected AD skin than from the
39 nasal cavity of healthy children. AD strains had increased ClfB ligand binding activity
40 compared to normal nasal carriage strains. Adherence of single *S. aureus* bacteria to
41 corneocytes from AD patients *ex vivo* was studied using atomic force microscopy. Bacteria
42 expressing ClfB recognised ligands distributed over the entire corneocyte surface. The
43 ability of an isogenic ClfB-deficient mutant to adhere to AD corneocytes was greatly reduced
44 compared to its parent clonal complex 1 clinical strain. ClfB from clonal complex 1 strains
45 had a slightly higher binding affinity for its ligand compared to ClfB from other clonal
46 complexes. Our results provide new insights into the first step in the establishment of *S.*
47 *aureus* colonisation in AD patients. ClfB is a key adhesion molecule for the interaction of
48 *S.aureus* with AD corneocytes and represents a target for intervention.

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54 Introduction

55 The skin of the majority of individuals with the inflammatory skin disease atopic dermatitis
56 (AD) is heavily colonised by *Staphylococcus aureus* (1). The healthy population has a much
57 lower rate of persistent carriage (~ 20%) and colonisation is usually confined to the nasal
58 cavity (2). *S. aureus* colonization of skin appears to post-date the development of AD, at
59 least in infant populations (3). In established AD the density of *S. aureus* on lesional and
60 non-lesional skin has a strong relationship to disease severity (4). A major risk factor for the
61 development of AD is loss-of-function mutations in the filaggrin (*FLG*) gene (5, 6) which
62 lead to a reduced level of natural moisturising factor (NMF) in the stratum corneum
63 accompanied by a skin epidermal barrier defect and an elevated pH (7). When the skin
64 barrier is compromised, factors produced by *S. aureus* exacerbate the symptoms of AD (8).
65 Several *S. aureus* factors have been linked to increased inflammation and disease severity in
66 AD including α -toxin (9), the staphylococcal superantigens (10) and δ -toxin (11).

67 *S. aureus* adheres to the cornified envelope of corneocytes in the stratum corneum.
68 Corneocytes from AD skin have an altered surface topology compared to corneocytes from
69 normal healthy skin, physical characteristics that are largely determined by levels of
70 expressed filaggrin and NMF (12). The factors promoting colonisation of atopic skin have
71 not yet been identified. Several *S. aureus* proteins are known to bind to host molecules, some
72 of which may be present on the surface of the stratum corneum of AD skin. For example,
73 compared with healthy skin, higher levels of fibronectin are found in the stratum corneum of
74 patients with AD and the expression of the *S. aureus* cell wall anchored (CWA) fibronectin
75 binding proteins (FnBPs) A and B enhances bacterial adherence to AD skin biopsy sections
76 (13). The CWA protein clumping factor B (ClfB) binds to the cornified envelope proteins
77 loricrin and cytokeratin 10 in the moist squamous epithelium promoting colonization of the

78 anterior nares (14, 15). ClfB and FnBPs are expressed at higher levels by bacteria grown *in*
79 *vitro* at the pH of AD skin than by bacteria grown at the low pH typical of healthy skin (16).
80 The binding of ClfB to its ligands is well understood at the molecular level. The X-ray crystal
81 structure of the ligand-binding region of ClfB has been solved both in the apo form and with
82 a peptide ligand bound (17). Binding occurs by the ‘dock, lock and latch’ mechanism
83 whereby a short peptide from loricrin or cytokeratin 10 binds to a hydrophobic trench located
84 between the separately folded N2 and N3 subdomains (18). A conformational change at the
85 C-terminus of N3 locks the ligand in place.

86 The aim of our study was to identify the bacterial factors that promote adherence of *S.*
87 *aureus* to corneocytes from AD skin. We studied clinically relevant strains of *S. aureus* from
88 the infected lesional skin of children with AD. Molecular typing methods revealed the
89 population structure of strains of *S. aureus* from AD skin infection and *in vitro* experiments
90 demonstrated that AD strains adhere more strongly to a ClfB ligand (the loricrin-derived
91 peptide L2v) than control strains of *S. aureus* isolated from the nares of a healthy cohort of
92 children with no history of AD. Isogenic *clfB* knockout mutants were generated in AD
93 strains representing the most common lineages of *S. aureus* isolated from paediatric patients.
94 The ability of an AD strain and its isogenic ClfB-deficient mutant to adhere to corneocytes
95 from patients with AD was studied *ex vivo* and using atomic force microscopy (19).

96

97 **Materials and Methods**

98 **Patient populations**

99 The AD patients enrolled in this study were presenting for the first time at a tertiary referral
100 centre at Our Lady's Children's Hospital, Crumlin, Dublin, Ireland between September 2012
101 and September 2014. An experienced paediatric dermatologist (MAMcA ADI, or both) made
102 the diagnosis and recorded the disease phenotype. All patients met the United Kingdom
103 diagnostic criteria for AD (20) and had moderate or severe disease. Exclusion criteria from
104 the study included patients who had pyrexial illness in the preceding 2 weeks and those who
105 had received immunosuppressive systemic therapy, such as oral corticosteroids, in the
106 preceding 3 months. The study was conducted in accordance with the Helsinki Declarations
107 and was approved by the Research Ethics Committee of Our Lady's Children's Hospital,
108 Dublin, Ireland. Full written informed consent was obtained from all patients' parents. The
109 children were treatment naive (for topical steroids, antibiotics or antimicrobials) at
110 presentation. Patient demographics and disease characteristics are summarized in Table S1.

111 Age-matched control subjects were children attending the Emergency Department of Temple
112 Street Children's University Hospital, Dublin, Ireland between July and August 2009.
113 Subjects were requested to complete a questionnaire and those whose reason for presentation
114 was not of an infective origin (i.e. trauma, accompanying sibling, etc.) and who had no
115 history of skin diseases (including AD), allergic rhinitis or bronchial asthma were selected for
116 inclusion in the study. Ethical permission was received from the Temple Street Children's
117 University Hospital Ethics Committee and full written informed consent was obtained from
118 all patients' parents. The mean age at recruitment was 30.48 months, and 53.06% of the
119 subjects were female.

120

121 **FLG genotyping**

122 All AD patients were screened for the 9 most common *FLG* mutations found in the Irish
123 population (R501X, Y2092X, 2282del4, R2447X, S3247X, R3419X, 3702delG, S1040X,
124 and G1139X) from DNA extracted from a blood sample. The methods used have been
125 previously described (21).

126

127 **Collection of strains and molecular typing of *S. aureus***

128 To collect AD strains, swabs were taken from a clinically infected site on the patient's skin
129 (Table S1) and streaked onto mannitol salt agar to select for *S. aureus*. *S. aureus* strains from
130 a healthy cohort (children with no history of AD and asymptomatic nasal carriage) were
131 recovered by rotational swabbing of the anterior nares of one nostril. In total 44 AD strains
132 and 49 nasal carriage isolates were studied. Strains were single colony purified on sheep
133 blood agar and a single colony isolated from each patient was *spa* typed. The *spa* types were
134 classified using two online nomenclature systems (Ridom and eGenomics) (22). One or more
135 isolates of each unique *spa* type were subjected to multilocus sequence typing (MLST), and
136 clonal complex (CC) was assigned by eBURST analysis of the MLST data.

137

138 **Bacterial growth conditions and strain construction**

139 *S. aureus* was grown in Tryptic Soy Broth (TSB) at 37°C. SH1000 is a commonly used
140 laboratory strain of *S. aureus* (23). Plasmid DNA isolated from *E. coli* strain SA08B (24)
141 was used to transform CC1 strains of *S. aureus* using standard procedures (25). Deletion of

142 the *clfB* gene was achieved by allelic exchange as described previously (14). The mutation
143 was confirmed by DNA sequencing of a PCR amplicon. The *clfB* mutant was phenotypically
144 indistinguishable from the parent strain in terms of growth rate and haemolysis on sheep
145 blood agar (data not shown). Plasmids pCU1 (26) and pCU1::*clfB* (27) were transformed
146 into AD08_{CC1} Δ *clfB*.

147

148 **Extraction of cell wall proteins and western immunoblotting**

149 Bacteria from an overnight culture were washed in TSB, diluted 1:200 and allowed to grow
150 to an OD₆₀₀ = 0.3 - 0.5 in TSB. Bacteria were washed in phosphate-buffered saline (PBS)
151 and resuspended to an OD₆₀₀ of 10 in lysis buffer (50 mM Tris/HCl, 20 mM MgCl₂, pH 7.5)
152 supplemented with raffinose (30% w/v, Sigma) and complete protease inhibitors (40 μ l/ml,
153 Roche). Cell wall proteins were solubilised by incubation with lysostaphin (100 μ g/ml;
154 AMBI, New York) for 8 min at 37°C. Protoplasts were removed by centrifugation at
155 16,000 $\times g$ for 5 min and the supernatant containing solubilised cell wall proteins was
156 aspirated and boiled for 10 min in final sample buffer. Proteins were separated by sodium
157 dodecyl sulfate polyacrylamide gel electrophoresis on 7.5% (w/v) polyacrylamide gels,
158 transferred onto polyvinylidene difluoride (Roche) and blocked in 10% (w/v) skimmed milk
159 proteins. Blots were probed with polyclonal rabbit antibodies against the ClfB A domain
160 (1:1000) and bound antibody was detected using horseradish peroxidase-conjugated protein A
161 (1:500, Sigma). Reactive bands were visualised using the LumiGLO reagent and peroxide
162 detection system (Cell Signalling Technology) using the ImageQuant Las 4000 imaging
163 system and ImageQuant TL software (GE Healthcare).

164

165 **Bacterial adherence to L2v**

166 Recombinant GST-tagged L2v was purified from *E. coli* as previously described (14) using a
167 GSTrap FF purification column (GE Healthcare) according to the manufacturer's instructions
168 and diluted in coating buffer (0.1 M NaHCO₃, 33 mM Na₂CO₃, pH 9.6). Wells of a microtitre
169 plate (Nunc maxisorb) were incubated with a solution of L2v (0.625 µg/ml) overnight at 4°C.
170 Wells were blocked with bovine serum albumin (BSA, 5% (w/v)) for 2 h at 37°C. *S. aureus*
171 was grown to an OD₆₀₀ = 0.3 - 0.5 in TSB. Washed bacteria were adjusted to an OD₆₀₀ of 1.0
172 in PBS, and 100 µl was added to each well and incubated for 1.5 h at 37 °C. Wells were
173 washed with PBS, and adherent cells fixed with formaldehyde (25% v/v), stained with crystal
174 violet and the A570 nm measured. Each experiment was performed three times.

175 **Sampling of the stratum corneum by tape stripping and transepidermal water loss** 176 **measurement**

177 A clinically unaffected site on the patient's volar forearm was used for transepidermal water
178 loss (TEWL) measurements and stratum corneum sampling using a previously described
179 method (28). TEWL was determined by using a Tewameter 300 (Courage and Khazaka
180 Electronic GmbH, Cologne, Germany). To sample the stratum corneum, circular adhesive
181 tape strips (3.8 cm², D-Squame; Monaderm, Monaco, France) were attached to volar forearm
182 skin and pressed for 10 seconds with a constant pressure (225 g/cm²) by using a D-Squame
183 Pressure Instrument D500 (CuDerm, Dallas, Tex). The tape strip was then gently removed
184 and placed in a closed vial. Eight consecutive tape strips were sampled, all from the same
185 site. The tape strips were immediately stored at -80°C until analysis. The fifth strip was
186 used for NMF measurements and the eighth strip for AFM.

187

188 **Natural moisturising factor measurement**

189 Natural moisturising factor analysis was performed on the fifth consecutive strip, according
190 to methods described in detail previously (29). Briefly, the tape strip was extracted with 25%
191 (wt/wt) ammonia solution. After evaporation of the ammonia extract, the residue was
192 dissolved in 250 μ l of pure water and analyzed by using high performance liquid
193 chromatography. The NMF concentration was normalized for the protein amount determined
194 with a Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, Ill; referred
195 to as the Pierce assay) to compensate for a variable amount of the stratum corneum on the
196 tape.

197

198 **Atomic Force Microscopy**

199 **Corneocyte imaging.** Atomic force microscopy imaging was performed on the eighth tape
200 strip in contact mode at a resolution of 512 lines, using SiNO₃ cantilevers (MSCT, Bruker,
201 nominal spring constant of 0.01 N/m), in Tris buffered saline (TBS, Tris 50 mM, NaCl 150
202 mM, pH = 7.4) at room temperature. For each condition, at least 3 different corneocytes were
203 imaged.

204

205 **Multiparametric imaging.** Multiparametric images of corneocytes were recorded in TBS
206 using the Quantitative ImagingTM mode available on the Nanowizard III AFM (JPK
207 Instruments, Germany). Images were obtained using a *S. aureus* AD08_{CC1} or AD08_{CC1} Δ *clfB*
208 cell probe (see below for cell probe preparation) at 128 pixels x 128 pixels, with an applied
209 force kept at 1.0 nN, and a constant approach/retract speed of 40.0 μ m/s (z-range of 1 μ m).
210 The cantilevers spring constants were determined by the thermal noise method. For each
211 condition, experiments were repeated for at least 3 different cell pairs.

212 **Single-cell force spectroscopy.** To prepare bacterial cell probes, colloidal probes were
213 obtained by attaching a single silica microsphere (6.1 μm diameter, Bangs Laboratories) with
214 a thin layer of UV-curable glue (NOA 63, Norland Edmund Optics) on triangular tipless
215 cantilevers (NP-O10, Bruker) and using a Nanowizard III AFM (JPK Instrument, Berlin,
216 Germany). Cantilevers were then immersed for 1 h in TBS (pH = 8.5) containing dopamine
217 hydrochloride (4 mg/ml, Sigma-Aldrich), rinsed in TBS, and used directly for cell probe
218 preparation. The nominal spring constant of the colloidal probe cantilever was determined by
219 the thermal noise method. Then, 50 μl of a diluted cell suspension was deposited into the
220 petri dish containing corneocytes, at a distinct location within the petri dish; 3 ml of PBS was
221 added to the system. The colloidal probe was brought into contact with an isolated bacterium
222 and retracted to attach the bacterial cell; proper attachment of the cell on the colloidal probe
223 was checked using optical microscopy. Cell probes were used to measure cell-cell interaction
224 forces at room temperature, using an applied force of 0.25 nN, a constant approach-retraction
225 speed of 1.0 $\mu\text{m/s}$ and a contact time of 100 ms. Data were analyzed using the Data
226 Processing software from JPK Instruments (Berlin, Germany). Adhesion forces values were
227 obtained by calculating the maximum adhesion force for each force curve recorded on
228 corneocytes and the data for five different cell pairs in each condition were pooled.

229 **Recombinant ClfB protein expression and purification.** DNA encoding the N2N3
230 subdomains of ClfB (residues 201-542) was amplified by PCR using genomic DNA from *S.*
231 *aureus* strain AD08_{CC1} or AD22_{CC30} as template and cloned into the vector pQE30. *E. coli*
232 TOPP3 carrying the recombinant plasmids was grown to late exponential phase ($\text{OD}_{600} = 0.6$)
233 and induced with IPTG. CC1 and CC30 ClfB harbouring an N-terminal hexahistidine tag
234 were purified using Ni^{2+} affinity chromatography.

235 **Surface plasmon resonance.** Surface plasmon resonance (SPR) was performed using the
236 BIAcore X100 system (GE Healthcare). Goat anti-GST IgG (30 $\mu\text{g/ml}$, GE Healthcare) was

237 diluted in 10 mM sodium acetate buffer at pH 5.0 and immobilized on CM5 sensor chips
238 using amine coupling. This was performed using 1-ethyl-3-(3-dimethylaminopropyl)
239 carbodiimide hydrochloride, followed by *N*-hydroxysuccinimide and ethanolamine
240 hydrochloride, as described by the manufacturer. Recombinant GST-tagged L2v (10–30
241 µg/ml) in PBS was passed over the anti-GST surface of one flow cell while recombinant GST
242 (10–30 µg/ml) was passed over the other flow cell to provide a reference surface. Increasing
243 concentrations of rClfB N2N3 in PBS were passed in over the surface of the chip without
244 regeneration. All sensorgram data were subtracted from the corresponding data from the
245 reference flow cell. The response generated from injection of buffer over the chip was also
246 subtracted from each sensorgram. Data was analysed using the BIAevaluation software
247 version 3.0. A plot of the level of binding (response units) at equilibrium against
248 concentration of rClfB N2N3 was used to determine the K_D . The data shown is representative
249 of 3 individual experiments.

250 **Statistical Tests**

251 Statistical significance was determined with the Student's *t*-test, using GRAPHPAD software.

252 Results

253 Population structure of atopic dermatitis strains of *S. aureus*.

254 The skin of AD patients is frequently colonised by *S. aureus*. This study aimed to identify
255 bacterial factors that promote adherence of AD strains of *S. aureus* to corneocytes from AD
256 skin. To facilitate this, strains of *S. aureus* were collected from infected skin lesions of
257 children with AD (AD strains). The paediatric patients were presenting for the first time at a
258 tertiary referral centre in Dublin, Ireland and were treatment naïve (Table S1). Molecular
259 typing methods were used to assign each strain to a clonal complex so that the general
260 population structure of the AD strains could be examined. The most common clonal complex
261 (CC) in the AD cohort was CC1 (9 isolates, 20.45%) followed by CC45 (7 isolates, 15.9%),
262 CC8 (6 isolates, 13.63%) and CC5 (6 isolates, 13.63%) (Fig. 1A). Commensal *S. aureus*
263 strains from a healthy cohort in Dublin, Ireland (children with no history of AD and
264 asymptomatic nasal carriage) were also typed to the same level (control strains, Fig. 1B)
265 Only 4.08% and 2.04% of the control strains belonged to CC1 and CC8, respectively,
266 showing that although CC1 and CC8 strains are frequently recovered from AD skin lesions
267 they rarely occur as commensal strains in the nares of healthy children in the community
268 (Fig. 1). In contrast, while CC30 was the most prevalent clonal complex among the control
269 strains (33% of strains) only 6.81% of the AD strains belonged to CC30 showing a
270 statistically significant underrepresentation of CC30 in the AD cohort. Thus the strains
271 causing skin infection in paediatric AD patients are different to the commensal strains
272 circulating in the community. There were no associations between the *S. aureus* clonal
273 complex and NMF levels in the patient's stratum corneum or the clinical phenotype (Table
274 S1).

275

276 **Ligand binding activity of AD strains.**

277 Disease causing strains of *S. aureus* often have increased ability to adhere to host molecules
278 or increased toxin production compared to commensal strains (30, 31). We hypothesised that
279 adhesion to corneocytes is an important first step in the colonisation and infection of AD
280 skin. Therefore we employed a phenotypic screening method to study the activity of the *S.*
281 *aureus* adhesin ClfB, a *S. aureus* CWA protein that binds to loricrin and cytokeratin 10 (14,
282 15). We examined the in vitro adherence of AD strains to the ClfB ligand L2v, a loricrin
283 derived peptide fused to GST (Fig. 2). The well characterised *S. aureus* strain SH1000 was
284 included as a control since adherence of SH1000 to L2v is solely dependent on ClfB (14).
285 The amount of adherence for each clinical strain was expressed as a percentage of the
286 adherence value measured for SH1000. The commensal control strains were also tested.
287 Each data point in Fig. 2 represents the mean adherence level of a single strain. All AD
288 strains adhered to immobilized L2v with a median level of adherence relative to SH1000 of
289 157% (Fig. 2). The median level of adherence for the control strains was significantly lower
290 (111%, $p < 0.0001$). These results indicate that all *S. aureus* strains that infect the skin of AD
291 patients display ClfB ligand binding activity and that this is higher than the *S. aureus* nasal
292 carriage control isolates from healthy individuals. These data suggested that ClfB could be
293 an important adhesin for AD skin. In addition AD strains from CC1 (red), the most common
294 clonal complex in the AD cohort, adhered very strongly to L2v with a median adherence
295 value of 196% (Fig. 2). For the CC30 strains (green) adherence values ranged between 0 and
296 155% for the control strains while for the three CC30 AD strains, adherence values ranged
297 between 144 and 191%. Adherence values for the CC8 strains (blue) were highly variable
298 ranging from 41 – 204%.

299

300

301 **Adherence of *S. aureus* to corneocytes from AD patients *ex vivo* is ClfB-dependent.**

302 The results described above suggested that ClfB ligand binding activity was a universal
303 feature of our paediatric AD strains. In order to investigate if adherence of *S. aureus* to AD
304 corneocytes requires ClfB, the *clfB* gene was deleted in a representative AD strain from CC1
305 (AD08_{CC1}, Table S1). Western immunoblotting using anti-ClfB IgG indicated that while the
306 parent strain AD08 expressed ClfB, bands corresponding to ClfB were not detected for the
307 ClfB-deficient mutant (AD08_{CC1}Δ*clfB*, Fig. 3A). Complementation of the *clfB* mutant with
308 plasmid pCU1::*clfB* restored the expression of ClfB (Fig. 3A). The *clfB* mutant did not
309 adhere to L2v while the parent strain adhered in a dose-dependent and saturable manner (Fig.
310 3B). Complementation of AD08_{CC1}Δ*clfB* with pCU1::*clfB* restored the ability of the mutant
311 to adhere to L2v while AD08_{CC1}Δ*clfB* carrying empty plasmid pCU1 could not adhere (Fig.
312 3C) showing that the ability of AD08_{CC1} to adhere strongly to L2v can be attributed to the
313 activity of ClfB.

314 AD08_{CC1} and its isogenic ClfB-deficient mutant were used in single cell atomic force
315 microscopy (AFM) experiments to determine if ClfB is important for the adherence of *S.*
316 *aureus* to AD skin. Corneocytes were collected by tape-stripping the unaffected skin of two
317 AD patients with low NMF levels and concurrent *S. aureus* infection at a different site
318 (patients 1434 and 1473, Table S1). Previous work has shown that low levels of NMF are
319 associated with changes in corneocyte morphology in AD skin compared to normal healthy
320 skin leading to the appearance of villus-like projections (12). We generated topographic
321 images of corneocytes from both patients in buffer, using contact mode imaging with silicon
322 nitride tips (Fig. S1) and found that the corneocytes from FLG AD patients displayed large

323 numbers of villus protrusions, about 200 nm in height, consistent with the morphology
324 expected at low NMF (12).

325 To investigate if ClfB is important for the adherence of *S. aureus* to corneocytes from AD
326 skin, AD08_{CC1} and its isogenic ClfB-deficient mutant (AD08_{CC1} Δ ClfB) were used in single-
327 cell force spectroscopy experiments (32, 33) (Fig. 4). A single *S. aureus* cell was immobilised
328 on an AFM cantilever and this was used as a probe to measure the binding forces between *S.*
329 *aureus* adhesins and corneocytes. The average binding force between *S. aureus* AD08_{CC1} and
330 AD corneocytes was similar for both patients (1186 pN and 1109 pN, Fig. 4A). In both cases
331 there was a statistically significant reduction in binding force when AD08_{CC1} Δ ClfB was used
332 as a probe on the same AD corneocytes (670 pN and 749 pN, Fig. 4C). These data provide
333 direct evidence that ClfB is involved in mediating attachment of *S. aureus* to AD
334 corneocytes.

335 Multiparametric imaging was then used to localise ligands recognised by *S. aureus* on the
336 surface of corneocytes from patients 1434 and 1473 (34, 35). Multiparametric imaging is a
337 newly developed AFM technique that enables researchers to simultaneously map the
338 structure, biophysical properties and molecular interactions of biological samples (19). AFM
339 cantilevers were functionalised with a single cell of AD08_{CC1} or AD08_{CC1} Δ ClfB. Force curves
340 were recorded across the corneocyte surface at high frequency, generating maps of structure
341 and adhesion. Multiple adhesion events with a mean force of $\sim$ 600 pN occurred between
342 AD08_{CC1} and the corneocyte surface indicating that many ligands for *S. aureus* are exposed
343 in the stratum corneum of AD patients (Fig. 5). In contrast, much fewer adhesion events with
344 a weaker strength ($\sim$ 200 pN) occurred with AD08_{CC1} Δ ClfB indicating that in the absence of
345 ClfB, *S. aureus* is less well able to adhere to corneocytes (Fig. 5). Importantly, the number of
346 ligands supporting *S. aureus* adherence to the surface of corneocytes was greatly reduced for

the ClfB mutant. This demonstrates that ClfB is a major adhesin mediating the interaction of *S. aureus* with AD skin.

Ligand binding by ClfB from AD strains

All AD strains adhered to L2v and the median adherence level was high (Fig. 2B). ClfB is the only *S. aureus* factor that binds to L2v (Fig. 3, (14). Variation in the amino-acid sequence of the ligand binding domains of ClfB has been reported previously (36). More sequence differences in ClfB are likely to occur between clonal complexes of *S. aureus* than within a clonal complex (36). Amino acid sequence variation in ClfB could confer a higher affinity for the ligand. We isolated genomic DNA from all AD strains and used PCR to amplify DNA encoding the ligand binding region of ClfB (N2 and N3 subdomains). All AD strains carried the *clfB* gene (data not shown). DNA sequencing was carried out on the *clfB* amplicon that was generated for all of the CC1, CC8 and CC30 strains. The deduced amino-acid sequences were aligned. The ClfB N2N3 subdomains of CC1 strains shared 100% amino acid identity with each other. The amino acid sequences of ClfBN2N3 from CC8 strains were 92% identical to the CC1 sequence. The three CC30 AD strains had identical ClfBN2N3 sequences that shared 94% identity with CC1 ClfB N2N3. Molecular modelling was carried out to allow visualisation of the position of the variant residues on the crystal structure of ClfB N2N3 (Fig. 6A). This indicated that the residues that vary between CC1 and CC30 are located well away from the trench between N2 and N3. Thus they are unlikely to alter the affinity of ligand binding to the trench by the dock, lock and latch mechanism. However it is possible that an additional, unknown ligand binding site could be located outside of the trench region. For example it has recently been shown for the related protein ClfA that high-affinity binding of ClfA to its ligand fibrinogen involves both the dock, lock and latch ligand-binding trench and a second interaction site at the top of the N3 subdomain

372 (37). Thus to investigate if sequence differences outside of the trench region in CC1 and
373 CC30 ClfB might alter the affinity for L2v, 6x histidine-tagged recombinant ClfB N2N3
374 proteins (rClfB N2N3) were purified from *E. coli*. The affinity of rClfB N2N3 with CC1 or
375 CC30 sequence for L2v was studied using surface plasmon resonance. GST-tagged L2v was
376 captured on the surface of a sensor chip that had been coated with anti-GST IgG and
377 increasing concentrations of rClfB N2N3 proteins were passed over the surface of the coated
378 sensor chip. To calculate the K_D the amount of binding at equilibrium was plotted against
379 each rClfB N2N3 concentration and the K_D of the interaction was calculated (Fig. 6).
380 Previously the affinity of CC8 rClfB N2N3 for L2v was calculated to be 2.21 μ M (14). CC1
381 rClfB N2N3 had a slightly higher affinity for GST-L2v ($1.91 \pm 0.17 \mu$ M, Fig. 6B) than CC30
382 rClfB N2N3 ($3.26 \pm 0.36 \mu$ M, Fig. 6C). The small but statistically significant difference in
383 affinity of CC1 and CC30 ClfB for L2v ($p < 0.001$) may partially explain why AD strains
384 from CC1 adhere more strongly to L2v.

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389 **Discussion**

390 The skin of AD patients differs in several respects from normal skin and provides an
391 environment where *S. aureus* can proliferate. By mutating the *clfB* gene we have shown the
392 importance of ClfB in mediating bacterial adherence to corneocytes taken from AD patients
393 known to be susceptible to *S. aureus*. This study used clinically relevant strains isolated from
394 AD patients, rather than relying on laboratory strains, thus increasing the clinical relevance of
395 our findings. Single cell AFM is an important recent technical advance which has allowed
396 measurement of the forces involved in binding of single bacterial cells to corneocytes (34).
397 Here we show that a *clfB* mutant bound significantly less strongly to corneocytes from two
398 AD patients with low NMF compared to the wild-type *S. aureus* parent strain. This
399 demonstrates the importance of ClfB-mediated adherence to ligands exposed on the
400 corneocyte surface. The cornified envelope proteins loricrin and cytokeratin 10 are ligands
401 for ClfB and it may be through binding to these proteins that ClfB mediates bacterial
402 attachment to corneocytes. Alternatively, given the altered corneocyte morphology and
403 protein expression profiles in AD skin, where NMF levels are low (12, 38, 39) it is possible
404 that ClfB is binding to other protein ligands. Additional *S. aureus* factors are likely to
405 contribute to the adhesion of *S. aureus* to corneocytes and colonisation of AD skin. We
406 believe that AFM could be used in future research to gain further insight into the molecular
407 basis of skin colonisation and infection in AD.

408 There were differences in the overall population structure of *S. aureus* strains from AD skin
409 lesions compared to strains isolated from a control population (commensal strains). This is
410 not entirely surprising given that the niche occupied by the bacteria is very different (AD skin
411 lesion versus nasal cavity). Nevertheless the control strains provided a useful comparison.
412 Since ClfB is a major determinant of nasal colonisation (14) it was surprising to find that
413 ClfB ligand binding activity was lower among commensal control strains compared to AD

414 strains. However nasal colonisation is a multifactorial process involving several
415 staphylococcal factors (40) and the relative contribution of each factor may differ from strain
416 to strain. We hypothesise that efficient colonisation of AD skin is also likely to require
417 additional factors. Currently there is no explanation as to why there are few CC30 strains
418 isolated from skin lesions in AD patients while the proportion of CC1 and CC8 strains is
419 high. Other studies have found similar trends in AD strain populations from different
420 geographic locations (41, 42). A slightly higher binding affinity was measured for the ClfB
421 from CC1 compared to CC30. We hypothesise that this difference could be amplified when
422 multiple copies of ClfB are present on the surface of *S. aureus* leading to an increase in
423 avidity. In addition it is possible that there are strain-dependent differences in the genetic
424 regulation of *clfB* expression which could account for differences in the amount of ClfB on
425 the surface of different strains. Genetic diversity between strains within a CC can often be
426 missed using molecular typing approaches (43). Whole genome sequencing allows a more
427 detailed analysis of the relationship between strains. It is likely that the ability to colonize
428 and to proliferate on AD skin is due to many factors in addition to the ability to adhere to
429 corneocytes. A detailed proteomic analysis or transcriptional profiling of clinical isolates
430 will give full details of the repertoire of virulence and colonisation factors expressed by
431 strains infecting AD lesions. Here we have identified a crucial role for ClfB in promoting
432 adherence of clinically relevant strains of *S. aureus* to corneocytes from AD skin. This
433 finding advances our understanding of the interaction between *S. aureus* and the altered
434 environment of AD skin and may inform targeted therapies to reduce colonisation and
435 infection in AD patients.

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439 **Acknowledgements**

440 OMF, McMcA and ADI are funded by the National Children's Research Centre, Dublin,
441 Ireland. AMT and JAG are supported by a Research Award from the British Skin Foundation.
442 WHIM, PGF and ADI were supported by Wellcome Trust (Programme grant
443 092530/Z/10/Z). Work at the Université catholique de Louvain was supported by the
444 European Research Council (ERC) under the European Union's Horizon 2020 research and
445 innovation programme (grant agreement No [693630]), the National Fund for Scientific
446 Research (FNRS), the FNRS-WELBIO (Grant n°WELBIO-CR-2015A-05), the Université
447 catholique de Louvain (Fonds Spéciaux de Recherche), the Federal Office for Scientific,
448 Technical and Cultural Affairs (Interuniversity Poles of Attraction Programme), and the
449 Research Department of the Communauté française de Belgique (Concerted Research
450 Action). Y.F.D. and C. F.-D. are respectively Research Director and postdoctoral researcher
451 of the FNRS. D.A.R. was supported by grant GM080602 from the National Institutes of
452 Health. The collection and molecular typing of the carriage isolates was supported by funds
453 from Temple Street Children's University Hospital. We gratefully acknowledge the
454 participation of our patients and their families, without this the study would not have been
455 possible. Many thanks to our research nurse Nuala Alyward for her assistance in sample
456 collection and to Stephanie MacCallum and Linda Campbell for *FLG* genotyping of our
457 patient series.

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622 **Figure Legends**

623 **Fig. 1. Clonal complex assignments of AD strains and control strains of *S. aureus*.**

624 Swabs were taken from a clinically infected site on the AD patient's skin (A, AD strains) or
625 from the anterior nares of asymptomatic carriers (B, control strains). A single colony isolate
626 from each patient was *spa* typed. One or more isolates of each unique *spa* type were
627 subjected to multilocus sequence typing and clonal complex (CC) was assigned by eBURST
628 analysis.

630 **Fig. 2. Adherence of *S. aureus* strains to L2v.** Control strains (■) or AD strains (●) were
631 grown to exponential phase, adjusted to an $OD_{600} = 1.0$ and incubated in wells coated with
632 L2v at 37°C. Following incubation wells were washed, adherent bacteria were stained with
633 crystal violet and the absorbance was read at 570 nm. Each data point represents the mean
634 adherence value of a single strain from three independent experiments expressed as a
635 percentage of the absorbance value measured for strain SH1000. Strains from CC1 are
636 shown in red, CC8 in blue, CC30 in green and other CCs in black. The horizontal lines
637 represent the median adherence value for the population. Statistical analysis was performed
638 using an unpaired *t*-test, *** $p < 0.001$.

640 **Fig. 3. Characterisation of a ClfB-deficient mutant of *S. aureus* AD08_{CC1}.** A) The *S.*
641 *aureus* strain AD08_{CC1} (1), its isogenic ClfB-deficient mutant AD08_{CC1}Δ*clfB* (2) and
642 complemented mutant AD08_{CC1}Δ*clfB* (pCU1::*clfB*) (3) were grown to exponential phase.
643 Cell wall extracts were separated on 7.5 % acrylamide gels, blotted onto PVDF membranes
644 and ClfB was detected using polyclonal rabbit antibodies. Bound antibody was detected
645 using horseradish peroxidase-conjugated protein A. Size markers (kDa) are indicated.

646 B) *S. aureus* strain AD08_{CC1} (●) and AD08_{CC1}Δ*clfB* (■) were grown to exponential phase,
647 washed and incubated in microtiter plates coated with L2v. Adherent cells were stained with
648 crystal violet and the absorbance was read at 570 nm. The graph shown is representative of
649 three independent experiments.

650 C) *S. aureus* strain AD08_{CC1} (red) or mutants AD08_{CC1}Δ*clfB* (blue), AD08_{CC1}Δ*clfB* (pCU1)
651 (white) and AD08_{CC1}Δ*clfB* (pCU1::i*clfB*) (green) were grown to exponential phase, washed
652 and incubated in microtiter plates coated with L2v (0.625 μg/ml). Adherent cells were
653 stained with crystal violet and the absorbance was read at 570 nm. Error bars represent the
654 standard error of the mean values obtained from three independent experiments. Statistical
655 significance was determined with Student's unpaired *t*-test, ***P* = 0.0059, ****P* < 0.0001.

656

657 **Fig. 4. Strength of the *S. aureus*-corneocyte interaction.** (a) Box-chart of the adhesion
658 forces recorded between *S. aureus* AD08_{CC1} or *S. aureus* AD08_{CC1}Δ*clfB* and corneocytes
659 from patient 1434. (b) Representative force curves recorded in each condition. (c) Box-chart
660 of the adhesion force between *S. aureus* AD08_{CC1} or *S. aureus* AD08_{CC1}Δ*clfB* and
661 corneocytes from patient 1473. (d) Representative force curves recorded in each condition.
662 All curves were obtained using an applied force of 250 pN and an approach and retraction
663 speed of 1.0 μm/s. In each case, values calculated from force curves recorded for 5 different
664 *S. aureus*-corneocyte pairs were pooled and represented as box-charts, showing mean
665 adhesion (full circle), median, first and third quartiles (box), range of data without outliers
666 (whiskers), 99th percentile (open circle) and extreme outliers (triangles). Statistical analysis
667 was performed using an unpaired *t*-test, *** *p* < 0.0001.

668

669 **Fig. 5. Nanoscale multiparametric imaging of corneocytes using single *S. aureus* probes.**

670 (a) Height image of a corneocyte coming from patient 1434 recorded in PBS using a *S.*
671 *aureus* AD08_{CC1} cell probe, and (b) corresponding adhesion image. (c) Height image of a
672 corneocyte coming from patient 1434 recorded in PBS using a *S. aureus* AD08_{CC1} Δ *clfB* cell
673 probe, and (d) corresponding adhesion image. For both conditions, similar data were
674 obtained in two other independent experiments.

675

676 **Fig. 6. Binding of rClfB N2N3 to L2v.** A) Ribbon representation of the crystal structure of
677 the N2N3 subdomains of ClfB showing the residues that vary between the CC1 and CC30
678 sequences (red). The ClfB ligand bound in the trench between subdomains N2 and N3 is
679 shown in stick format (blue). Representative sensorgrams showing binding of rClfB N2N3 to
680 L2v in a single cycle kinetics assay. GST-tagged L2v was captured on a CM5 chip coated
681 with anti-GST IgG and increasing concentrations of rClfB N2N3 with the CC1 (B) or CC30
682 (C) sequence were passed over the surface. Binding was plotted as response units against
683 time. The affinities were calculated from curve fitting to a plot of the response unit values
684 against concentrations of rClfB N2N3. The data shown is representative of 3 individual
685 experiments.

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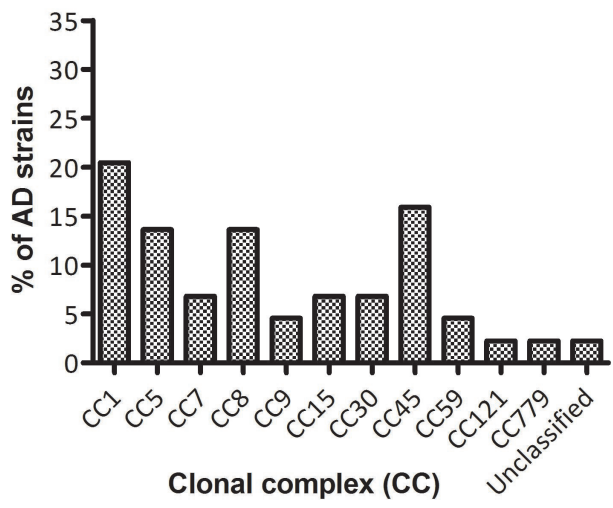
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