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

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Bacterial cells are surrounded by cell wall, whose main component is peptidoglycan (PG), a macromolecule that withstands the internal turgor of the cell. PG composition can vary considerably between species. The Gram-positive pathogen *Staphylococcus aureus* possesses highly crosslinked PG due to the presence of cross bridges containing five glycines, which are synthesised by the FemXAB protein family. FemX adds the first glycine of the cross bridge, while FemA and FemB add the second and the third, and the fourth and the fifth glycines, respectively. Of these, FemX was reported to be essential. To investigate the essentiality of FemAB, we constructed a conditional *S. aureus* mutant of the femAB operon. Depletion of femAB was lethal, with cells appearing as pseudomulticellular forms that eventually lyse due to extensive membrane rupture. This deleterious effect was mitigated by drastically increasing the osmolarity of the medium, indicating that pentaglycine crosslinks are required for *S. aureus* cells to withstand internal turgor. Despite the absence of canonical membrane targeting domains, FemA has been shown to localise at the membrane. To study its mechanism of localisation, we constructed mutants in key residues present in the putative transferase pocket and the $\alpha_6$ helix of FemA, possibly involved in tRNA binding. Mutations in the $\alpha_6$ helix led to a sharp decrease in protein activity *in vivo* and *in vitro* but did not impair correct membrane localisation, indicating that FemA activity is not required for localisation. Our data indicates that, contrarily to what was previously thought, *S. aureus* cells do not survive in the absence of a pentaglycine cross bridge.

*S. aureus* is one of the main pathogens responsible for life-threatening infections worldwide, particularly hospital- and community-acquired methicillin resistant *S. aureus* strains (HA-MRSA and CA-MRSA, respectively), which constitute a major challenge to antibiotic therapy1,2. Most of the widely used, and more potent antibiotics, target steps in the biosynthesis of peptidoglycan (PG), the core component of the bacterial wall. PG is a macromolecule composed of glycan chains, where each unit is constituted of $N$-acetylmuramic acid (MurNAc) and $N$-acetylglucosamine (GlcNAc) sugars, with a stem peptide attached to MurNAc. Glycan chains are connected (crosslinked) through flexible species-specific peptide bridges, creating a mesh-like structure that envelops the cell3. The structural features of PG confer both robustness and flexibility to the cell envelope, which are necessary to withstand high pressure derived from intracellular turgor4.

MRSA strains are resistant to $\beta$-lactams, which irreversibly acylate the transpeptidase domain of Penicillin-Binding Proteins (PBPx), enzymes responsible for the last steps of PG biosynthesis1. In these strains, the major determinant of methicillin resistance is the acquired meca gene, which encodes for PBP2A, an enzyme insensitive to $\beta$-lactam acylation1. However, high-level $\beta$-lactam resistance is in fact dependent on several additional elements, which were initially identified by transposon mutagenesis and termed fem (factor essential for methicillin resistance) or aux (auxiliary) genes6,7. Approximately 30 fem/aux determinants have been identified so far and most are housekeeping genes, involved in a variety of cellular processes and probably present in every *S. aureus* strain8. Three closely related factors - *fmhB* and the co-transcribed *femA* and *femB* genes, encode for the
FemX, FemA and FemB proteins, respectively, peptidyl transferases which synthesise the pentaglycine bridges used to crosslink glycan chains in S. aureus. During the inner membrane steps of PG synthesis (see Pinho et al. for a review), the Fem proteins sequentially transfer five glycine residues to the stem peptide of the PG precursor lipid II using glycolyl-charged tRNA molecules. Importantly, in vivo and in vitro studies have shown that each Fem protein has strict substrate specificity: FemX adds the first glycine, FemA adds the second and the third and FemB adds the fourth and fifth glycines, and each Fem cannot substitute for another. Although fmhB was shown to be an essential gene, mutants carrying transposon inactivated femA or femB grew poorly but were viable, suggesting that S. aureus can survive with a PG composed of monoglycine crossbridges. However, HPLC analysis of the PG composition in these mutants revealed an overall reduction, but not absence of crosslinked muropeptides.

A second study on the essentiality of femAB was done by Strandén and colleagues, who constructed a femAB mutant, AS145, by allelic replacement of the femAB operon by a tetracycline resistance marker. AS145 showed impaired growth, methicillin hypersusceptibility, accumulation of monoglycyl substituted PG monomers and drastically reduced crosslinking of glycan strands, when compared to the parental strain. Cis-complementation of the femAB mutation in AS145 with wild-type femAB restored synthesis of the pentaglycine crossbridge and methicillin resistance, but the growth rate remained low. Therefore the authors postulated that survival of AS145 required compensatory or suppressor mutations. Transcriptional analysis revealed that AS145 underwent severe metabolic adaptations to survive, including upregulation of membrane transporters associated with glycerol uptake (an osmoprotectant), upregulation of the arginine-deiminase pathway (an alternative for ATP production) and alterations in nitrogen metabolism. Collectively these data suggested that femAB mutants adapted to survive with shortened crossbridges by reducing metabolic activity to alleviate internal turgor.

The femAB operon and the pentaglycine crossbridges are unique features of staphylococci. This makes FemAB proteins potentially interesting targets for MRSA-specific drug design. In this work we wanted to investigate if depletion of the FemAB proteins is lethal in an MRSA strain and to determine the phenotypic defects associated with lack of femAB expression.

Results and Discussion

The femAB operon is essential for the viability of S. aureus. Previous S. aureus femAB null mutants likely had compensatory mutations. To evaluate the essentiality of femAB, as well as the phenotypes resulting from FemAB depletion in a background without the existence of compensatory mutations, we constructed a conditional femAB mutant. The femAB operon of the clinically relevant CA-MRSA strain MW2 was placed under the control of the IPTG inducible Pspac promoter. As the Pspac promoter is known to be leaky, a plasmid-encoded lacI repressor gene was provided to decrease the basal transcription of femAB. The resulting strain was named MW2-iFemAB.

Growth of MW2-iFemAB in liquid medium supplemented with IPTG at 0, 10, 25 and 500 µM was followed for 10 hours. In the presence of 500 µM of IPTG, growth of the conditional mutant was similar to the parental strain MW2 (Fig. 1a), therefore this concentration of inducer was used in subsequent assays. The growth rate of MW2-iFemAB decreased with decreasing IPTG concentrations and, surprisingly, no bacterial growth was observed in the absence of IPTG, indicating that this operon is essential for survival (Fig. 1a), contrarily to what was previously thought. In order to confirm the essentiality of femAB, we measured colony forming units (CFUs) in cultures of MW2-iFemAB incubated in the absence of IPTG, and found severe loss of viability when compared to IPTG-induced cultures or to parental MW2 (Supplementary Fig. 1). To evaluate the possibility of polar effects on genes downstream of femAB, we introduced a plasmid encoding femAB under the control of the cadmium-inducible promoter Pcad into MW2-iFemAB. Expression of plasmid-encoded femAB was able to restore growth in cells of MW2-iFemAB background depleted of endogenous femAB (Supplementary Fig. 2).

We also tested the essentiality of the femAB operon in an HA-MRSA background – strain COL – and likewise no growth of COL-iFemAB was observed in the absence of IPTG (Supplementary Fig. 3).

To assess the effect of loss of FemAB activity on PG composition, we analysed the cell wall of MW2-iFemAB cells incubated with 500, 10 or 0 µM of IPTG until bacterial growth was arrested in the non-induced culture (see Methods). As expected, the muropeptide profiles in cells depleted of FemAB show a massive accumulation of pentaglycine crossbridges (peaks 11, 15, 16, etc.). This degree of peptidoglycan structural organisation might be the minimum to ensure cell viability.

Loss of FemAB activity leads to membrane damage. The morphology of cells depleted of FemAB was analysed by super resolution structured illumination microscopy (SIM). MW2-iFemAB was grown with or without IPTG (500 µM). Following growth arrest of the non-induced culture (Supplementary Fig. 5, arrow), cells were stained with membrane dye FM 4–64, PG dye Van-FL and DNA dye Hoechst 33342. In the presence of IPTG, MW2-iFemAB cells divided normally with DNA segregation preceding the synthesis of a division septum at mid-cell (Fig. 2a, top row). Cells containing multiple septa were rarely observed (0.3 ± 0.6%, N = 300 septating cells).
cells - Fig. 2b, left panel). In contrast, FemAB depleted cells often appeared as pseudomulticellular forms with two or more perpendicular septa (46.7 ± 9.9%, N = 300 septating cells), suggesting that a second round of division starts before daughter cell separation is completed (Fig. 2b, right panel arrows). Furthermore, the nucleoid morphology of FemAB depleted cells was altered, with the presence of cells containing condensed DNA (Fig. 2a, bottom row asterisks, 43.7 ± 5.5% in FemAB− vs 0.7 ± 0.6% in FemAB+, N = 300 cells for each condition). Our results are in agreement with previous reports showing that suppressed fem mutants show irregular placement of cross walls and retarded cell separation16. This phenotype can be a consequence of either multiple septation or defective cell splitting.

When cells depleted of FemAB were incubated for longer periods, we noticed a decrease in culture density, suggesting cell lysis (Supplementary Fig. 5). We therefore imaged cells 2 hours after growth arrest and observed extensive membrane damage, characterised by bulges and invaginations (Fig. 3a, arrow, 68.9 ± 2.0% in FemAB− vs 4.0 ± 2.6% in FemAB+, N = 300) and the presence of anucleate cells, indicative of loss of viability (Fig. 3a, asterisks, 48.0 ± 8.8% in FemAB− vs 1.0 ± 1.0% in FemAB+, N = 300). These results suggest that the inability of S. aureus to survive with shortened crossbridges could be because the three-dimensional structure of this alternate PG does not confer sufficient robustness and/or flexibility to bear the internal osmotic pressure, in these conditions, causing the cells to rupture. In order to test this hypothesis we incubated MW2-iFemAB in the absence of pentaglycine crosslinks could be because the three-dimensional structure of this alternate PG does not confer sufficient robustness and/or flexibility to bear the internal osmotic pressure, in these conditions, causing the cells to rupture. In order to test this hypothesis we incubated MW2-iFemAB in the absence of IPTG (to deplete FemAB expression) with increasing concentrations of NaCl added to the medium, to alleviate turgor. MW2-iFemAB was able to grow in the presence of NaCl in a dose dependent manner (Fig. 3b), confirming that in the absence of pentaglycine crosslinks, the PG layer is not able to withstand the internal pressure exerted on the membrane. Supplementing the medium with a different osmolyte, sucrose, also rescued cells depleted of FemAB (Supplementary Fig. 6). These results are in accordance with data from Hübscher and colleagues18, who showed by transcriptome analysis that the femAB null mutant AS145 adapted to the FemAB deficit by tuning its metabolic pathways, presumably to reduce turgor. It is likely that monoglycyl-substituted muropeptides are not suitable substrates for transpeptidation by S. aureus PBPs in vivo and thus crosslinking of glycan chains is stalled after FemAB depletion. Accordingly, solid-state NMR data obtained by Kim et al.22 indicated that monoglycyl crossbridges would be too short to connect the glycan chains of the S. aureus PG, and that crosslinking with such a reduced bridge length would require a major rearrangement of the tertiary structure of PG22.

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

In order to investigate the mechanism of localisation of FemA, we identified possible key regions in FemA required for activity, based on the known crystal structure of FemA and on homology to the FemX protein from *Weisella viridescens*. We decided to focus on the putative transferase pocket that contains Arg220, Phe224 and Tyr327, which are conserved across the Fem family. We also mined the sequence of FemA for regions which could bind DNA/RNA using DP-Bind, in order to identify the putative tRNA-binding site. We found that the region with the highest probability of binding to RNA corresponded to the α6 helix (aa 176–188) of FemA, rich in Lys/Arg residues with polar and charged sidechains exposed to the solvent, which could stabilise the entering tRNA. Specifically, amino acids Lys180 and Arg181 showed >96% probability of binding DNA/RNA in each of three individual prediction algorithms performed by DP-Bind (see Methods), and therefore were selected for mutagenesis.

**Figure 2.** Loss of FemAB activity inhibits daughter cell separation during division. (a) SIM images of MW2-iFemAB cells grown in the presence (+) or absence (−) of IPTG and labelled with cell wall dye Van-FL and DNA dye Hoechst 33342. (b) 3D-SIM projections of MW2-iFemAB cells grown in the presence (+) or absence (−) of IPTG and stained with cell wall dye Van-FL. IPTG-induced cells divide normally with DNA segregation preceding the synthesis of the division septum at mid-cell (Panel a, top row and Panel b, left column). In contrast, FemAB-depleted cells often had condensed nucleoids (Panel a, bottom row, asterisks) and appeared as pseudo multicellular forms with two perpendicular septa (Panel b, white arrows). Scale bars, 1 μm.
To assess if the selected mutations had an effect on FemA transferase activity, we cloned wild-type femA into the pET-24b expression vector and performed site-directed mutagenesis on femA residues to obtain FemA mutants where the target residues were replaced by alanines. In this way, we constructed pET-FemARF220AA and pET-FemAY327A, in order to express mutants in the transferase domain and pET-FemAKR180AA to express a mutant of the predicted tRNA binding helix. We purified recombinant FemAwt, FemAKR180AA, FemARF220AA and FemAY327A with C-terminal histidine tags and synthesised the FemA substrate lipid II-Gly1 in vitro (see Methods). As recombinant FemARF220AA was very unstable and readily precipitated, we could not use it for further assays. Lipid II-Gly1 was trapped in Triton X-100 micelles and incubated with either FemAwt, FemAKR180AA or FemAY327A in the presence of [U-14C]-glycine charged tRNA. After 30, 60 or 90 minutes the lipid fraction was extracted and separated by thin layer chromatography and radioactive glycine transfer to lipid II-Gly1 was measured. Both mutants FemAKR180AA and FemAY327A transferred less [U−14C]-glycine to their substrate than FemAwt, consistent with a reduction of enzyme activity (Fig. 4a and Supplementary Fig. 7).

In order to both confirm decrease of FemA activity in vivo and study protein localisation, we used the backbone of pFemABwt, a replicative vector encoding a femA-mCherry fusion followed by femB (both under the control of a cadmium inducible promoter), to generate femA-mCherry alleles with the mutations described above. These expression plasmids were transformed into MW2-iFemAB, allowing us to deplete native femAB expression (in the absence of IPTG) and express mutant alleles (in the presence of cadmium). We were able to complement the lack of femAB expression from the native locus by expressing femA-mCherry-femB from pFemABwt in the presence of cadmium (0.1 µM), as assessed by growth rates, morphology, lysostaphin (an enzyme that cuts pentaglycine bridges) and oxacillin MICs and muropeptide composition (Table 1). Expression of the catalytic site mutants FemARF220AA and FemAY327A caused a reduction of the pentaglycine substituted monomer content in peptidoglycan (Fig. 4b), although morphology was similar to wild-type and no major differences in lysostaphin and oxacillin MICs were observed (Table 1). In contrast, the double mutation in the α6 helix of FemA caused severe loss of FemA activity. The FemAKR180AA mutant showed a marked reduction in growth rate, increased lysostaphin and decreased oxacillin resistances and a pseudomulticellular morphology when observed by microscopy (Table 1), similar to what was observed when depleting femAB expression. Furthermore, analysis of the muropeptide content in this mutant revealed a pronounced accumulation of monoglycyl substituted pentapeptides and concomitant reduction in pentaglycine crosslinked species (Fig. 4b and Table 1). Nevertheless, FemAKR180AA still localised to the membrane, similarly to FemAwt, indicating that FemA localisation is independent of protein activity (Fig. 4c). Because loss of activity in FemAKR180AA is likely due to a
deficit in tRNA binding, it is possible that FemAKR180AA could still localise to the membrane through recognition of the lipid-linked peptidoglycan precursor.

The mutations in the substrate binding pocket had a minor effect on FemA localisation, since FemAY327A and FemARF220AA appeared dispersed in the cytoplasm in a fraction of the cell population (Fig. 4c, white arrows). Nevertheless, as these mutations did not seem to decrease protein activity \textit{in vivo} to a great extent, we could not conclude that the mechanism of FemA localisation to the membrane is via substrate recognition. An alternative possibility is that the recruitment of FemA to the membrane is mediated by protein-protein interactions with the membrane associated eukaryotic-type serine/threonine kinase Stk, a global cell wall synthesis regulator, which was recently shown to interact with FemA and FemB by bacterial two hybrid\cite{31}. However, the same study could not find interactions between Stk and FemX, which initiates Lipid II crossbridge synthesis, or between FemX and FemA/B\cite{31}. Further experiments are necessary to clarify the interactions of Fem proteins with each other and with their substrates, in order to understand how the localisation and timing of PG crossbridge synthesis is modulated during the cell cycle.

**Figure 4.** Selected mutations decrease FemA activity. (a) Recombinant FemA<sup>wt</sup>, FemA<sup>KR180AA</sup> and FemA<sup>Y327A</sup> were incubated with lipid II-Gly<sub>i</sub> in the presence of [U-<sup>14</sup>C]-glycine charged tRNA, for either 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 II-Gly<sub>i</sub> when compared to FemA<sup>wt</sup>, indicating reduced FemA activity. Columns denote mean values and error bars represent standard deviation from 3 independent experiments. For 90 min samples, \( P \) value = 0.013 for FemA<sup>wt</sup> vs FemA<sup>KR180AA</sup> and 0.007 for FemA<sup>wt</sup> vs FemA<sup>Y327A</sup> (b) Muropeptide profiles of MW2-iFemAB cells depleted of native FemAB expression and containing ectopically expressed wild-type FemA-mCherry and FemB (FemA<sup>wt</sup>) or derivatives with mutations in FemA-mCherry (FemA<sup>KR180AA</sup>, FemA<sup>RF220AA</sup> and FemA<sup>Y327A</sup>) and wild-type FemB, from the cadmium-inducible promoter \( P_{cad} \). Ectopic expression of FemA<sup>wt</sup> complemented the lack of native FemAB expression, while expression of FemA<sup>KR180AA</sup> led to accumulation of monoglycine monomer species (peak 4) with concomitant reduction in higher-order pentaglycine crosslinked species (peaks 11, 15, 16 and black arrow, see Supplementary Fig. 4 for peak assignment). Expression of FemA<sup>Y327A</sup> or FemA<sup>RF220AA</sup> led to similar phenotypes, albeit to a lesser extent. (c) Fluorescence microscopy images of strains described in (b). FemA<sup>KR180AA</sup> localised to the membrane in >95% of the cells, similarly to FemA<sup>wt</sup>. FemA<sup>Y327A</sup> and FemA<sup>RF220AA</sup> appeared dispersed in the cytoplasm in a fraction of the population (27% and 10%, respectively, white arrows). \( N = 400 \) cells for each strain.

**Table 1.** \textit{In vivo} activity profiles of FemA mutants.

|               | Doubling time (min) | MIC lysostaphin (µg/ml) | MIC oxacillin (µg/ml) | Morphology | Gly5/Gly1 monomer species ratio |
|---------------|---------------------|------------------------|-----------------------|------------|--------------------------------|
| 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                            |
Concluding Remarks

The structural features of the staphylococcal PG seem remarkably unique in nature, as pentaglycine crosslinks have not been observed outside of the genus. These long bridges likely confer high flexibility to *S. aureus* PG that allows a high level of PG crosslinking, which in turn allows the cell to withstand high internal turgor. Accordingly, *femAB* mutants isolated in the past adapted to life with shortened crossbridges by reducing metabolic activity\(^\text{16}\). Moreover, the nature and length of PG branching has been implicated in resistance to β-lactams, not only in *S. aureus*, but also in other bacteria such as *Streptococcus pneumoniae*\(^\text{32–34}\).

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

Methods

Bacterial growth conditions. Strains and plasmids constructed for this study are listed in Supplementary Table 1. *S. aureus* strains were grown in trypsic soy broth (TSB, Difco) at 200 r.p.m with aeration at 37 °C or on trypsic soy agar (TSA, Difco) at 30 or 37 °C. *Escherichia coli* strains were grown in Luria–Bertani broth (Difco) with aeration, or Luria–Bertani agar (Difco) at 37 or 30 °C. When necessary, antibiotics ampicillin (100 μg/ml), erythromycin (10 μg/ml), kanamycin (30 μg/ml), neomycin (50 μg/ml) or chloramphenicol (30 μg/ml) were added to the media. Unless stated otherwise, isopropyl β-D-1-thiogalactopyranoside (IPTG, Apollo Scientific) was used at 500 μM to induce expression of constructs under the control of the Psac promoter. Cadmium chloride (Sigma-Aldrich) was used at 0.1 μM when required to induce expression of constructs under the control of the Pcad promoter.

Construction of *S. aureus* strains. In order to construct an *S. aureus* strain with the femAB operon under the control of an inducible promoter, a fragment containing the first 400 bp of *femA* was amplified from *S. aureus* MW2 DNA with primer pair spacfemab_P1 EcoRI/spacfemab_P2 BamHI (see Supplementary Table 2 for primer sequences), cut with EcoRI and BamHI restriction enzymes and cloned into pMUTIN4\(^\text{35}\), downstream of the promoter of an inducible promoter, a fragment containing the first 400 bp of *femB* (see Supplementary Table 2 for primer sequences), cut with EcoRI and BamHI restriction enzymes and cloned into pMUTIN4\(^\text{35}\), downstream of the promoter of an inducible promoter. The resulting strains contain a truncated copy of *femA* under the control of the *femAB* native promoter, and the *femAB* operon under the control of Psac. Multicopy plasmid pMGPII\(^\text{36}\), which encodes Psac repressor LacI, was then transduced into these strains, giving rise to MW2-iFemAB and COL-iFemAB.

To construct *S. aureus* strains expressing mutated alleles of FemA-mCherry together with wild-type FemB, first a fragment containing *femA*-mCherry-STOP codon-femB was amplified from pMADfemAmChm\(^\text{39}\) using primers pcentfemab_P1 BamHI and pcentfemab_P2 EcoRI. This fragment was cut with BamHI and EcoRI and cloned into replicative vector pCNX\(^\text{37}\), under the control of Pcad, giving plasmid pFemAB\(^\text{38}\). pFemAB\(^\text{38}\) was then used as the template for site-directed mutagenesis using Phusion polymerase (Thermo Scientific) following manufacturer’s instructions. Primers fema_kr180aa_fw/ fema_kr180aa_rev were used to generate pFemAKR180AB, encoding both KR180AA and KR180AA mutations; and primers fema_y327aa_fw/ fema_y327aa_rev were used to generate pFemAY327AB, encoding the Y327A mutation. Each plasmid was sequenced to confirm the presence of the mutations. pFemAB\(^\text{38}\), pFemAKR180AAB, pFemAY327AB, and empty vector pCNX were propagated in DC10B, electroporated into electrocompetent RN4220 cells, and transduced (using phage 80α) to MW2 and COL, where it was integrated in the *femAB* locus by homologous recombination. The resulting strains contain a truncated copy of *femA* under the control of the *femAB* native promoter, and the *femAB* operon under the control of Psac. Multicopy plasmid pMGPII\(^\text{36}\), which encodes Psac repressor LacI, was then transduced into these strains, giving rise to MW2-iFemAB and COL-iFemAB.

Growth curves of *S. aureus* strains. To assess whether the *femAB* operon is essential for growth, or the effects of *femA* mutations on growth rates, overnight cultures of MW2, MW2-iFemAB, COL, COL-iFemAB, MW2pFemAB\(^\text{38}\), MW2pFemAKR180AAB, MW2pFemARF220AAB, and MW2pFemAY327A were grown in TSB with 500 μM of IPTG, with the appropriate antibiotics (when applicable, at the concentrations described above), back-diluted 1:500 in the same medium and grown until the cultures reached an OD\(_{600}\) of 0.7. At this point the cultures were washed three times to remove IPTG and back-diluted to an OD\(_{600}\) of 0.007 in fresh TSB containing either 0, 10, 25 or 50 μM of IPTG, or 0.06, 0.12 or 0.25 M of NaCl, or 0.25% (w/v) sucrose, in the case of MW2-iFemAB and COL-iFemAB. In the case of MW2pFemAB\(^\text{40}\), MW2pFemAKR180AAB, MW2pFemARF220AAB, MW2pFemAY327A and MW2pCNX, cells were incubated without IPTG and cadmium chloride was added at 0.1 μM to drive the expression of either wild-type or mutant *femA* alleles from the pCNX-based plasmids.

Growth of all strains was monitored for 10 hours in a Bioscreen C Analyzer (Growth Curves USA), at 37 °C with shaking with OD\(_{600}\) readings taken every 15 minutes. Growth curves were obtained from three independent experiments done with three biological replicates.

Viability Assays. To assess the effect of *femAB* depletion on *S. aureus* cell viability, overnight cultures of MW2 and MW2-iFemAB grown with IPTG and the appropriate antibiotics were washed three times to remove the IPTG and back-diluted 1:2000 in fresh TSB supplemented with 500 μM of IPTG or no IPTG. Bacterial growth was monitored for 8 hours by measuring OD\(_{600}\) and 100 μl of appropriate dilutions were plated on TSA (MW2) or TSA supplemented with IPTG at 500 μM (MW2-iFemAB), at 1 hour intervals for 8 hours. Following overnight incubation, colony forming units (CFUs) on each plate were counted. In each experiment, CFUs were counted in a total of 6 plates, corresponding to three technical replicates for each of 2 relevant dilutions. These assays were done in triplicate.
Minimum inhibitory concentration (MIC) assays. MICs of lysostaphin and oxacillin were determined by broth microdilution in sterile 96-well plates. The medium used was TSB, containing a series of two-fold dilutions of each compound. Cultures of *S. aureus* strains and mutants were added at a final density of ~5 × 10^5 CFU ml^-1^ to each well. Wells were reserved in each plate for sterility control (no cells added) and cell viability (no compound added). Plates were incubated at 37 °C. Endpoints were assessed visually after 48 h and the MIC was determined as the lowest concentration that inhibited growth. All assays were done in triplicate.

Purification and analysis of *S. aureus* muropeptides. To evaluate changes in the peptidoglycan composition caused by the depletion of the femA operon, or caused by the expression of mutant FemA proteins, cells of MW2, MW2-iFemAB, MW2pFemA^wt^, MW2pFemA^K180AA^, MW2pFemA^K220AA^ and MW2pFemA^Y327A^ were first grown overnight in TSB supplemented with 500 μM of IPTG and the applicable antibiotics. Cultures were then washed three times to remove the IPTG and back-diluted 1:500 in fresh TSB with 0, 10 or 500 μM of IPTG, in the case of MW2 and MW2-iFemAB, or in the presence of cadmium chloride and absence of IPTG, in the case of MW2pFemA^wt^, MW2pFemA^K180AA^, MW2pFemA^K220AA^ and MW2pFemA^Y327A^. Cells were collected at mid-exponential phase and PG was purified as described by Filipe et al.8. Muropeptides were prepared from PG samples by digestion with mutanolysin (0.135 μg of PG, from Sigma-Aldrich) and analysed by reverse phase HPLC using a Hypersil ODS (C18) column (Thermo-Fisher Scientific). Muropeptide species were eluted in 0.1 M sodium phosphate, pH 2.0, with a gradient of 5–30% methanol for 155 minutes and detected at 206 nm. The proportion of pentaglycine to monoglycine substituted monomers (Gly5/Gly1 ratio) in muropeptide preparations was calculated using the areas corresponding to half of the width of peaks 5 (Gly5) and 4 (Gly1) from the obtained chromatogram. To determine the molecular weights of muropeptides in peaks 4 and 5, muropeptide samples were separated by reverse phase HPLC using two Chromolith® Performance RP-8 endcapped 100–4.6 monolithic columns (Merck Millipore) in tandem with a linear gradient from 0.01% (v/v) Trifluoroacetic acid (TFA) to 0.01% (v/v) TFA 15% (v/v) acetonitrile over 58 minutes followed by a wash step at 0.01% (v/v) TFA 50% (v/v) acetonitrile for 6 minutes at a flow of 1 mL/min at 30 °C. Sample elution was monitored by recording the absorbance at 206 nm. Muropeptide peaks 4 and 5 were collected, the eluent was evaporated at 30 °C using a SpeedVac and then resuspended in water, prior to MS analysis by MicroLC-MS using an Eksigent LC4500 coupled to TripleTOF 6600 with Dual spray ion source.

*S. aureus* imaging by fluorescence microscopy. To evaluate changes in morphology caused by the depletion of the FemA operon, or to investigate the localisation of mutant FemA proteins, cells of MW2, MW2-iFemAB, MW2pFemA^wt^, MW2pFemA^K180AA^, MW2pFemA^K220AA^ and MW2pFemA^Y327A^ were first grown overnight in TSB supplemented with 500 μM of IPTG and the applicable antibiotics. Cultures were then washed three times to remove the IPTG and back-diluted 1:500 in fresh TSB with 0, 25 or 500 μM of IPTG, in the case of MW2 and MW2-iFemAB, or in the presence of cadmium chloride and absence of IPTG, in the case of MW2pFemA^wt^, MW2pFemA^K180AA^, MW2pFemA^K220AA^ and MW2pFemA^Y327A^. Cells were grown to an OD600nm of approximately 0.6 at which point IPTG was added (final concentration 1 mM) and incubated for 3 hours with shaking (150 rpm) at 30 °C. Cells were harvested by centrifugation and washed with 50 mM sodium phosphate buffer (pH 7.5) containing 300 mM NaCl and 20% glycerol. Afterwards, cells were suspended in the same buffer, containing phenylmethylsulfonyl fluoride (PMSF, final concentration, 0.1 mM) and lysozyme (final concentration, 1 mg/mL), and incubated on ice for 30 min. Cells were then disrupted three times in an ultrasonicator and centrifuged for 30 min at 4 °C to precipitate cell debris. The resulting supernatant was purified by affinity chromatography using a Ni-NTA column (Qiagen), following manufacturer’s instructions. Protein concentration was assessed using a BCA Protein Assay Kit (Pierce).

Overexpression and purification of recombinant His-tagged proteins. Recombinant proteins were purified essentially as described by Rohrer et al.39, with some modifications. Single colonies of BL21 (DE3) expression strains containing either plasmid pET-FemA^wt^, pET-FemA^K180AA^, pET-FemA^K220AA^ or pET-FemA^Y327A^ were isolated from LA plates with kanamycin and used to inoculate LB (1 L) containing kanamycin. Cultures were grown to an OD600 of approximately 0.6 at which point IPTG was added (final concentration 1 mM) and incubated for 3 hours with shaking (150 rpm) at 30 °C. Cells were harvested by centrifugation and washed with 50 mM sodium phosphate buffer (pH 7.5) containing 300 mM NaCl and 20% glycerol. Afterwards, cells were suspended in the same buffer, containing phenylmethylsulfonyl fluoride (PMSF, final concentration, 0.1 mM) and lysozyme (final concentration, 1 mg/mL), and incubated on ice for 30 min. Cells were then disrupted three times in an ultrasonicator and centrifuged for 30 min at 4 °C to precipitate cell debris. The resulting supernatant was purified by affinity chromatography using a Ni-NTA column (Qiagen), following manufacturer’s instructions. Protein concentration was assessed using a BCA Protein Assay Kit (Pierce).

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

**FemA enzymatic activity assay.** In order to compare the activity of wild-type FemA to selected FemA mutants, enzymatic reactions were performed as described previously. Briefly, 100 µl reactions were prepared containing 2.5 nmol of lipid II–Glyγ, 10 µg of glycyl-tRNA synthetase (GlyS), 25 µg of tRNA, 2 mM ATP and 50 nmol [14C]-glycine in Tris buffer (100 mM Tris-HCl, 20 mM MgCl2, pH 7.5, and 0.8% Triton X-100). Then 2.7 µg of wild-type FemA or FemA mutant protein was added and the reaction mixtures were incubated for 30, 60 or 90 minutes at 30°C. Lipid intermediates were then extracted and analysed by thin layer chromatography, as described above. Finally, the amount of [14C]-glycine transferred to lipid II–Glyγ, was quantified using phospho-imaging in a STORM system (GE Healthcare). Enzymatic assays were done in triplicate.

**Identification of FemA residues possibly involved in tRNA binding.** Identification of FemA residues which could bind glycyl-charged tRNA was performed using DP-Bind [28,29](http://lcg.rit.albany.edu/dp-bind/), a sequence-based web server which predicts DNA/RNA binding domains in proteins based on biochemical properties of amino acids and evolutionary information. Probability maps were generated using PSI-BLAST position-specific scoring matrix (PSSM) and three distinct machine learning methods that use evolutionary information: support vector machine (PSSM-SVM), kernel logistic regression (PSSM-KLR), and penalized logistic regression (PSSM-PLR). FemA residues K180 and R181 were identified as possibly part of DNA-binding domains based on strict consensus between the three methods. SwissPdb viewer/Deep view ([http://www.expasy.org/spdbv/](http://www.expasy.org/spdbv/)) was used to evaluate the structure of FemA, using file 1LRZ (doi: 10.2210/pdb1LRZ/pdb) deposited in the RCSB PDB by Benson et al.

**Data Availability**

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

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**Acknowledgements**
This study was funded by the European Research Council through grant ERC-2017-CoG 771709 (to M.G.P.), by Project LISBOA-01-0145-FEDER-007660 Microbiologia Molecular, Estrutural e Celular (to ITQB/NOVA), by the German Research Foundation (DFG; SCHN1284/1-2) to T.S. and FCT fellowship SFRH/BD/71993/2010 (J.M.M.). Mass spectrometry data of muropeptides was acquired by the Mass Spectrometry Unit (UniMS), ITQB/iBET, Oeiras, Portugal.

**Author Contributions**
J.M.M., H.G.S. and M.G.P. designed the research. J.M.M. constructed all strains and performed all experiments with the exception of the glycine incorporation assays, which were performed by D.R. and muropeptide purification for mass spec analysis and viability curves, which were performed by G.C. J.M.M., G.C., D.R., T.S., S.R.F. and M.G.P. analysed the data. J.M.M. and M.G.P. wrote the manuscript.

**Additional Information**
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-41461-1.

**Competing Interests:** The authors declare no competing interests.

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