

# 1 **The pentaglycine bridges of *Staphylococcus aureus* peptidoglycan are essential for** 2 **cell integrity**

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10

## 11 **Abstract**

12 Bacterial cells are surrounded by cell wall, whose main component is peptidoglycan  
13 (PG), a macromolecule that withstands the internal turgor of the cell. PG composition can vary  
14 considerably between species. The Gram-positive pathogen *Staphylococcus aureus* possesses  
15 highly crosslinked PG due to the presence of cross bridges containing five glycines, which are  
16 synthesised by the FemXAB protein family. FemX adds the first glycine of the cross bridge,  
17 while FemA and FemB add the second and the third, and the fourth and the fifth glycines,  
18 respectively. Of these, FemX was reported to be essential. To investigate the essentiality of  
19 FemAB, we constructed a conditional *S. aureus* mutant of the *femAB* operon. Depletion of  
20 *femAB* was lethal, with cells appearing as pseudomulticellular forms that eventually lyse due to  
21 extensive membrane rupture. This deleterious effect was mitigated by drastically increasing  
22 the osmolarity of the medium, indicating that pentaglycine crosslinks are required for *S. aureus*  
23 cells to withstand internal turgor. Despite the absence of canonical membrane targeting  
24 domains, FemA has been shown to localise at the membrane. To study its mechanism of  
25 localisation, we constructed mutants in key residues present in the putative transferase pocket  
26 and the  $\alpha 6$  helix of FemA, possibly involved in tRNA binding. Mutations in the  $\alpha 6$  helix led to a  
27 sharp decrease in protein activity *in vivo* and *in vitro* but did not impair correct membrane  
28 localisation, indicating that FemA activity is not required for localisation. Our data indicates  
29 that, contrarily to what was previously thought, *S. aureus* cells do not survive in the absence of  
30 a pentaglycine cross bridge.

## 31 **Introduction**

32 *S. aureus* is one of the main pathogens responsible for life-threatening infections  
33 worldwide, particularly hospital- and community-acquired methicillin resistant *S. aureus*

34 strains (HA-MRSA and CA-MRSA, respectively), which constitute a major challenge to antibiotic  
35 therapy<sup>1,2</sup>. Most of the widely used, and more potent antibiotics, target steps in the  
36 biosynthesis of peptidoglycan (PG), the core component of the bacterial wall. PG is a  
37 macromolecule composed of glycan chains, where each unit is constituted of N-acetylmuramic  
38 acid (MurNAc) and N-acetylglucosamine (GlcNAc) sugars, with a stem peptide attached to  
39 MurNAc. Glycan chains are connected (crosslinked) through flexible species-specific peptide  
40 bridges, creating a mesh-like structure that envelops the cell<sup>3</sup>. The structural features of PG  
41 confer both robustness and flexibility to the cell envelope, which are necessary to withstand  
42 high pressure derived from intracellular turgor<sup>4</sup>.

43 MRSA strains are resistant to  $\beta$ -lactams, which irreversibly acylate the transpeptidase  
44 domain of Penicillin Binding Proteins (PBPs), enzymes responsible for the last steps of PG  
45 biosynthesis<sup>1</sup>. In these strains, the major determinant of methicillin resistance is the acquired  
46 *mecA* gene, which encodes for PBP2A, an enzyme insensitive to  $\beta$ -lactam acylation<sup>5</sup>. However,  
47 high-level  $\beta$ -lactam resistance is in fact dependent on several additional elements, which were  
48 initially identified by transposon mutagenesis and termed *fem* (factor essential for methicillin  
49 resistance) or *aux* (auxiliary) genes<sup>6,7</sup>. Approximately 30 *fem/aux* determinants have been  
50 identified so far and most are housekeeping genes, involved in a variety of cellular processes  
51 and probably present in every *S. aureus* strain<sup>8</sup>. Three closely related factors - *fmhB* and the co-  
52 transcribed *femA* and *femB* genes, encode for the FemX, FemA and FemB proteins,  
53 respectively, peptidyltransferases which synthesise the pentaglycine bridges used to crosslink  
54 glycan chains in *S. aureus*<sup>9,10</sup>. During the inner membrane steps of PG synthesis (see Pinho et  
55 al.<sup>11</sup> for a review), the Fem proteins sequentially transfer five glycine residues to the PG  
56 precursor lipid II using glycyl-charged tRNA molecules<sup>12</sup>. Importantly, *in vivo* and *in vitro*  
57 studies have shown that each Fem protein has strict substrate specificity: FemX adds the first  
58 glycine, FemA adds the second and the third and FemB adds the fourth and fifth glycines, and  
59 each Fem cannot substitute for another<sup>13,14</sup>. Although *fmhB* was shown to be an essential  
60 gene<sup>15</sup>, mutants carrying transposon inactivated *femA* or *femB* grew poorly but were viable,  
61 suggesting that *S. aureus* can survive with a PG composed of monoglycine crossbridges<sup>9,16</sup>.  
62 However, HPLC analysis of the PG composition in these mutants revealed an overall reduction,  
63 but not absence of crosslinked species and, importantly, monoglycyl-substituted oligomers  
64 were never found<sup>17</sup>.

65 A second study on the essentiality of *femAB* was done by Strandén and colleagues,  
66 who constructed a *femAB* mutant, AS145, by allelic replacement of the *femAB* operon by a  
67 tetracycline resistance marker<sup>18</sup>. AS145 showed impaired growth, methicillin

68 hypersusceptibility, accumulation of monoglycyl substituted PG monomers and drastically  
69 reduced crosslinking of glycan strands, when compared to the parental strain<sup>18</sup>. *Cis*-  
70 complementation of the *femAB* mutation in AS145 with wild-type *femAB* restored synthesis of  
71 the pentaglycine crossbridge and methicillin resistance, but the growth rate remained low<sup>19</sup>.  
72 Therefore the authors postulated that survival of AS145 required compensatory or suppressor  
73 mutations<sup>19</sup>. Transcriptional analysis revealed that AS145 underwent severe metabolic  
74 adaptations to survive, including upregulation of membrane transporters associated with  
75 glycerol uptake (an osmoprotectant), upregulation of the arginine-deiminase pathway (an  
76 alternative for ATP production) and downregulation of nitrogen metabolism. Collectively these  
77 data suggested that *femAB* mutants adapted to survive with shortened crossbridges by  
78 drastically reducing metabolic activity to alleviate internal turgor<sup>19</sup>.

79         The *femAB* operon and the pentaglycine crossbridges are unique features of *S. aureus*  
80 among prokaryotes. This makes FemAB proteins potentially interesting targets for MRSA-  
81 specific drug design. In this work we wanted to investigate if full depletion of the *femAB*  
82 operon is lethal in an MRSA strain and to determine the phenotypic defects associated with  
83 lack of *femAB* expression.

84

## 85 **Results and Discussion**

### 86 **The *femAB* operon is essential for the viability of *S. aureus***

87         Previous *S. aureus femAB* null mutants likely had compensatory mutations<sup>16,17,19</sup>. To  
88 evaluate the essentiality of *femAB* as well as the phenotypes resulting from FemAB depletion  
89 in a background without the existence of compensatory mutations, we constructed a  
90 conditional *femAB* mutant. The *femAB* operon of the clinically relevant CA-MRSA strain MW2  
91 was placed under the control of the IPTG inducible *Pspac* promoter. As the *Pspac* promoter is  
92 known to be leaky<sup>20</sup>, a plasmid-encoded *lacI* repressor gene was provided to decrease the  
93 basal transcription of *femAB*. The resulting strain was named MW2-iFemAB.

94         Growth of MW2-iFemAB in liquid medium supplemented with IPTG at 0, 10, 25 and  
95 500  $\mu$ M was followed for 10 hours. In the presence of 500  $\mu$ M of IPTG, growth of the  
96 conditional mutant was similar to the parental strain MW2 (Fig. 1a), therefore this  
97 concentration of inducer was used in subsequent assays. The growth rate of MW2-iFemAB  
98 decreased with decreasing IPTG concentrations and, surprisingly, no bacterial growth was  
99 observed in the absence of IPTG, indicating that this operon is essential for survival (Fig. 1a),

100 contrarily to what was previously thought. We tested the essentiality of the *femAB* operon in  
101 an HA-MRSA background – strain COL – and likewise no growth of COL-iFemAB was observed  
102 in the absence of IPTG (Supplementary Fig. 1). To assess the effect of loss of FemAB activity on  
103 PG composition, we analysed the cell wall of MW2-iFemAB cells incubated with 500, 25 or 0  
104  $\mu\text{M}$  of IPTG until bacterial growth was arrested in the non-induced culture (see Methods). As  
105 expected, the muropeptide profiles in cells depleted of FemAB show a massive accumulation  
106 of peak 4 (see Supplementary Fig. 2 for peak assignment), which was previously identified as  
107 the monomeric pentapeptide substituted with a single glycine residue<sup>21</sup>, the substrate of FemA  
108 (Fig.1b, [IPTG] 0  $\mu\text{M}$ ). This was in contrast to cells where *femAB* expression was fully induced  
109 (Fig.1b, [IPTG] 500  $\mu\text{M}$ ), where the major monomeric form present was the pentaglycine  
110 substituted monomer (peak 5). Accordingly, lack of FemAB also prevented the formation of  
111 pentaglycine crosslinked forms such as dimers (peak 11), trimers (peak 15), tetramers (peak  
112 16) and higher order forms, which co-elute near the end of the run (Fig. 1b, arrow). Low  
113 FemAB expression levels, just enough to sustain bacterial growth ([IPTG] 25  $\mu\text{M}$ ), resulted in  
114 the presence of some pentaglycine crosslinked forms (peaks 11, 15, 16, etc.). This degree of  
115 peptidoglycan structural organisation might be the minimum to ensure cell viability.

#### 116 **Loss of FemAB activity leads to membrane damage**

117 The morphology of cells depleted of FemAB was analysed by super resolution  
118 structured illumination microscopy (SIM). MW2-iFemAB was grown with or without IPTG (500  
119  $\mu\text{M}$ ). Following growth arrest of the non-induced culture (Supplementary Fig. 3, arrow), cells  
120 were stained with membrane dye FM 4-64, PG dye Van-FL and with DNA dye Hoechst 33342.  
121 In the presence of IPTG, MW2-iFemAB cells divided normally with DNA segregation preceding  
122 the synthesis of a division septum at mid-cell (Fig. 2a, top row). Cells containing multiple septa  
123 were rarely observed (<1%, N = 466 septating cells - Fig. 2b, left panel). In contrast, FemAB  
124 depleted cells often appeared as pseudomulticellular forms with two or more perpendicular  
125 septa (56%, N = 480 septating cells), suggesting that a second round of division starts before  
126 daughter cell separation is completed (Fig. 2b, right panel arrows). Furthermore, the nucleoid  
127 morphology of FemAB depleted cells was altered, with the presence of cells containing  
128 condensed DNA (Fig. 2a, bottom row asterisks). Our results are in agreement with previous  
129 reports that suppressed *fem* mutants show irregular placement of cross walls and retarded cell  
130 separation<sup>16</sup>. This phenotype can be a consequence of either multiple septation or defective  
131 splitting.

132           When cells depleted of FemAB were incubated for longer periods, we noticed a  
133 decrease in culture density, suggesting cell lysis (Supplementary Fig. 3). We therefore imaged  
134 cells 2 hours after growth arrest and observed extensive membrane damage, characterised by  
135 bulges and invaginations (Fig. 3a, arrow) and the presence of anucleate cells, indicative of loss  
136 of viability (Fig. 3a, asterisks). These results suggest that the inability of *S. aureus* to survive  
137 with shortened crossbridges could be because the three-dimensional structure of this  
138 alternate PG does not confer sufficient robustness and/or flexibility to bear the internal  
139 osmotic pressure, in these conditions, causing the cells to rupture. In order to test this  
140 hypothesis we incubated MW2-iFemAB in the absence of IPTG (to deplete FemAB expression)  
141 with increasing concentrations of NaCl added to the medium, to alleviate turgor. MW2-iFemAB  
142 was able to grow in the presence of NaCl in a dose dependent manner (Fig. 3b), confirming  
143 that in the absence of pentaglycine crosslinks, the PG layer is not able to withstand the internal  
144 pressure exerted on the membrane. This is in accordance with data from Hübscher and  
145 colleagues<sup>19</sup>, who showed by transcriptome analysis that *femAB* null mutant AS145 adapted to  
146 the FemAB deficit by tuning its metabolic pathways, presumably to reduce turgor. It is likely  
147 that monoglycine crossbridges are not suitable substrates for transpeptidation by *S. aureus*  
148 PBPs *in vivo* and thus crosslinking of glycan chains was stalled after FemAB depletion.  
149 Accordingly, solid-state NMR data obtained by Kim et al.<sup>22</sup> indicated that monoglycyl  
150 crossbridges would be too short to connect the glycan chains of the *S. aureus* PG, and that  
151 crosslinking with such a reduced bridge length would require a major rearrangement of the  
152 tertiary structure of PG<sup>22</sup>.

### 153 **FemA activity is not required for membrane localisation**

154           The Fem proteins are non-ribosomal peptidyl transferases which use dedicated amino  
155 acid charged tRNA molecules as substrates, an interesting activity seldom seen in nature<sup>12</sup>. The  
156 mechanism of this transfer is still poorly understood, as binding sites for entering tRNA  
157 molecules have not been identified. In the case of Fem proteins which transfer two amino  
158 acids, such as FemA (and FemB), the transfer of both glycines to lipid II appears to occur  
159 simultaneously rather than sequentially, judging from *in vitro* data, which may indicate that  
160 these proteins act as homodimers<sup>23</sup>. We have recently reported that all three Fem proteins of  
161 *S. aureus* localise to the membrane throughout the entire cell cycle, which was unexpected  
162 given that these proteins lack canonical transmembrane domains<sup>24</sup>. Therefore a possible  
163 mechanism of Fem localisation to the membrane could be through protein activity, which is  
164 dependent of interactions with both the substrate lipid II and glycyl-charged tRNA molecules.

165 In order to investigate the mechanism of localisation of FemA, we identified possible  
166 key regions in FemA required for activity, based on the known crystal structure of FemA<sup>23</sup> and  
167 on homology to the FemX protein from *Weisella viridescens*<sup>25,26</sup>. We decided to focus on the  
168 putative transferase pocket that contains Arg220, Phe224 and Tyr327, which are conserved  
169 across the Fem family<sup>23,26</sup>. We also mined the sequence of FemA for regions which could bind  
170 DNA/RNA using DP-Bind<sup>27,28</sup>, in order to identify the putative tRNA-binding site. We found that  
171 the region with the highest probability of binding to RNA corresponded to the  $\alpha$ 6 helix (aa 176-  
172 188) of FemA, rich in Lys/Arg residues with polar and charged sidechains exposed to the  
173 solvent<sup>23</sup>, which could stabilise the entering tRNA. Specifically, amino acids Lys180 and Arg181  
174 showed >96% probability of binding DNA/RNA in each of three individual prediction algorithms  
175 performed by DP-Bind (see Methods), and therefore were selected for mutagenesis.

176 To assess if the selected mutations had an effect on FemA transferase activity, we  
177 cloned wild-type *femA* into the pET-24b expression vector and performed site-directed  
178 mutagenesis on *femA* residues to obtain FemA mutants where the target residues were  
179 replaced by alanines. In this way, we constructed pET-FemA<sup>RF220AA</sup> and pET-FemA<sup>Y327A</sup>, in order  
180 to express mutants in the transferase domain and pET-FemA<sup>KR180AA</sup> to express a mutant of the  
181 predicted tRNA binding helix. We purified recombinant FemA<sup>wt</sup>, FemA<sup>KR180AA</sup>, FemA<sup>RF220AA</sup> and  
182 FemA<sup>Y327A</sup> with C-terminal histidine tags and synthesised the FemA substrate lipid II-Gly<sub>1</sub> *in*  
183 *vitro* (see Methods). As recombinant FemA<sup>RF220AA</sup> was very unstable and readily precipitated,  
184 we could not use it for further assays. Lipid II-Gly<sub>1</sub> was trapped in Triton X-100 micelles and  
185 incubated with either FemA<sup>wt</sup>, FemA<sup>KR180AA</sup> or FemA<sup>Y327A</sup> in the presence of [U-<sup>14</sup>C]-glycine  
186 charged tRNA. After 30, 60 or 90 minutes the lipid fraction was extracted and separated by  
187 thin layer chromatography and radioactive glycine transfer to lipid II-Gly<sub>1</sub> was measured. Both  
188 mutants FemA<sup>KR180AA</sup> and FemA<sup>Y327A</sup> transferred less [U-<sup>14</sup>C]-glycine to their substrate than  
189 FemA<sup>wt</sup>, consistent with a reduction of enzyme activity (Fig. 4a).

190 In order to both confirm loss of FemA activity *in vivo* and study protein localisation, we  
191 used the backbone of pFemAB<sup>wt</sup>, a replicative vector encoding a *femA-mCherry* fusion followed  
192 by *femB* (both under the control of a cadmium inducible promoter), to generate *femA-mCherry*  
193 alleles with the mutations described above. These expression plasmids were transformed into  
194 MW2-iFemAB, allowing us to deplete native *femAB* expression (in the absence of IPTG) and  
195 express mutant alleles (in the presence of cadmium).

196 We were able to complement the lack of *femAB* expression from the native locus by  
197 expressing *femA-mCherry-femB* from pFemAB<sup>wt</sup> in the presence of cadmium (0.1  $\mu$ M), as

198 assessed by growth rates, morphology, lysostaphin (an enzyme that cuts pentaglycine  
199 bridges<sup>29</sup>) and oxacillin MICs and muropeptide composition (Table 1). Expression of the  
200 catalytic site mutants FemA<sup>RF220AA</sup> and FemA<sup>Y327A</sup> caused a reduction of the pentaglycine  
201 substituted monomer content in peptidoglycan (Fig. 4b), although morphology was similar to  
202 wild-type and no significant differences in lysostaphin and oxacillin MICs were observed (Table  
203 1). In contrast, the double mutation in the  $\alpha 6$  helix of FemA caused severe loss of FemA  
204 activity. The FemA<sup>KR180AA</sup> mutant showed a marked reduction in growth rate, increased  
205 lysostaphin and decreased oxacillin resistances and a pseudomulticellular morphology when  
206 observed by microscopy (Table 1), similar to what was observed when depleting *femAB*  
207 expression. Furthermore, analysis of the muropeptide content in this mutant revealed a  
208 pronounced accumulation of monoglycyl substituted pentapeptides and concomitant  
209 reduction in pentaglycine crosslinked species (Fig. 4b and Table 1). Nevertheless, FemA<sup>KR180AA</sup>  
210 still localised to the membrane, similarly to FemA<sup>wt</sup>, indicating that FemA localisation is  
211 independent of protein activity (Fig. 4c). Because loss of activity in FemA<sup>KR180AA</sup> is likely due to a  
212 deficit in tRNA binding, it is possible that FemA<sup>KR180AA</sup> could still localise to the membrane  
213 through recognition of the lipid-linked peptidoglycan precursor.

214 The mutations in the substrate binding pocket had a minor effect on FemA localisation,  
215 since FemA<sup>Y327A</sup> and FemA<sup>RF220AA</sup> appeared dispersed in the cytoplasm in a small percentage of  
216 the cell population (Fig. 4c, white arrows). Nevertheless, as these mutations did not seem to  
217 decrease protein activity *in vivo* to a great extent, we could not conclude that the mechanism  
218 of FemA localisation to the membrane is via substrate recognition. An alternative possibility is  
219 that the recruitment of FemA to the membrane is mediated by protein-protein interactions  
220 with the membrane associated eukaryotic-type serine/threonine kinase Stk, a global cell wall  
221 synthesis regulator, which was recently shown to interact with FemA and FemB by bacterial  
222 two hybrid<sup>30</sup>. However, the same study could not find interactions between Stk and FemX,  
223 which initiates Lipid II crossbridge synthesis, or between FemX and FemA/B<sup>30</sup>. Further  
224 experiments are necessary to clarify the interactions of Fem proteins with each other and with  
225 their substrates, in order to understand how the localisation and timing of PG crossbridge  
226 synthesis is modulated during the cell cycle.

## 227 **Concluding remarks**

228 The structural features of the staphylococcal PG seem remarkably unique in nature, as  
229 pentaglycine crosslinks have not been observed outside of the genus. These long bridges likely  
230 confer high flexibility to *S. aureus* PG that allows a high level of PG crosslinking, which in turn

231 allows the cell to withstand high internal turgor. Accordingly, *femAB* mutants isolated in the  
232 past adapted to life with shortened crossbridges by drastically reducing metabolic activity<sup>18</sup>.  
233 Moreover, the nature and length of PG branching has been implicated in resistance to  $\beta$ -  
234 lactams, not only in *S. aureus* but also in other bacteria such as *Streptococcus pneumoniae*<sup>7,31-</sup>  
235 <sup>33</sup>.

236 We have shown that the depletion of the *femAB* operon is lethal in CA-MRSA strain  
237 MW2 and in HA-MRSA strain COL, leading to the disruption of the cell envelope, causing cells  
238 to lose viability. This suggests that monoglycyl-substituted mucopeptides are not good  
239 substrates for transpeptidation *in vivo*, either because transpeptidases fail to recognise them  
240 or because different *S. aureus* glycan strands are too far apart to be crosslinked via  
241 crossbridges with only one glycine.

242

## 243 **Methods**

### 244 **Bacterial growth conditions**

245 Strains and plasmids constructed for this study are listed in Supplementary Table 1. *S.*  
246 *aureus* strains were grown in tryptic soy broth (TSB, Difco) at 200 r.p.m with aeration at 37 °C  
247 or on tryptic soy agar (TSA, Difco) at 30 or 37 °C. *Escherichia coli* strains were grown in Luria–  
248 Bertani broth (Difco) with aeration, or Luria–Bertani agar (Difco) at 37 or 30 °C. When  
249 necessary, antibiotics ampicillin (100  $\mu$ g/ml), erythromycin (10  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml),  
250 neomycin (50  $\mu$ g/ml) or chloramphenicol (30  $\mu$ g/ml) were added to the media. Unless stated  
251 otherwise, isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG, Apollo Scientific) was used at 500  $\mu$ M  
252 to induce expression of constructs under the control of the *Pspac* promoter. Cadmium chloride  
253 (Sigma-Aldrich) was used at 0.1  $\mu$ M when required to induce expression of constructs under  
254 the control of the *Pcad* promoter.

### 255 **Construction of *S. aureus* strains**

256 In order to construct an *S. aureus* strain with the *femAB* operon under the control of  
257 an inducible promoter, a fragment containing the first 400 bp of *femA* was amplified from *S.*  
258 *aureus* MW2 DNA with primer pair spacfemab\_P1 EcoRI/spacfemab\_P2 BamHI (see  
259 Supplementary Table 2 for primers sequences), cut with EcoRI and BamHI restriction enzymes  
260 and cloned into pMUTIN4<sup>34</sup>, downstream of the *Pspac* promoter, giving plasmid pFemABi,  
261 which was sequenced. pFemABi was then propagated in DC10B cells, electroporated into



262 electrocompetent RN4220 cells, and transduced (using phage 80 $\alpha$ ) to MW2 and COL, where it  
263 integrated in the *femAB* locus by homologous recombination. The resulting strain contains a  
264 truncated copy of *femA* under the control of the *femAB* native promoter, and the *femAB*  
265 operon under the control of *Pspac*. Multicopy plasmid pMGPII<sup>35</sup>, which encodes *Pspac*  
266 repressor Lacl, was then transduced into this strain, giving rise to MW2-iFemAB and COL-  
267 iFemAB.

268 To construct *S. aureus* strains expressing mutated alleles of FemA-mCherry together  
269 with wild-type FemB, first a *femA-mCherry-STOP* codon-*femB* was amplified from  
270 pMADfemAmch<sup>24</sup> using primers pcnfemab\_P1 BamHI and pcnfemab\_P2 EcoRI. This fragment  
271 was cut with BamHI and EcoRI and cloned into replicative vector pCNX<sup>36</sup>, under the control of  
272 *Pcad*, giving plasmid pFemAB<sup>wt</sup>. pFemAB<sup>wt</sup> was then used as the template for site-directed  
273 mutagenesis using Phusion polymerase (Thermo Scientific) following manufacturer's  
274 instructions. Primers fema\_kr180aa\_fw/ fema\_kr180aa\_rev were used to generate  
275 pFemA<sup>KR180AA</sup>, encoding both K180A and R181A mutations; Primers fema\_rf220aa\_fw/  
276 fema\_rf220aa\_rev were used to generate pFemA<sup>RF220AA</sup>, encoding both R220A and F224A  
277 mutations; and primers fema\_Y327a\_fw/ fema\_Y327a\_rev were used to generate pFemA<sup>Y327A</sup>,  
278 encoding the Y327A mutation. Each plasmid was sequenced to confirm the presence of the  
279 mutations. pFemAB<sup>wt</sup>, pFemA<sup>KR180AA</sup>, pFemA<sup>RF220AA</sup> and pFemA<sup>Y327A</sup> were propagated in DC10B,  
280 electroporated into RN4220 and transduced to MW2-iFemAB, giving strains MW2pFemAB<sup>wt</sup>,  
281 MW2pFemA<sup>KR180AA</sup>, MW2pFemA<sup>RF220AA</sup> and MW2pFemA<sup>Y327A</sup>, respectively.

## 282 **Growth curves of *S. aureus* strains**

283 To assess whether the *femAB* operon is essential for viability, or the effects of FemA  
284 mutations on growth rates, overnight cultures of MW2, MW2-iFemAB, COL, COL-iFemAB,  
285 MW2pFemAB<sup>wt</sup>, MW2pFemA<sup>KR180AA</sup>, MW2pFemA<sup>RF220AA</sup> and MW2pFemA<sup>Y327A</sup> grown in TSB with  
286 500  $\mu$ M of IPTG, with the appropriate antibiotics (when applicable, at the concentrations  
287 described above) were back-diluted 1:500 in the same medium and grown until the cultures  
288 reached an OD<sub>600</sub> of 0.7. At this point the cultures were washed three times to remove IPTG  
289 and back-diluted to an OD<sub>600</sub> of 0.007 in fresh TSB containing either 0, 10, 25 or 500  $\mu$ M of  
290 IPTG or 0.06, 0.12 or 0.25 of NaCl, in the case of MW2-iFemAB and COL-iFemAB. In the case of  
291 MW2pFemAB<sup>wt</sup>, MW2pFemA<sup>KR180AA</sup>, MW2pFemA<sup>RF220AA</sup> and MW2pFemA<sup>Y327A</sup>, cells were  
292 incubated without IPTG and cadmium chloride was added at 0.1  $\mu$ M to drive the expression of  
293 either wild-type or mutant *femA* alleles from the pCNX-based plasmids.

294 Growth of all strains was monitored for 10 hours in a Bioscreen C Analyzer (Growth  
295 Curves USA), at 37 °C with shaking with OD<sub>600</sub> readings taken every 15 minutes. Growth curves  
296 were obtained from three independent experiments done with three biological replicates.

### 297 **Minimum inhibitory concentration (MIC) assays**

298 MICs of lysostaphin and oxacillin were determined by broth microdilution in sterile 96-  
299 well plates. The medium used was TSB, containing a series of two-fold dilutions of each  
300 compound. Cultures of *S. aureus* strains and mutants were added at a final density of ~5x10<sup>5</sup>  
301 CFU ml<sup>-1</sup> to each well. Wells were reserved in each plate for sterility control (no cells added)  
302 and cell viability (no compound added). Plates were incubated at 37°C. Endpoints were  
303 assessed visually after 48 h and the MIC was determined as the lowest concentration that  
304 inhibited growth. All assays were done in triplicate.

### 305 **Purification and analysis of *S. aureus* muropeptides**

306 To evaluate changes in the peptidoglycan composition caused by the depletion of the  
307 *femAB* operon, or caused by the expression of mutant FemA proteins, cells of MW2, MW2-  
308 iFemAB, MW2pFemA<sup>wt</sup>, MW2pFemA<sup>KR180AA</sup>, MW2pFemA<sup>RF220AA</sup> and MW2pFemA<sup>Y327A</sup> were first  
309 grown overnight in TSB supplemented with 500 μM of IPTG and the applicable antibiotics.  
310 Cultures were then washed three times to remove the IPTG and back-diluted 1:500 in fresh  
311 TSB with 0, 10 or 500 μM of IPTG, in the case of MW2 and MW2-iFemAB, or in the presence of  
312 cadmium chloride and absence of IPTG, in the case of MW2pFemA<sup>wt</sup>, MW2pFemA<sup>KR180AA</sup>,  
313 MW2pFemA<sup>RF220AA</sup> and MW2pFemA<sup>Y327A</sup>. Cells were collected at mid-exponential phase and PG  
314 was purified as described by Filipe et al.<sup>37</sup>. Muropeptides were prepared from PG samples by  
315 digestion with mutanolysin (0.135 U/μg of PG, from Sigma-Aldrich) and analysed by reverse  
316 phase HPLC using a Hypersil ODS (C18) column (Thermo-Fisher Scientific). Muropeptide species  
317 were eluted in 0.1 M sodium phosphate, pH 2.0, with a gradient of 5–30% methanol for 155  
318 minutes and detected at 206 nm.

### 319 ***S. aureus* imaging by fluorescence microscopy**

320 To evaluate changes in morphology caused by the depletion of the *femAB* operon, or  
321 to investigate the localisation of mutant FemA proteins, cells of MW2, MW2-iFemAB,  
322 MW2pFemA<sup>wt</sup>, MW2pFemA<sup>KR180AA</sup>, MW2pFemA<sup>RF220AA</sup> and MW2pFemA<sup>Y327A</sup> were first grown  
323 overnight in TSB supplemented with 500 μM of IPTG and the applicable antibiotics. Cultures  
324 were then washed three times to remove the IPTG and back-diluted 1:500 in fresh TSB with 0,  
325 25 or 500 μM of IPTG, in the case of MW2 and MW2-iFemAB, or in the presence of cadmium

326 chloride and absence of IPTG, in the case of MW2pFemA<sup>wt</sup>, MW2pFemA<sup>KR180AA</sup>,  
327 MW2pFemA<sup>RF220AA</sup> and MW2pFemA<sup>Y327A</sup>. Cells were grown to an OD<sub>600 nm</sub> of 0.4-0.6,  
328 harvested and then washed with phosphate buffer saline (PBS). Subsequently, cells were  
329 washed with PBS, mounted on microscope slides covered with a thin layer of 1% agarose in  
330 PBS and imaged by fluorescence microscopy.

331 To evaluate defects in cell morphology, cells were incubated with membrane dye Nile  
332 Red (10 µg/ml, Invitrogen), Hoechst 33342 (10 µg/ml, Invitrogen) and a mixture containing  
333 equal amounts of vancomycin (Sigma) and a BODIPY FL conjugate of vancomycin (Van-FL,  
334 Molecular Probes) to a final concentration of 0.8 µg/ml, for 5 minutes at room temperature.  
335 Cells were then washed three times with PBS before being spotted on the agarose pads.

336 Super-resolution Structured Illumination Microscopy (SIM) imaging was performed  
337 using an Elyra PS.1 microscope (Zeiss) with a Plan-Apochromat 63x/1.4 oil DIC M27 objective.  
338 SIM images were acquired using five grid rotations, unless stated otherwise, with 34 µm  
339 grating period for the 561 nm laser (100 mW), 28 µm period for 488 nm laser (100 mW) and  
340 23 µm period for 405 nm laser (50 mW). Images were captured using a Pco.edge 5.5 camera  
341 and reconstructed using ZEN software (black edition, 2012, version 8.1.0.484) based on a  
342 structured illumination algorithm, using synthetic, channel specific optical transfer functions  
343 and noise filter settings ranging from -6 to -8.

344 Wide-field fluorescence microscopy was performed using a Zeiss Axio Observer  
345 microscope with a Plan-Apochromat 100x/1.4 oil Ph3 objective. Images were acquired with a  
346 Retiga R1 CCD camera (QImaging) using Metamorph 7.5 software (Molecular Devices).

### 347 **Overexpression and purification of recombinant His-tagged proteins**

348 Recombinant proteins were purified essentially as described by Rohrer et al.<sup>38</sup>, with  
349 some modifications. Single colonies of BL21 (DE3) expression strains containing either plasmid  
350 pFemA<sup>B<sup>wt</sup></sup>, pFemA<sup>KR180AA</sup>B, pFemA<sup>RF220AA</sup>B or pFemA<sup>Y327A</sup>B were isolated from LA plates with  
351 kanamycin and used to inoculate LB (1 L) containing kanamycin. Cultures were grown to an  
352 OD<sub>600nm</sub> of approximately 0.6 at which point IPTG was added (final concentration 1 mM) and  
353 incubated for 3 hours with shaking (150 rpm) at 30 °C. Cells were harvested by centrifugation  
354 and washed with 50 mM sodium phosphate buffer (pH 7.5) containing 300 mM NaCl and 20%  
355 glycerol. Afterwards, cells were suspended in the same buffer, containing PMSF (final  
356 concentration, 0.1 mM) and lysozyme (final concentration, 1 mg/mL), and incubated on ice for  
357 30 min. Cells were then disrupted three times in an ultrasonicator and centrifuged for 30 min

358 at 4°C to precipitate cell debris. The resulting supernatant was purified by affinity  
359 chromatography using a Ni-NTA column (Qiagen), following manufacturer's instructions.  
360 Protein concentration was assessed using a BCA Protein Assay Kit (Pierce).

### 361 **Synthesis and purification of lipid II and lipid II-Gly<sub>1</sub>**

362 Lipid II was prepared by reacting undecaprenyl phosphate (Larodan), UDP-MurNAc-  
363 pentapeptide from *Staphylococcus simulans*, UDP-GlcNAc (Sigma) and membrane proteins of  
364 *Micrococcus luteus* as previously described<sup>14</sup>. Monoglycyl lipid II was synthesised by reacting  
365 lipid I with tRNA preparations, in the presence of enzymes FemX and GlyS, according to the  
366 method described by Schneider et al.<sup>14</sup>. Lipid intermediates were extracted from reaction  
367 mixtures with an equal volume of butanol/pyridine acetate (2:1; vol:vol; pH 4.2). Extracts were  
368 then purified by anion-exchange chromatography using a Hi-Trap DEAE FF-agarose column  
369 (Amersham Biosciences) by reverse-phase HPLC and eluted in a linear gradient from  
370 chloroform–methanol–water (2:3:1) to chloroform–methanol-300 mM ammonium  
371 bicarbonate (2:3:1). The fractions containing lipid species were identified by thin layer  
372 chromatography with chloroform–methanol–water–ammonia (88:48:10:1) as solvent<sup>39</sup>. The  
373 concentration of purified lipids was calculated by measuring inorganic phosphates released  
374 after the treatment with perchloric acid, as described previously<sup>40</sup>.

### 375 **FemA enzymatic activity assay**

376 In order to compare the activity of wild-type FemA to selected FemA mutants,  
377 enzymatic reactions were performed as described previously<sup>14</sup>. Briefly, 100 µl reactions were  
378 prepared containing 2.5 nmol of lipid II-Gly<sub>1</sub>, 10 µg of glycyl-tRNA synthetase (GlyS), 25 µg of  
379 tRNA, 2 mM ATP and 50 nmol [<sup>14</sup>C]-glycine in Tris buffer (100 mM Tris-HCl, 20 mM MgCl<sub>2</sub>, pH  
380 7.5, and 0.8% Triton X-100). Then 2.7 µg of wild-type FemA or FemA mutant protein was added  
381 and the reaction mixtures were incubated for 30, 60 or 90 minutes at 30°C. Lipid intermediates  
382 were then extracted and analysed by thin layer chromatography, as described above. Finally,  
383 the amount of [<sup>14</sup>C]-glycine transferred to lipid II-Gly<sub>1</sub> was quantified using phosphoimaging in  
384 a STORM system (GE Healthcare). Enzymatic assays were done in triplicate.

### 385 **Identification of FemA residues possibly involved in tRNA binding**

386 Identification of FemA residues which could bind glycyl-charged tRNA was performed  
387 using DP-Bind<sup>27,28</sup> (<http://lcg.rit.albany.edu/dp-bind/>), a sequence-based web server which  
388 predicts DNA/RNA binding domains in proteins based on biochemical properties of amino acids  
389 and evolutionary information. Probability maps were generated using PSI-BLAST position-

390 specific scoring matrix (PSSM) and three distinct machine learning methods that use  
391 evolutionary information: support vector machine (PSSM-SVM), kernel logistic regression  
392 (PSSM-KLR), and penalized logistic regression (PSSM-PLR). FemA residues K180 and R181 were  
393 identified as possibly part of DNA-binding domains based on strict consensus between the  
394 three methods. SwissPdb viewer/Deep view (<http://www.expasy.org/spdbv/>) was used to  
395 evaluate the structure of FemA, using file 1LRZ (doi: [10.2210/pdb1LRZ/pdb](https://doi.org/10.2210/pdb1LRZ/pdb)) deposited in the  
396 RCSB PDB by Benson et. al.

397

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## 403 Author Contributions

404 J.M.M., H.G.S. and M.G.P. designed the research. J.M.M. constructed all strains and performed  
405 all experiments with the exception of the glycine incorporation assays, which were performed  
406 by D.M. J.M.M., D.M., T.S., S.R.F. and M.G.P. analysed the data. J.M.M. and M.G.P. wrote the  
407 manuscript.

## 408 Competing interests statement

409 The authors declare no competing interests of any nature.

## 410 Data Availability

411 Source data are available from the corresponding author upon reasonable request.

412

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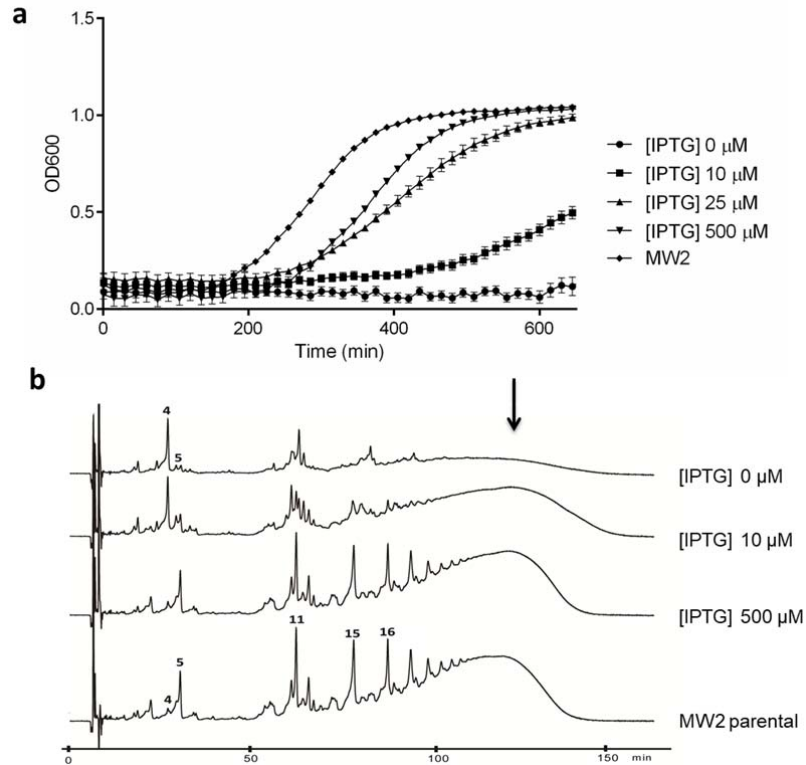
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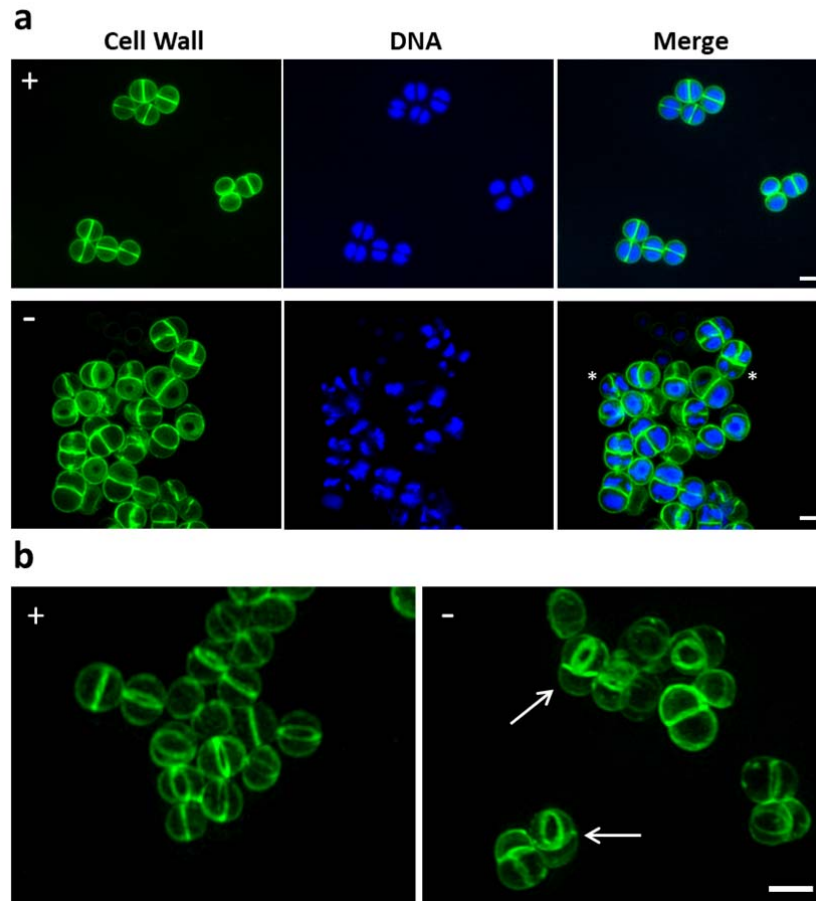




522

523 **Figure 1. FemAB are essential for cell viability in *S. aureus*.** (a), Growth curves of MW2-iFemAB strain  
524 with IPTG-inducible *femAB* operon. In the presence of high IPTG concentrations ([IPTG] 500  $\mu\text{M}$ ), growth  
525 was similar to the parental strain (MW2). Cell growth was reduced with decreasing IPTG concentrations.  
526 In the absence of IPTG ([IPTG] 0  $\mu\text{M}$ ), no cell growth was detected. Symbols indicate means and error  
527 bars indicate standard deviation from three biological replicates. (b), Muropeptide HPLC profiles of  
528 MW2-iFemAB grown in the presence of different levels of IPTG. Depletion of FemAB led to the  
529 accumulation of monomeric pentapeptides substituted with one glycine (peak 4), in contrast to  
530 pentaglycine forms (peak 5) seen in fully induced ([IPTG] 500  $\mu\text{M}$ ) or parental strain (MW2 parental)  
531 profiles (see Supplementary Fig. 2 for peak assignment). Loss of FemAB activity also impaired the  
532 formation of pentaglycine crosslinked forms such as di-, tri- and tetramers (peaks 11, 15 and 16,  
533 respectively) and higher order oligomers (black arrow). Muropeptide profiles shown are representative  
534 of three independent experiments.

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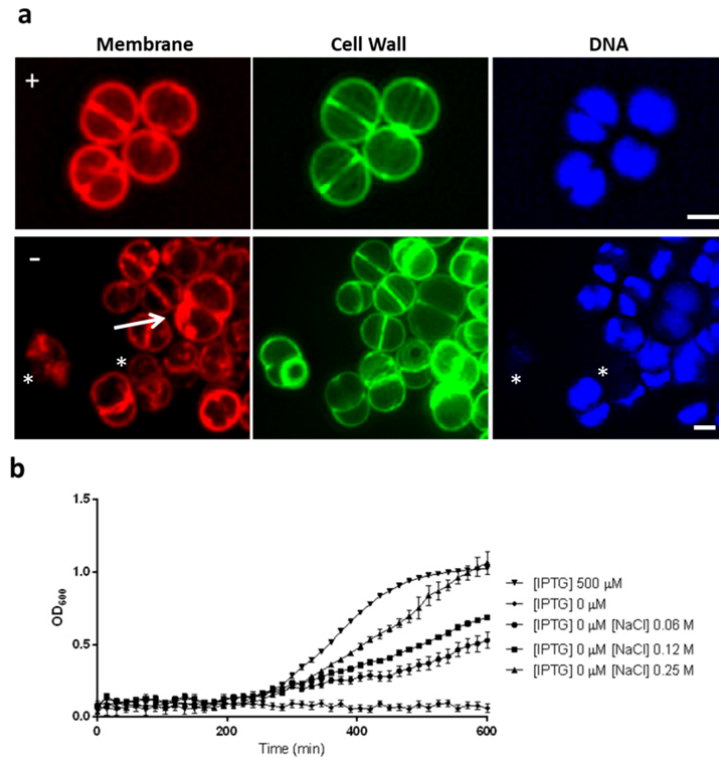


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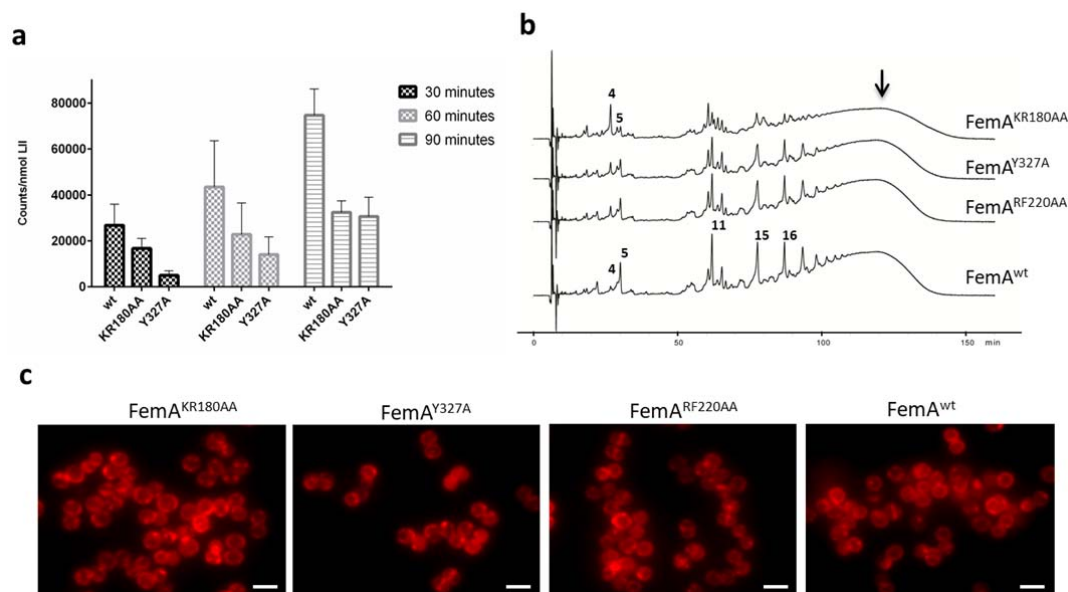
538 **Figure 2. Loss of FemAB activity inhibits daughter cell separation during division. (a)**, SIM images of  
539 MW2-iFemAB cells growing in the presence (+) or absence (-) of IPTG and labelled with cell wall dye Van-  
540 FL and DNA dye Hoechst 33342. **(b)**, 3D-SIM projections of MW2-iFemAB cells growing in the presence  
541 (+) or absence (-) of IPTG and stained with cell wall dye Van-FL. IPTG-induced cells divide normally with  
542 DNA segregation preceding the synthesis of the division septum at mid-cell (Panel **a**, top row and Panel  
543 **b**, left column). In contrast, FemAB depleted cells often had condensed nucleoids (Panel **a**, bottom row  
544 asterisks) and appeared as pseudo multicellular forms with two perpendicular septa (Panel **b**, white  
545 arrows). Scale bars, 1  $\mu\text{m}$

546



547

548 **Figure 3. FemAB activity is required to withstand internal turgor.** MW2-iFemAB cells depleted of  
549 FemAB for a period of 2 hours following growth arrest were stained with membrane dye FM 4-64, cell  
550 wall dye Van-FL and DNA dye Hoechst 33342 and imaged by SIM. FemAB depleted cells (-) show loss of  
551 membrane integrity characterised by bulging and invagination (white arrow) as well as absence of DNA  
552 staining (asterisks), indicative of loss of viability, when compared to IPTG-induced cells (+). Scale bars, 1  
553  $\mu$ m. **(b)**, Growth rates of MW2-iFemAB incubated in the presence ([IPTG] 500  $\mu$ M) or absence ([IPTG] 0  
554  $\mu$ M) of IPTG, or in the absence of IPTG with increasing NaCl concentrations. Addition of NaCl to the  
555 medium allowed cells to grow in the absence of FemAB expression, in a dose dependent manner.  
556 Symbols indicate means and error bars indicate standard deviation from three biological replicates.



557

558 **Figure 4. Selected mutations decrease FemA activity.** (a), Recombinant FemA<sup>wt</sup>, FemA<sup>KR180AA</sup> and  
 559 FemA<sup>Y327A</sup> were incubated with lipid II-Gly<sub>1</sub> in the presence of [U-<sup>14</sup>C]-glycine charged tRNA, for either  
 560 30, 60 or 90 minutes. Both FemA<sup>KR180AA</sup> and FemA<sup>Y327A</sup> showed decreased [U-<sup>14</sup>C]-glycine transfer to lipid  
 561 II-Gly<sub>1</sub> when compared to FemA<sup>wt</sup>, indicating reduced FemA activity. Columns denote mean values and  
 562 error bars represent standard deviation from 3 independent experiments. (b), muropeptide profiles of  
 563 MW2-iFemAB cells depleted of native FemAB expression and containing ectopically expressed wild-type  
 564 FemA-mCherry and FemB (FemA<sup>wt</sup>) or derivatives with mutations in FemA-mCherry (FemA<sup>KR180AA</sup>,  
 565 FemA<sup>RF220AA</sup> and FemA<sup>Y327A</sup>) and wild-type FemB, from the cadmium-inducible promoter *Pcad*. Ectopic  
 566 expression of FemA<sup>wt</sup> complemented the lack of native FemAB expression, while expression of  
 567 FemA<sup>KR180AA</sup> led to accumulation of monoglycine monomer species (peak 4) with concomitant reduction  
 568 in higher-order pentaglycine crosslinked species (peaks 11, 15, 16 and black arrow, see Supplementary  
 569 Fig. 2 for peak assignment). Expression of FemA<sup>Y327A</sup> or FemA<sup>RF220AA</sup> led to similar phenotypes, albeit to a  
 570 lesser extent. (c), fluorescence microscopy images of strains described in (b). FemA<sup>KR180AA</sup> localised to  
 571 the membrane in >95% of the cells, similarly to FemA<sup>wt</sup>. FemA<sup>Y327A</sup> and FemA<sup>RF220AA</sup> appeared dispersed  
 572 in the cytoplasm in a fraction of the population (27% and 10%, respectively, white arrows). N = 400 cells  
 573 for each strain.

574

575 **Table 1. *In vivo* activity profiles of FemA mutants.**

	Doubling time (min)	MIC lysostaphin (µg/ml)	MIC oxacillin (µg/ml)	Morphology	Gly5/Gly1 monomer species fraction
MW2pFemA <sup>KR180AA</sup>	52	2.5	0.4	defective	1:3
MW2pFemA <sup>RF220AA</sup>	30	0.15	1.6	wt	2:1
MW2pFemA <sup>Y327A</sup>	27	0.08	0.8	wt	2:1
MW2pFemA <sup>wt</sup>	27	0.08	3.2	wt	6:1
Parental MW2	25	0.08	1.6	wt	6:1

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578