Mutation of lipoprotein processing pathway gene *lspA* or inhibition of LspA activity by globomycin increases MRSA resistance to β-lactam antibiotics

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Running title: Lipoprotein processing and β-lactam susceptibility in MRSA

Abstract

The *Staphylococcus aureus* cell envelope comprises numerous components, including peptidoglycan (PG), wall teichoic acids (WTA), lipoteichoic acids (LTA), targeted by antimicrobial drugs. MRSA resistance to methicillin is mediated by the *mecA*-encoded β-lactam-resistant transpeptidase, penicillin binding protein 2a (PBP2a). However, PBP2a-dependent β-lactam resistance is also modulated by the activity of pathways involved in the regulation or biosynthesis of PG, WTA or LTA. Here, we report that mutation of the lipoprotein signal peptidase II gene, *lspA*, from the lipoprotein processing pathway, significantly increased β-lactam resistance in MRSA. Mutation of *lgt*, which encodes diacylglycerol transferase (Lgt) responsible for synthesis of the LspA substrate did not impact β-lactam susceptibility. Consistent with previous reports, *lgt* and *lspA* mutations impaired growth in chemically defined media, but not in complex broth. MRSA exposure to the LspA inhibitor globomycin also increased β-lactam resistance. Mutation of *lgt* in an *lspA* background restored β-lactam resistance to wild type. The *lspA* mutation had no effect on PBP2a expression, PG composition or autolytic activity indicating a potential role for WTA or LTA. The *lspA* and *lgt* mutants exhibited marginally increased resistance to the D-alanine pathway inhibitor D-cycloserine. In addition, mutation of *lgt* and multicopy *lspA* expression, but not mutation of *lspA,* significantly increased susceptibility to the lipoteichoic acid synthase inhibitor Congo red revealing complex interplay between lipoprotein processing mutations and the expression/stability of cell surface glycopolymers. These findings indicate that accumulation of the LspA substrate, diacylglyceryl lipoprotein, increases MRSA resistance to β-lactam antibiotics through impacts on cell envelope components other than PG.

44 **Introduction**

45 The cell envelope of *Staphylococcus aureus* comprises a cytoplasmic membrane surrounded
46 by a thick peptidoglycan layer, cell wall-anchored proteins, lipoteichoic acids (LTA), wall
47 teichoic acids (WTA) and cell surface proteins. Accurate biosynthesis, assembly and stability
48 of these cell envelope components is essential for the growth and pathogenesis of *S. aureus*,
49 and is the target of numerous antimicrobial agents (1). The peptidoglycan layer determines
50 cell shape and protects the cell from osmotic lysis, cell surface proteins have important roles
51 in adhesion, biofilm formation, and immune evasion, and teichoic acids are involved in
52 protecting the cell from the activity of cationic antimicrobial peptides.
53 Methicillin resistance in MRSA is mediated by the *mecA*-encoded, low-affinity penicillin-
54 binding protein 2a (PBP2a) carried on the mobile staphylococcal cassette chromosome *mec*
55 resistance (HeR) under laboratory growth conditions. HeR strains can become highly or
56 homogeneously resistant (HoR) after selection on elevated β-lactam concentrations via
57 poorly understood mechanisms, which require accessory mutations at other chromosomal
58 loci frequently associated with activation of the stringent response, cyclic-di-adenosine
59 monophosphate (c-di-AMP) signalling pathway (2-5), the activity of RNA polymerase (6) and
60 the ClpXP chaperone-protease complex (7,8). In addition, methicillin susceptible *S. aureus*
61 strains (MSSA) lacking *mecA* can also acquire low-level resistance through adaptive mutations
62 impacting the c-di-AMP signalling pathway and ClpXP activity (9).
63 Bacterial lipoproteins are a class of lipid-modified membrane proteins, involved in a range of
64 diverse functions such as; nutrient acquisition (10), signal transduction (11), respiration (12),
65 protein folding (13), virulence (14), antibiotic resistance (15) and host invasion (16). Mature
66 lipoproteins are composed of lipid moieties, specifically acyl groups, linked to the N-terminal
67 of a protein. The hydrophobic nature of the acyl groups serves as a membrane anchor for the
68 lipoprotein (17). In Gram-negative bacteria, lipoproteins reside in both the cytoplasmic and
69 outer membranes, while in Gram-positive bacteria they are anchored in the outer leaflet of
70 the cytoplasmic membrane and the protein portion may extend into the cell wall and beyond
71 (18).
72 Lipoprotein genes are estimated to comprise 1-3% to all genes in bacterial genomes (18).
73 While many lipoproteins have been identified and experimentally validated, others are
74 putatively identified using predictive software, thus their functions remain unknown (19). A

75 recent bioinformatic evaluation of Staphylococcal lipoproteins in the MRSA strain USA300
76 identified 67 lipoproteins, comprising 2.57% of all genes (18). When grouped by function, 25
77 of the 67 lipoproteins were implicated in ion (notably iron) and nutrient transport, 8 were
78 ascribed miscellaneous functions including sex pheromone biosynthesis, respiration,
79 chaperone-folding, and protein translocation and 15 were classified as tandem lipoproteins,
80 of which 9 are "lipoprotein-like" lipoproteins, known to play a role in host cell invasion (16).
81 The remaining 19 lipoproteins were not assigned any known function.
82 Lipoproteins are synthesised in a precursor form called preprolipoproteins. The N-terminal
83 domain includes a type II signal peptide, approximately 20 amino acids in length (20), which
84 enables translocation of preprolipoproteins to the cytoplasmic membrane, predominantly via
85 the general secretory (Sec) pathway (21). The signal peptide has 3 distinct domains; a
86 positively charged N domain, a hydrophobic H domain and a C-terminal lipobox. The lipobox
87 is comprised of a conserved 3-amino acid sequence $[LVI]_{-3}$ $[ASTVI]_{-2}$ $[GAS]_{-1}$ in front of an
88 invariant cysteine residue $[C]_{+1}$ (22,23). This lipobox serves as a recognition site for enzymes
89 of the lipoprotein processing pathway, enabling lipid modification of the cysteine residue, and
90 cleavage of the signal peptide between the amino acid at position -1 and the +1 cysteine (22).
91 The first enzyme in the lipoprotein processing pathway is diacylglycerol transferase (Lgt)
92 which covalently attaches a diacylglycerol molecule from phosphatidyl glycerol onto the
93 sulfhydryl group of the invariant cysteine, resulting in a prolipoprotein (24). This diacylglycerol
94 serves as a membrane anchor. Next, the type II lipoprotein signal peptidase (Lsp) cleaves the
95 signal peptide between the amino acid at position -1 and +1, leaving the invariant cysteine
96 residue as the new terminal amino acid (25). Lgt and Lsp are conserved in all bacterial species.
97 In Gram-negative bacteria, a third step is catalysed by the enzyme N-acyl transferase (Lnt),
98 which transfers an N-acyl group onto the invariant cysteine residue at the N-terminal of the
99 protein (26). Lnt homologs have been identified in high-GC Gram-positive bacteria (27) but
100 not in low-GC Firmicutes. Despite the lack of an apparent Lnt homolog, N-acylated
101 lipoproteins have been identified in *S. aureus* (28) and recent work has identified two novel
102 non-contiguous genes *lnsA* and *lnsA* which catalyse the N-terminal acylation of lipoproteins
103 in *S. aureus* (29). The membrane metalloprotease Eep and the EcsAB transporter were shown
104 to be involved in the processing and export of linear peptides, including the signal peptide
105 cleaved by LspA in the lipoprotein processing pathway (30-32). Lgt and Lsp are essential for
106 the viability of Gram-negative bacteria (33). In contrast, *lgt* and *lspA* mutations do not impact

107 viability in Gram-positive bacteria (34), but are associated with changes in growth,
108 immunogenicity (35) and virulence (14) phenotypes.
109 In this study, we characterised the impact of *lspA and lgt* mutations, alone and in
110 combination, on susceptibility to β-lactams, D-cycloserine and Congo red, growth, PBP2a
111 expression, peptidoglycan structure, and autolytic activity in MRSA. The impact of
112 globomycin, which is known to inhibit LspA activity, on β-lactam susceptibility was also
113 characterised. Our data suggest that accumulation of the LspA substrate, diacylglyceryl-
114 prolipoprotein, modulates resistance to β-lactam antibiotics in MRSA.

115 **Results**
116
117 **Mutation of *lspA* in MRSA increases resistance to β-lactam antibiotics.** The Nebraska
118 Transposon Mutant Library (NTML) (36) was screened to identify mutants exhibiting altered
119 susceptibility to cefoxitin, which is recommended as a surrogate for measuring *mecA*-
120 mediated oxacillin resistance in clinical laboratories, in accordance with Clinical and
121 Laboratory Standards Institute (CLSI) guidelines for disk diffusion susceptibility assays.
122 Mutants identified by this screen included NE869 (*yjbH)* (37), NE1909 (*sagA*) (38) and NE810
123 (*cycA*)(39), all of which have previously been implicated in β-lactam resistance. A new mutant
124 identified in this screen was NE1757 (*lspA*::Tn), which exhibited increased resistance to
125 cefoxitin (Fig 1A, B). PCR was used to confirm the presence of *lspA*::Tn allele in NE1757 (data
126 not shown). Using E-test strips, as described in the methods, oxacillin MIC of the *lspA* mutant
127 NE1757 was found to be 128 - 256 µg/ml, compared to 32 - 64 µg/ml for JE2 (Fig. 1C). Two
128 JE2 transductants carrying the *lspA*::Tn allele from NE1757 also exhibited increased resistance
129 to oxacillin (Fig. 1C).
130 The increased oxacillin resistance phenotype of NE1757 was also complemented by the
131 introduction of a plasmid (pLI50)-borne copy of the wild type *lspA* gene (p*lspA*) into the
132 mutant. Growth of JE2, NE1757, NE1757 pLI50 and NE1757 p*lspA* on MHA 2% NaCl
133 supplemented with oxacillin 32 µg/ml visually demonstrated that carriage of the
134 complementation plasmid reversed the increased oxacillin resistance phenotype of NE1757
135 (Fig. S1). Measurement of oxacillin MICs by agar dilution showed that NE1757 and NE1757
136 pLI50 had MICs of 256 µg/ml, while JE2 and the complemented strain NE1757 p*lspA* had MICs
137 of 64 µg/ml (Table 1).
138 Comparison of JE2 and NE1757 growth in MHB, MHB 2% NaCl, TSB and TSB 0.5 mg/ml oxacillin
139 revealed no significant differences (Fig. S2). Similarly, population analysis profiling revealed
140 that the heterogeneous pattern of oxacillin resistance expressed by JE2 was unchanged in
141 NE1757 (Fig. S3). These observations indicate that the increased β-lactam resistance
142 phenotype of NE1757 was not attributable to any growth advantage or change in the
143 heterogeneous/homogeneous oxacillin resistance profile.
144 Comparative WGS analysis confirmed that the only change in the NE1757 genome was the
145 insertion of the *Bursa aurealis* transposon in the *lspA* gene at position 1192002 (Table S1) and
146 there were no SNPs present. The NE1757 genome was also checked manually for zero

147 coverage regions to confirm the absence of any large deletions and insertions. Taken together
148 these data indicate that mutation of *lspA*, which encodes lipoprotein signal peptidase II in the
149 lipoprotein processing pathway (Fig. 2), increases resistance to oxacillin and cefoxitin in JE2.
150
151 **Mutation of *lspA* does not affect PBP2a expression or peptidoglycan structure and**
152 **crosslinking.** One of the numerous lipoproteins processed by LspA is PrsA, a chaperone and
153 foldase protein, reported to play a role in PBP2a folding and β-lactam resistance (40). To
154 investigate if improper processing of PrsA, or another lipoprotein, in the *lspA* mutant
155 impacted on PBP2a, Western blotting was used to compare PBP2a expression in JE2, NE1757
156 and NE1757 p*lspA.* The MSSA strain 8325-4 was included as a *mecA*-negative control. Growth
157 of JE2 and NE1757 in MHB without 2% NaCl at 37°C (data not shown) or MHB with 2% NaCl
158 at 35°C supplemented with 0.5 μg/ml oxacillin (Fig. 3A) revealed similar levels of PBP2a
159 expression in all strains.
160 Quantitative peptidoglycan compositional analysis was performed using UPLC analysis of
161 muramidase-digested muropeptide fragments extracted from exponential or stationary
162 phase cultures of JE2 and NE1757 grown in MHB or MHB supplemented with oxacillin 3 mg/ml
163 or 32 μg/ml. The PG profile of JE2 and the *lspA* mutant NE1757 were similar under all growth
164 conditions tested (Fig. 3B). Thus, supplementation of MHB with oxacillin was associated with
165 a significant changes in muropeptide oligomerization and reduced crosslinking, but these
166 effects were the same in both JE2 and NE1757 (Fig. S4). The total PG concentrations extracted
167 from JE2 and NE1757 cell pellets was also the same (data not shown). Comparison of Triton
168 X-100-induced autolysis in JE2 and NE1757 also revealed identical autolytic profiles (Fig. S5).
169 Finally, the NaCl tolerance phenotypes of JE2 and NE1757 were also similar (Fig. S6), indicating
170 that c-di-AMP signalling, which has previously been implicated in the control of β-lactam
171 resistance, autolytic activity and NaCl tolerance (4,5) was unaffected by the *lspA* mutation.
172 These data indicate that increased β-lactam resistance in the *lspA* mutant was not associated
173 with significance changes in PG abundance, structure, crosslinking, c-di-AMP signalling or
174 autolytic activity.
175
176 **Exposure to the LspA inhibitor globomycin also increases β-lactam resistance.** Globomycin
177 is a natural peptide antibiotic, first discovered in 1978, produced by 4 different strains of
178 actinomycetes (41,42). It is an inhibitor of LspA and works by sterically blocking the active site

179 of the enzyme (43). Globomycin has moderate to strong antibacterial activity against many
180 Gram-negative species and has been proposed to cause disruption of cell surface integrity
181 (44). However, despite its ability to inhibit LspA, globomycin does not have significant
182 antimicrobial activity against Gram-positive bacteria including *S. aureus*, with MICs >100
183 μg/ml (41,42,45).
184 Because disruption of *lspA* increased resistance to β-lactams, we hypothesized that chemical
185 inhibition of LspA by globomycin may also be associated with increased β-lactam resistance.
186 To test this hypothesis, the susceptibility of JE2 and NE1757 to oxacillin was determined in
187 the presence or absence of globomycin. A series of JE2 and NE1757 cultures grown in MHB
188 2% NaCl were used to determine that oxacillin 40 μg/ml inhibited growth of JE2 but not
189 NE1757 (Fig. 4A) as predicted. Next, JE2 MHB 2% NaCl 40 μg/ml oxacillin cultures were further
190 supplemented with globomycin 10, 20, 30, 40 or 50 μg/ml (Fig. 4B). Oxacillin-induced
191 inhibition of JE2 growth was rescued globomycin, optimally at 10, 20 and 30 μg/ml (Fig. 4B).
192 Growth of JE2 in oxacillin 40 μg/ml and globomycin 50 μg/ml was substantially impacted
193 compared to lower globomycin concentration (Fig. 4B) indicating that the antagonism of
194 oxacillin by globomycin was dose dependent.
195 These experiments were extended to USA300_FPR3757, from which JE2 is derived and
196 ATCC43300, a SCC*mec* type II MRSA clinical isolate. Oxacillin concentrations of 50 μg/ml and
197 30 μg/ml inhibited growth of USA300 and ATCC4330, respectively (Fig. 5). Globomycin
198 concentrations (determined empirically for each strain) of 20 μg/ml for USA300 (Fig. 4C) and
199 30 μg/ml for ATCC43300 (Fig. 4D) rescued growth in the presence of oxacillin. These data
200 demonstrate that the increased oxacillin resistance phenotype observed in the *lspA* mutant
201 NE1757, can be replicated by globomycin-induced inhibition of LspA activity in wild type JE2
202 and other MRSA strains.
203 In contrast to the observation that globomycin increased β-lactam resistance in wild type JE2,
204 USA300 and ATCC43300, the growth of NE1757 in a range of globomycin concentrations from
205 10 – 50 μg/ml had a dose-dependent and negative effect on growth in the presence of
206 oxacillin 40μg/ml (Fig. S7). Taken together, these data suggest that globomycin antagonises
207 β-lactam antibiotics, increasing MRSA resistance to oxacillin and cefotaxime in LspA-
208 dependent manner. However, in the absence of *lspA*, the combination of globomycin and

209 oxacillin interferes with growth of JE2, particularly at higher concentrations of globomycin,
210 perhaps due to off-target effects.
211 To determine if globomycin could also increase resistance to other classes of β-lactam
212 antibiotics, its effect on cefotaxime resistance in JE2, USA300 and ATCC43300 was evaluated.
213 Cefotaxime was chosen because it is a 3rd generation cephalosporin with broad spectrum
214 activity against Gram-positive and Gram-negative bacteria commonly used in the clinic,
215 whereas oxacillin is a narrow-spectrum penicillin antibiotic. Cefotaxime 40 µg/ml inhibited
216 growth of JE2 and USA300 (Fig. 5A and B), while 30 µg/ml inhibited growth of ATCC43300 (Fig.
217 5C). Globomycin (30 µg/ml) rescued growth of all three strains in oxacillin (Fig. 5A, B and C)
218 suggesting that globomycin-mediated inhibition of LspA may antagonise the activity of many
219 or all β-lactam antibiotics.
220
221 **Mutation of *lgt* increases susceptibility to the lipoteichoic acid synthase inhibitor Congo**
222 **red.** Congo red was recently identified as a selective inhibitor of lipoteichoic acid synthase
223 (LtaS) activity (46). To investigate the possible involvement of LTA expression or stability in
224 the increased resistance of the *lspA* mutant to β-lactams serial dilutions of overnight *lspA*, *lgt*
225 and *lspA*/*lgt* mutant cultures were spotted onto TSA 0.125% Congo Red. Our data showed
226 that mutation of *lgt* dramatically increased susceptibility to the selective lipoteichoic acid
227 synthase (LtaS) inhibitor Congo red (Fig. 6) suggesting that impaired lipoprotein processing
228 affects the expression or stability of LTA. However, while the *lgt*/*lspA* double mutant was even
229 more susceptible to Congo red than the single *lgt* mutant, the single *lspA* mutations in NE1757
230 and NE1757 MM did not significantly alter Congo red susceptibility and complementation of
231 NE1757 was also associated with increased susceptibility (Fig. 6). These data show that
232 mutation of *lgt* or multicopy expression of *lspA* both increase susceptibility to this LTA
233 inhibitor indicating that the lipoprotein processing pathway impacts LTA synthesis/stability in
234 a complex manner. The mutations in the *lgt* and *lspA* genes alone, and in particular when
235 combined, significantly increased resistance to the alanylation pathway inhibitor D-
236 cycloserine (DCS)(Table 1). However lack of correlation between the effects of *lgt* and *lspA*
237 mutations on susceptibility to Congo red, DCS and β-lactams indicates that further analysis is
238 needed to better understand the interactions between lipoprotein processing pathway
239 intermediates and the expression/stability of LTA and WTA.
240

241 **Mutation of *lgt* in the *lspA* background restores wild type levels of β-lactam resistance.** LspA
242 catalyses the second major step in the lipoprotein processing pathway (Fig. 2). To probe the
243 contribution of lipoprotein processing to LspA-controlled oxacillin resistance, we compared
244 the impact of *lgt, lspA* and *lgt/lspA* mutants on growth and resistance to oxacillin, as well as
245 cefotaxime, nafcillin and vancomycin. Lgt catalyses the addition of a diacylglycerol moiety
246 onto preprolipoproteins, from which the signal peptide is then cleaved by LspA (Fig. 2). To
247 construct a *lspA/lgt* double mutant the erythromycin resistance marker of the *lspA*::Tn allele
248 in NE1757 was first exchanged for a markerless transposon to generate a strain designated
249 NE1757 MM into which the erythromycin-marked *lgt*::Tn allele from NE1905 was transduced.
250 Comparison of growth of JE2, NE1757, NE1757 MM, NE1905 and the *lgt/lspA* double mutant
251 NE1757/NE1905 revealed no significant differences MHB, MHB with 2% NaCl or TSB (Fig. 7A,
252 B, C). However, consistent with previous analysis of a *lgt* mutant (35), the *lspA, lgt* and in
253 particular the *lgt*/*lspA* mutants exhibited impaired growth in CDM (Fig. 7D), suggesting that
254 aberrant lipoprotein processing may affect nutrient acquisition under substrate-limiting
255 conditions.
256 The *lgt* mutant NE1905 exhibited no changes in susceptibility to oxacillin, cefotaxime, nafcillin
257 or vancomycin (Table 1). As observed for oxacillin, the *lspA* mutant NE1757 was more
258 resistant to cefotaxime and nafcillin and these phenotypes were complemented by the p*lspA*
259 plasmid (Table 1). Neither the *lspA* nor *lgt* mutations increased resistance to vancomycin
260 (Table 1). Oxacillin and nafcillin MICs were restored to wild type levels in the *lspA*/*lgt* double
261 mutant, and the cefotaxime MIC was significantly reduced (Table 1).
262 Taken together, these data indicate that while mutation of *lspA* or *lgt* or both impact growth
263 in CDM, only mutation of *lspA* alone is associated with increased β-lactam resistance. The *lgt*
264 mutation and possible accumulation of unprocessed prolipoproteins (Fig. 8A,B) does not
265 increase β-lactam resistance, whereas the possible accumulation of diacylglyceryl-lipoprotein
266 in a LspA mutant (Fig. 8C, D) is associated with this phenotype. The oxacillin, cefotaxime and
267 nafcillin MICs of the *ecsB* mutant from the NTML were the same as wild type (Table 1)
268 indicating that downstream processing of the LspA-cleaved signal peptide is not associated
269 with altered β-lactam resistance.

270 **Discussion**

271 Advances in our understanding of the accessory factors that control levels of *mecA*/PBP2a-
272 dependent resistance to methicillin has the potential to reveal new therapeutic targets and
273 drugs that may facilitate the reintroduction of other β-lactam antibiotics for the treatment of
274 MRSA infections. In this study, we demonstrated that mutation of the lipoprotein processing
275 pathway gene *lspA*, or inhibition of LspA with globomycin increased resistance to β-lactam
276 antibiotics. Although numerous mutations impacting the stringent response (ppGPpp) and c-
277 di-AMP signalling are associated with the transition from a heterogeneous to homogeneous
278 pattern of resistance and elevated PBP2a expression (2,3), our data show that the *lspA*
279 mutation was not associated with a HoR phenotype, increased PBP2a expression, or altered
280 NaCl tolerance (which is controlled by c-di-AMP) (4,5). On the other hand, changes in β-lactam
281 resistance independent of altered PBP2a regulation has long been known (47-49), and several
282 auxiliary factors known to influence β-lactam resistance in MRSA have been described (49-
283 54). In addition to unchanged PBP2a expression, no evidence of peptidoglycan remodelling
284 was observed in NE1757 after growth in the presence or absence of oxacillin, potentially
285 implicating wall teichoic acid (WTA) or lipoteichoic acid (LTA) synthesis or stability in the *lspA*
286 mutant phenotype. Inhibition of WTA synthesis was previously shown to decrease β-lactam
287 resistance in a PBP2a-independent manner (50). Reduced LTA stability, as evidenced by
288 Western blotting and increased susceptibility to the selective lipoteichoic acid synthase
289 inhibitor Congo red, was recently correlated with a PBP2a-independent reduction in β-lactam
290 resistance in auxiliary factor *auxA* and *auxB* mutants (49). Interestingly AuxA is structurally
291 similar to SecDF (49) and may interact with Sec pathway and lipoprotein processing (Fig. 8).
292 The *lgt* mutant was significantly more susceptible to Congo red than the *lspA* mutant and
293 multicopy expression of *lspA* also increased Congo red susceptibility suggesting that although
294 the lipoprotein processing pathway modulates LTA synthesis/stability, the relationship
295 between these two pathways appears to be complex.

296 Consistent with previous studies of lipoprotein pathway processing mutants in *S. aureus* (35),
297 *Listeria monocytogenes* (55) and *Streptococcus agalactiae* (56), our analysis also showed that
298 the *lgt, lspA* and *lgt*/*lspA* double mutants all exhibited impaired growth in CDM but not in TSB
299 or MHB indicating that the impact of lipoprotein processing pathway mutations on nutrient
300 acquisition and growth under nutrient limiting conditions can be compensated in rich media.

301 Mutation of the *lgt* gene from the lipoprotein processing pathway (Figs. 2, 7) did not affect β-
302 lactam resistance and introduction of the *lgt* mutation into a *lspA* mutant restored wild type
303 levels of resistance. These data implicate accumulation of diacylglycerol lipoprotein in
304 elevated β-lactam resistance. Consistent with this possibility, lipoproteins were retained in
305 the membrane of a *S. agalactiae lspA* mutant, but were released into the supernatant in large
306 concentrations by *lgt* and *lgt/lspA* mutants (56). Lipoproteins synthesised by the *S. agalactiae*
307 *lspA* mutant retained signal peptide, which was absent in a *lgt* mutant with LspA activity.
308 Importantly, signal peptide processing also occurred in the *lgt/lspA* double mutant, albeit
309 with cleavage occurring between different amino acids, implicating the involvement of an
310 alternative signal peptidase. Our analysis revealed no change in oxacillin susceptibility in the
311 MRSA *lgt*/*lspA* double mutant, indicating that even if an alternative peptidase can cleave the
312 signal peptide, this may not impact β-lactam resistance. Furthermore, mutation of *ecsB*,
313 which has recently been implicated in export of linear peptides from the lipoprotein
314 processing pathway in *S. aureus* (32), did not change the oxacillin MIC in JE2 (Table 1) also
315 indicating that downstream processing of signal peptides cleaved from lipoproteins by LspA
316 is not associated with altered β-lactam resistance. In *L. monocytogen*es, deletion of *lgt* also
317 led to significant release of lipoproteins into the supernatant. However, treatment of the *L.*
318 *monocytogenes lgt* mutant with globomycin (inhibiting LspA activity) resulted in enhanced
319 lipoprotein retention in the membrane (55), suggesting that the impact of globomycin and
320 *lspA* mutation on lipoprotein processing is not necessarily the same. In a *S. aureus lgt* mutant,
321 the Götz group reported that the majority of lipoprotein (lacking signal peptide) was released
322 into the supernatant (35). Taken together, the data suggest that accumulation of membrane-
323 anchored diacylglycerol lipoprotein with uncleaved signal peptide, or lipoprotein that is mis
324 localised or released due to aberrant signal peptide processing by an alternative peptidase, is
325 accompanied by increased β-lactam resistance in MRSA.
326

327 **Experimental procedures**

328 **Bacterial strains and culture conditions**. Bacterial strains and plasmids used in this study are
329 listed in Table S2. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or agar (LBA).
330 *S. aureus* strains were grown in Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA) or chemically
331 defined medium (CDM) (57). Muller Hinton Broth (MHB) or Muller Hinton Agar (MHA) (Oxoid)
332 supplemented with 2% NaCl where indicated, were used for antimicrobial susceptibility
333 testing (AST). Antibiotic concentrations used were 10 µg/ml erythromycin, 10 µg/ml
334 chloramphenicol, 75 µg/ml kanamycin, 100 µg/ml ampicillin.

335 Two hundred and fifty ml flasks were filled with 25 ml growth media, and overnight cultures
336 were used to inoculate the media at a starting OD_{600} of 0.05. Overnight cultures were grown
337 in TSB, and washed once in 5 ml PBS before being used to inoculate the CDM cultures. Flasks
338 were incubated at 37°C shaking at 200 rpm. OD_{600} readings were measured at 1 - 2 h intervals.
339 Colony forming units (CFU) were enumerated in serially diluted 20 µl aliquots removed from
340 flask cultures. Three independent biological replicates were performed for each strain and
341 the resulting data plotted using GraphPad Prism software.

342 Data from growth experiments in a Tecan Sunrise microplate instrument were recorded by
343 Magellan software. Overnight cultures were adjusted to an OD_{600} of 1 in fresh media and 10
344 µl inoculated into 200 µl media per well before being incubated at 37°C for 24 h with shaking.
345 OD_{600} was recorded every 15 min. For CDM, overnight TSB cultures were first washed in 5 ml
346 PBS and adjusted to OD_{600} of 1 in PBS.

347

348 **Genetic manipulation of *S. aureus*.** Phage 80α transduction was used to verify the association
349 between antibiotic resistance phenotypes and transposon insertion-marked mutations from
350 the NTML as described previously (39). Transductants were verified by PCR amplification of
351 the target locus using primers listed in Table S3. The plasmid pTnT, which contains a
352 truncated, markerless transposon was used to construct a markerless *lspA* mutant designated
353 NE1757 MM, as described previously (58). A double *lgt/lspA* double mutant was subsequently
354 constructed using phage 80α to transduce the *lgt*::Tn allele from NE1905 into NE1757 MM. A
355 1324 bp fragment encompassing the *lspA* gene was PCR amplified from JE2 genomic DNA
356 using primers NE1757_INF#3_Fwd and NE1757_INF#3_Rev (Table 2), purified with GenElute™
357 PCR Clean-Up Kit and cloned into the *E. coli* - *Staphylococcus* shuttle vector pLI50 digested
358 with *Eco*RI (New England Biolabs) using In-Fusion® HD Cloning Kit (Clontech) to generate

359 p*lspA.* Using electroporation, p*lspA* was transformed sequentially into Stellar™ (*E. coli* HST08)
360 (Clontech), *S. aureus* RN4220 and finally NE1757.
361
362 **Disk diffusion susceptibility assays.** Cefoxitin disk diffusion susceptibility assays were
363 performed in accordance with CLSI guidelines. Briefly, isolates were grown at 37°C on MHA
364 for 24 h and 5 - 6 colonies were resuspended in 0.85% saline to OD_{600} of 0.08 - 0.1 (0.5
365 McFarland; 1×10^8 CFU/ml) and swabbed onto MHA plates with a uniform agar depth of 4
366 mm. A 30 µg cefoxitin disk (Oxoid) was applied, the plate incubated at 35°C for 16 - 18 h and
367 the zone of inhibition diameter measured. Strains were classified as sensitive, intermediate,
368 or resistant, according to CLSI criteria.
369
370 **Minimum inhibitory concentration (MIC) measurements.** For oxacillin M.I.C.Evaluators
371 (Oxoid) several colonies from 24 h MHA plates were resuspended in 0.85% saline to OD_{600} of
372 0.08 - 0.1, (0.5 McFarland standard) and evenly swabbed onto MHA 2% NaCl (4 mm agar
373 depth). An M.I.C.Evaluator strip was applied and the plate incubated at 35°C for 24 h. Three
374 biological repeats were performed for each strain.
375 For broth microdilution MIC measurements using 96-well plates, each plate row was used to
376 prepare two-fold dilutions of antibiotic in MHB, typically ranging from 256 - 0.5 µg/ml across
377 10 wells. For oxacillin and nafcillin MIC measurements, MHB 2% NaCl was used. Several
378 colonies from 24 h MHA plates were resuspended in 0.85% saline to OD_{600} of 0.08 - 0.1 (0.5
379 McFarland standard), diluted 1:20 in 0.85% saline and 10 µl of this cell suspension used to
380 inoculate each well (approximately 5×10^4 CFU/well) in a final volume of 100 µl. The plates
381 were incubated at 35°C for 16 - 20 h, or 24 h incubation for oxacillin and nafcillin. The MIC
382 was the lowest concentration of antimicrobial agent that completely inhibited growth.
383 Freshly prepared MHA plates (with 2% NaCl when using oxacillin and nafcillin) were
384 supplemented with antimicrobial agents at 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 µg/ml to
385 perform agar dilution MIC measurements. The inoculum was prepared as described for the
386 broth microdilution method above before being further diluted 1:20 in 0.85% saline and 4 µl
387 spot-inoculated onto each plate (approximately 10^4 CFU per 5-8 mm diameter spot). The MIC
388 was the lowest concentration of antimicrobial agent that completely inhibited growth after
389 16-18 h (24 h for oxacillin and nafcillin) at 35°C, disregarding a single colony or a faint haze
390 associated with the inoculum.

391 Congo red susceptibility assays were performed as described previously (59). Briefly,
392 overnight cultures grown in TSB were adjusted to an OD_{600} of 1 in PBS, serially diluted and
393 spotted onto TSA plates supplemented with 1% aqueous Congo red solution (VWR) to a final
394 concentration of 0.125%. Plates were incubated for 24 h at 37°C. These assays were
395 performed with at least 3 biological repeats and representative image is shown for TSA
396 0.125% Congo red.
397
398 **Autolytic activity assays.** Overnight cultures (20 µl) were inoculated into 20 ml TSB, grown at
399 37°C (200 rpm) to OD_{600} of 0.5, washed with 20 ml cold PBS, resuspended in 1 ml cold PBS
400 and finally adjusted to OD_{600} of 1. Triton X-100 was added at a final concentration of 0.1% v/v
401 and the cell suspension incubated at 37°C with shaking (200 rpm). OD_{600} was recorded every
402 30 min for 4 h. Autolytic activity was expressed as a percentage of the initial OD_{600}. NE406
403 (*atl*::Tn) was used as a control and at least 3 biological replicates performed for each strain.
404
405 **Globomycin and β-lactam antibiotic synergy/antagonism assays.** One hundred µl of MHB
406 cefotaxime or MHB 2% NaCl oxacillin was added to the individual wells of 96-well plates. The
407 oxacillin or cefotaxime concentration chosen for each strain was based on approximate MICs
408 i.e. the lowest antibiotic concentration that inhibited growth. Overnight MHB cultures were
409 resuspended in PBS at OD_{600} of 0.1 (0.5 McFarland standard) and then further diluted 1:20
410 before 10 µl (approximately 5×10^5 CFU/ml) was added to each well and the plates were
411 incubated at 35°C with shaking on a Tecan Sunrise microplate instrument for 20 h
412 (cefotaxime) or 24 h (oxacillin). Globomycin ranging from 10 - 100 µg/ml was then added to
413 the cefotaxime or oxacillin cultures to measure potential synergism or antagonism. Three
414 independent biological replicates were performed for each strain and antibiotic combination.
415
416 **PBP2a western blot analysis.** Overnight MHB cultures were used to inoculate 25 ml of MHB
417 2% NaCl, with or without 0.5 µg/ml oxacillin to a starting OD_{600} of 0.05, incubated at 35°C (200
418 rpm shaking) until an OD_{600} of 0.8 was reached before the cells were pelleted and
419 resuspended in PBS to an OD_{600} of 10. Six µl of lysostaphin (10 µg/ml) and 1 µl of DNase (10
420 µg/ml) was added to 500 µl of this concentrated cell suspension before being incubated at
421 37°C for 40 min. Next, 50 µl of 10% SDS was added and the incubation continued for a further
422 20 min. The lysed cells were then pelleted in a microcentrifuge for 15 min, following which
423 the protein-containing supernatant was collected and total protein concentration

424 determined using the Pierce® BCA Protein Assay Kit. Samples containing 8 µg total protein
425 were mixed 1:1 with protein loading buffer (2x) (National Diagnostics) and incubated at 95°C
426 for 5 min and loaded onto a 7.5% Tris-Glycine gel and separated at 120 V for 60 mins.
427 Electrophoretic transfer to a PVDF membrane was carried out at 30 V for 30 min on the Trans-
428 Blot Turbo Transfer System (Biorad). The PVDF membrane was blocked overnight in 5% skim
429 milk powder in PBS at 4°C. The following day, the membrane was washed in fresh PBS. Anti-
430 PBP2a (Abnova) was diluted 1:1000 in PBS-Tween 20 (0.1%) and incubated with the
431 membrane for 1 h at room temperature. The membrane was washed in PBS to remove
432 unbound antibody. The secondary antibody, HRP-rec-Protein G (Invitrogen) was diluted
433 1:2000 in PBS-Tween 20 (0.1%) and incubated with the membrane at room temperature for
434 1 h. Visualisation of the membrane was performed with the Opti-4CN Substrate kit (Biorad).
435 Three independent experiments were performed and representative images of the
436 developed PVDF membrane were recorded.
437
438 **Population level antibiotic resistance profile analysis.** Characterisation of the population
439 resistance profile was performed as described previously (60). Overnight cultures were grown
440 in TSB, adjusted to an OD_{600} of 1, 10-fold serially diluted from 10^{-1} to 10^{-7}, and a 20 µl aliquot
441 of each dilution plated onto a series of TSA agar plates supplemented with oxacillin 0.25, 0.5,
442 1, 2, 4, 8, 16, 32, 64 and 128 µg/ml. CFUs were enumerated after overnight incubation at 37°C
443 and the results were expressed as CFU/ml at each oxacillin concentration. Three independent
444 experiments were performed for each strain.
445
446 **Antibiotic tolerance assay.** Tolerance assays were performed as described previously (61).
447 Briefly overnight TSB cultures were sub-cultured into 25 ml of fresh TSB in 250 ml flasks at a
448 starting OD_{600} of 0.05 and grown to an OD_{600} of 0.5 at 37°C with 200 rpm shaking. At this time
449 (T_0) an aliquot was removed for CFU enumeration and 12.5 µg/ml oxacillin promptly added
450 before the cultures were re-incubated. Antibiotic tolerance was expressed as the % CFU/ml
451 after 2, 4, 6, 8 and 24 h growth in the antibiotic compared to the CFU/ml at T_0. The results
452 represent 3 biological replicates of each strain.
453
454 **Salt tolerance assay.** Overnight TSB cultures were adjusted to an OD_{600} of 1 in fresh TSB and
455 serially diluted from 10^{-1} to 10^{-7}. Four µl of each dilution was spot-inoculated onto TSA 2.2M
456 NaCl plates and incubated at 37°C overnight.

457
458 **Genomic DNA extraction and Whole Genome Sequencing (WGS).** Genomic DNA (gDNA)
459 extractions were performed using the Wizard® Genomic DNA Purification Kit (Promega)
460 following pre-treatment of *S. aureus* cells with 10 µg/ml lysostaphin (Ambi Products LLC) at
461 37°C for 30 min. WGS was performed by MicrobesNG (http://www.microbesng.uk) using an
462 Illumina sequencing platform with 2x250 bp paired-end reads. CLC Genomics Workbench
463 software (Qiagen) was used for genome sequencing analysis of strains. As a reference
464 genome, a contig was produced for wild type JE2 by mapping Illumina reads onto the closely
465 related USA300 FPR3757 genome sequence (RefSeq accession number NC_07793.1). The
466 Illumina short read sequences from NE1757 were then mapped onto the assembled JE2
467 sequence and the presence of the transposon insertion confirmed. Single Nucleotide
468 Polymorphisms (SNPs), deletions or insertions were mapped in the NE1757 genome and
469 presence of large deletions ruled out by manually searching for zero coverage regions using
470 the CLC Genomics Workbench software.
471
472 **Peptidoglycan analysis.** For each strain and growth condition tested, independent
473 quadruplicate 50 ml cultures were grown to an OD_{600} of 0.5, harvested and resuspended in 5
474 ml PBS before peptidoglycan was extracted as described previously (39,62). Mass
475 spectrometry was performed on a Waters XevoG2-XS QTof mass spectrometer. Structural
476 characterization of muropeptides was determined based on their MS data and MS/MS
477 fragmentation pattern, matched with PG composition and structure reported previously (63-
478 66).
479
480
481 **Acknowledgements**
482
483 This study was funded by grants from the Health Research Board (HRA-POR-2015-1158 and
484 ILP-POR-2019-102) (www.hrb.ie), the Irish Research Council (GOIPG/2016/36)
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487 Gallagher for assistance and advice throughout this study. The illustrations in Figures 2 and 8
488 were created with Biorender.com.
489

Table and Figures

500

501 **Table 1.** Antibacterial activity of oxacillin, cefotaxime, nafcillin vancomycin and D-cycloserine
502 (MIC measurements; µg/ml[1]) against ATCC 29213 (control), JE2, NE1757 (*lspA*::Tn), NE1757
503 p*lspA*, NE1757 pLI50, NE1757 MM (markerless *lspA* mutant), NE1757 MM p*lspA,* JE2 *lspA*::Tn
504 #2 (transductant), NE1905 (*lgt*::Tn) and NE1757/NE1905 double mutant *lspA*/*lgt*.

Strain	Oxacillin	Cefotaxime	Nafcillin	Vancomycin	D-Cycloserine
ATCC 29213	≥1	2	0.5	1	32
JE2	32 - 64	64	32	1	32
NE1757 (*lspA*)	128 - 256	256	64	1	16 - 32
NE1757 p*lspA*	64	64	32	1	16 - 32
NE1757 pLI50	256	256	-	1	16 - 32
NE1757 MM	128 - 256	256	64	-	-
NE1757 MM p*lspA*	64	64	32	-	-
JE2 *lspA*::Tn #2	128 - 256	256	-	1	16 - 32
NE1905 (*lgt*)	64	64	32	1	16 - 32
NE1757/NE1905 (*lspA*/*lgt*)	64	128	32	1	16
NE107 (*ecsB*)	64	64	32	1	16 - 32

505 [1] MIC measurements (µg/ml) were performed by MH agar dilution in accordance with CLSI
506 standards.
507

508

509
510
511

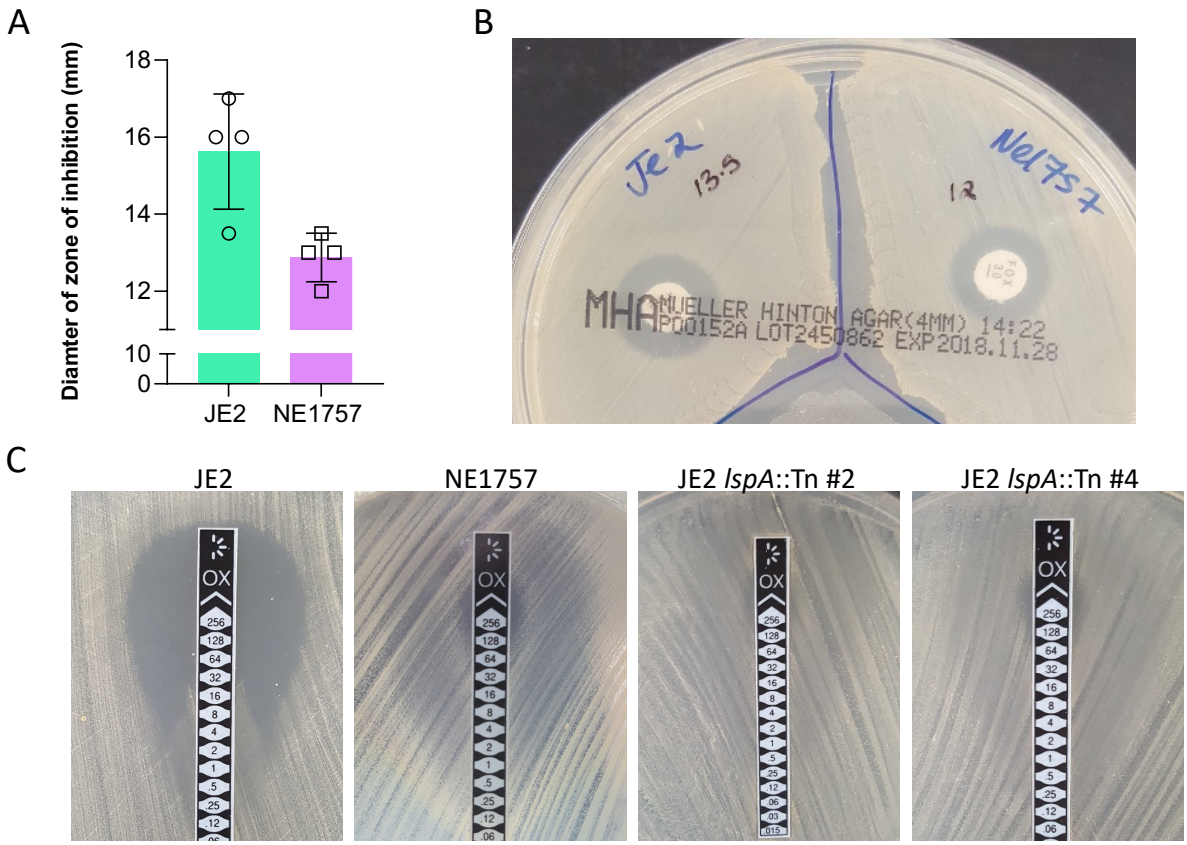
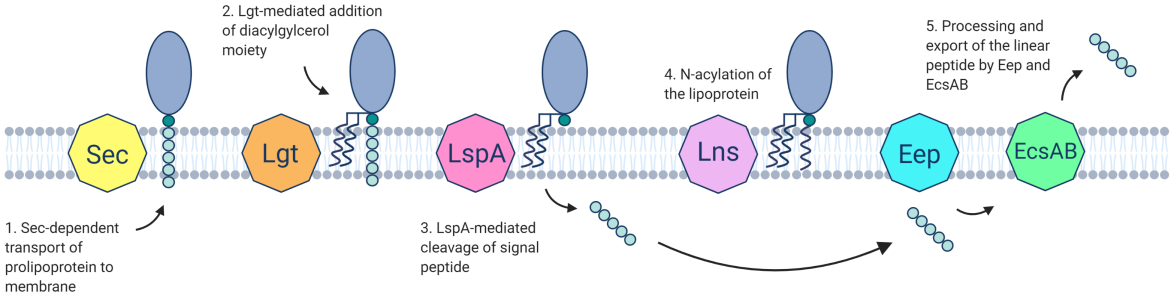


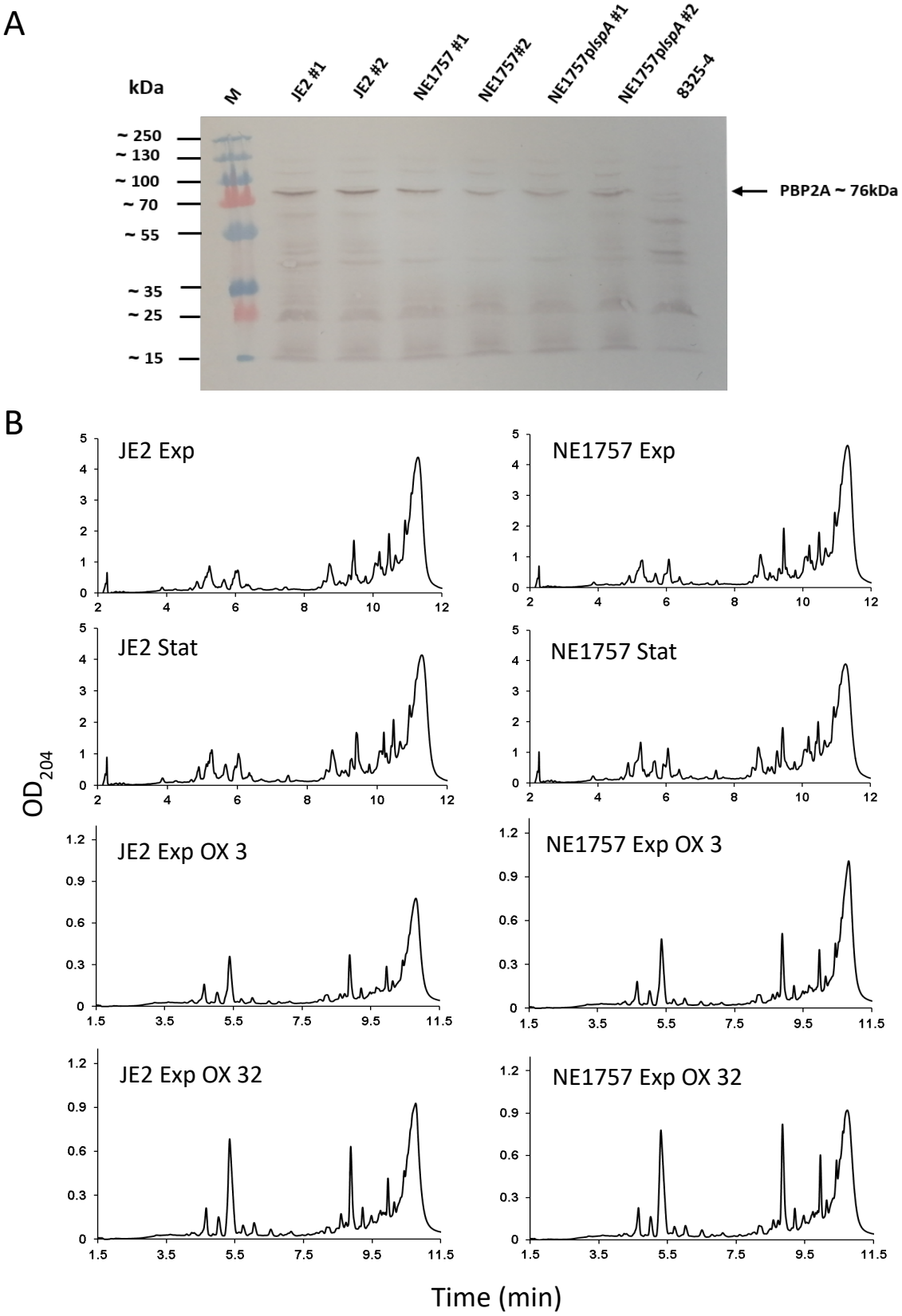
Fig. 1. Mutation of *lspA* increases resistance to cefoxitin and oxacillin. (A) Average diameters of the cefoxitin disk zones of inhibition for JE2 and NE1757 (*lspA*::Tn) from 4 independent experiments, plotted using Prism software (GraphPad). **(B)** Representative image of JE2 (left) and NE1757 (right) grown on MH agar with a cefoxitin 30 µg disk. **(C)** M.I.C.Evaluator measurement of oxacillin minimum inhibitory concentrations (MICs) in JE2, NE1757 (*lspA*::Tn), and two independent JE2 transductants (#2 and #4) carrying the *lspA*::Tn allele grown on MHB 2% NaCl agar. This assay was repeated 3 independent times for each strain and a representative image is shown.

527



528
529
530 **Fig. 2. Role of LspA in the proposed model of lipoprotein processing in Gram-positive**
531 **bacteria.** Lipoproteins are synthesized in the cytoplasm as precursors. **1.** Prolipoprotein is
532 translocated usually via the Sec machinery through recognition of its signal peptide (SP). This
533 sequence also contains the lipobox specific for bacterial lipoproteins. **2.** Diacylglyceryl
534 transferase (Lgt) catalyzes the transfer of a diacylglyceryl group from phosphatidylglycerol
535 onto the prolipoprotein resulting in diacylglyceryl-prolipoprotein. **3.** Lipoprotein signal
536 peptidase II (LspA) recognizes the diacylglyceryl-modified signal peptide and cleaves between
537 the amino acid at position-1 and the lipid-modified cysteine residue at +1. **4.** The lipoprotein
538 N-acylation transferase system (Lns) converts diacyl lipoproteins to triacylated lipoprotein
539 (29). **5.** Eep and EcsAB play roles in the processing and secretion of linear peptides (32).
540

541

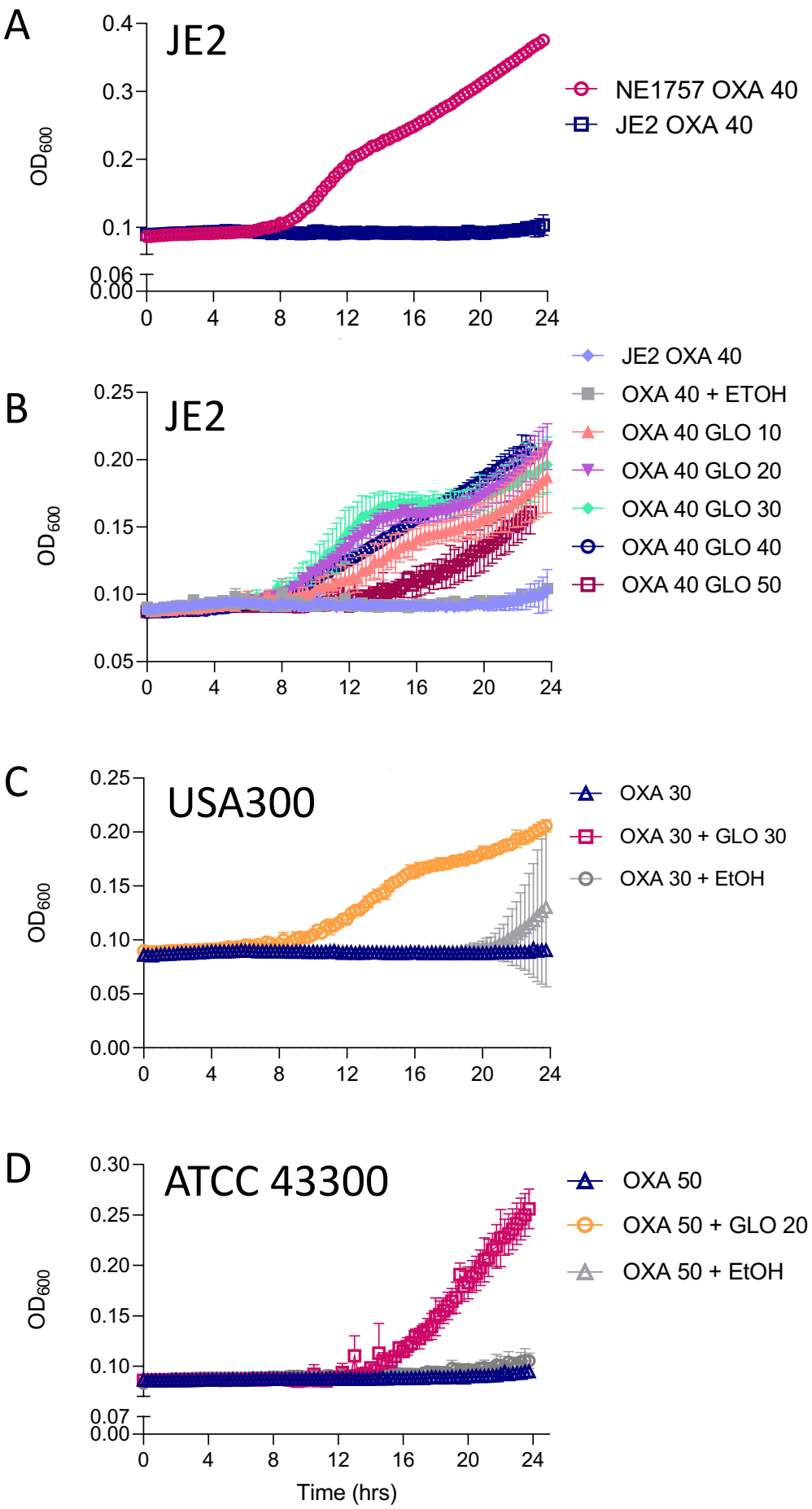


542

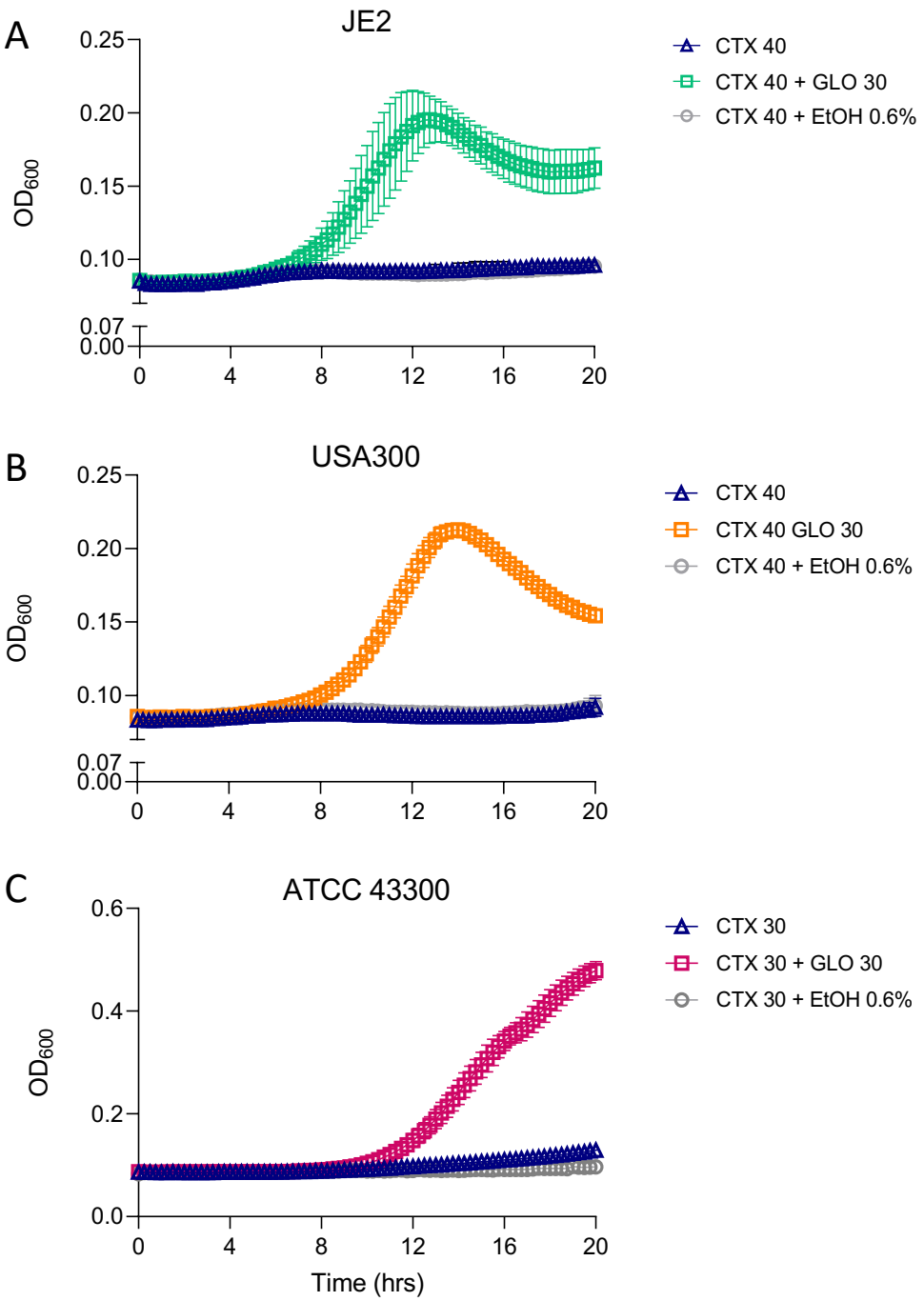
543 **Fig. 3. Mutation of *lspA* does not affect PBP2a expression levels or peptidoglycan structure**
544 **and crosslinking. (A)** Western blot of PBP2a protein in JE2, NE1757 (*lspA*), NE1757 p*lspA* and
545 MSSA strain 8325-4 (negative control). Cells were grown to exponential stage in MHB 2% NaCl

546 supplemented with 0.5 μg/ml oxacillin, with the exception of 8325-4 which was grown in MHB
547 2% NaCl. For each sample, 8 μg total protein was run on a 7.5% Tris-Glycine gel, transferred
548 to a PVDF membrane and probed with anti-PBP2a (1:1000), followed by HRP-conjugated
549 protein G (1:2000) and colorimetric detection with Opti-4CN Substrate kit. Three independent
550 experiments were performed and a representative image is shown. (**B)** Representative UV
551 chromatograms of peptidoglycan extracted from JE2 and NE1757 collected from cultures
552 grown to exponential or stationary phase in MHB or MHB supplemented with oxacillin 3
553 μg/ml or 32 μg/ml. Each profile shown is a representative of 3 biological replicates.

554


A
JE2
OD600
0.4
0.3
0.2
0.1
0.06
0.00
0
4
8
12
16
20
24
NE1757 OXA 40
JE2 OXA 40
B
JE2
OD600
0.25
0.20
0.15
0.10
0.05
JE2 OXA 40
OXA 40 + ETOH
OXA 40 GLO 10
OXA 40 GLO 20
OXA 40 GLO 30
OXA 40 GLO 40
OXA 40 GLO 50
C
USA300
OD600
OXA 30
OXA 30 + GLO 30
OXA 30 + EtOH
D
ATCC 43300
OD600
0.30
0.07
OXA 50
OXA 50 + GLO 20
OXA 50 + EtOH
Time (hrs)

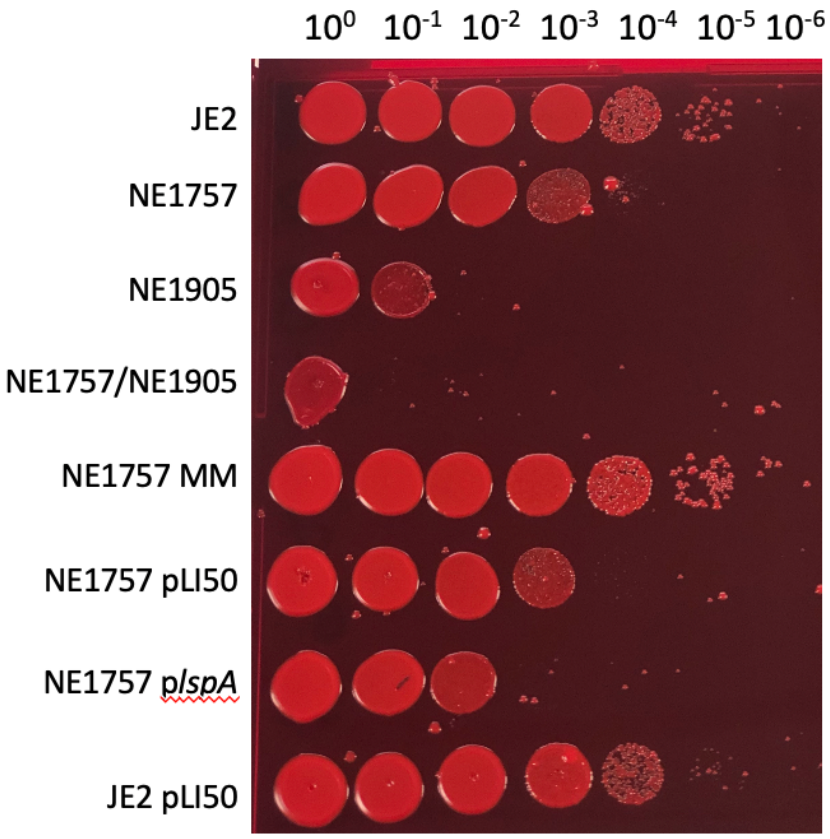
555 **Fig. 4. Mutation of *lspA* or exposure to globomycin increases oxacillin resistance. (A)** JE2 and
556 NE1757 (*lspA*) grown in MHB 2% NaCl supplemented with oxacillin 40 µg/ml. **(B)** JE2 grown in
557 MHB 2% NaCl supplemented with oxacillin 40 µg/ml and globomycin concentrations ranging
558 from 10-50 µg/ml or MHB 2% NaCl supplemented with oxacillin 40 µg/ml and 0.6% ethanol
559 (control solvent for globomycin). **(C)** USA300 grown in MHB 2% NaCl supplemented with
560 oxacillin 50 µg/ml, MHB 2% NaCl supplemented with oxacillin 50 µg/ml and globomycin 20
561 µg/ml or MHB 2% NaCl supplemented with oxacillin 50 µg/ml and 0.4% ethanol (control
562 solvent for globomycin). **(D)** ATCC 43300 grown in MHB 2% NaCl supplemented with oxacillin
563 30 µg/ml, MHB 2% NaCl supplemented with oxacillin 30 µg/ml and globomycin 30 µg/ml or
564 MHB 2% NaCl supplemented with oxacillin 50 µg/ml and 0.6% ethanol (control solvent for
565 globomycin). The oxacillin and globomycin concentrations used in these experiments were
566 determined empirically for each strain. Cultures were grown in a Tecan Sunrise incubated
567 microplate reader for 24 h at 35°C. OD_{600} was recorded at 15 min intervals and growth curves
568 were plotted in Prism software (GraphPad). The data presented are the average of 3
569 independent biological replicates, and error bars represent standard deviations.
570



571

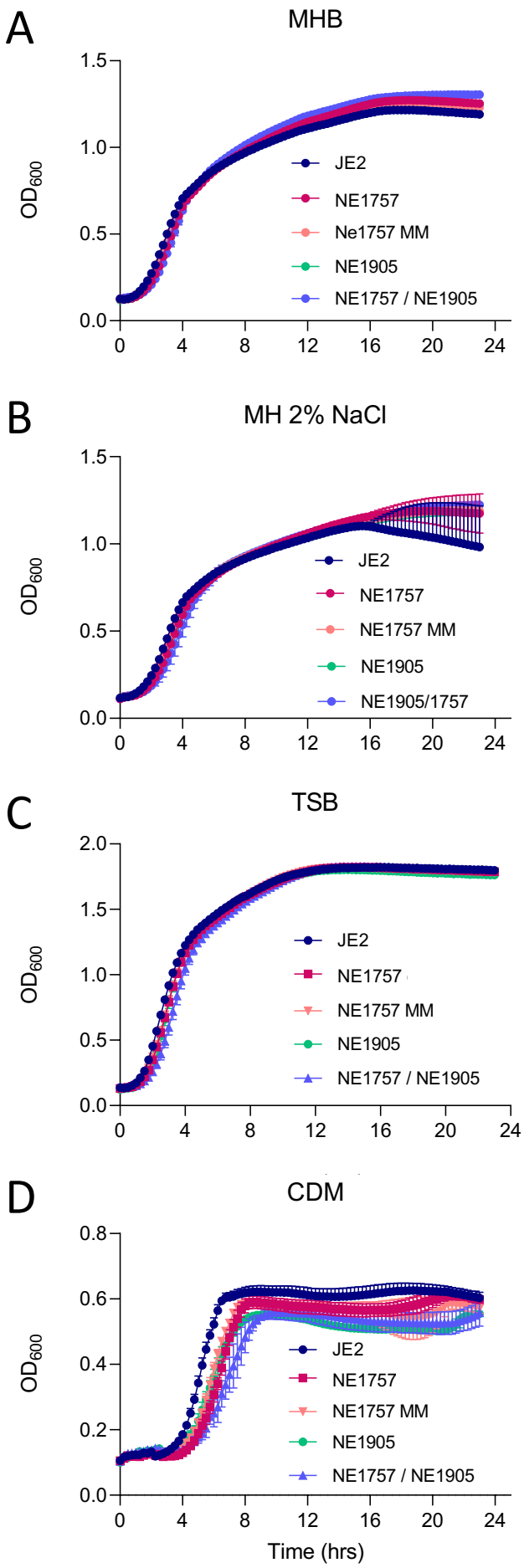
572 **Fig. 5. Globomycin increases cefotaxime resistance in JE2, USA300 and ATCC43300. (A)** JE2
573 was grown in MHB 2% NaCl supplemented with cefotaxime (CTX) 40 µg/ml, MHB 2% NaCl
574 supplemented with CTX 40 µg/ml and globomycin 30 µg/ml or MHB 2% NaCl supplemented
575 with CTX 40 µg/ml and 0.6% ethanol (control solvent for globomycin). **(B)** USA300 was grown
576 in MHB 2% NaCl supplemented with CTX 40 µg/ml, MHB 2% NaCl supplemented with CTX 40
577 µg/ml and globomycin 30 µg/ml or MHB 2% NaCl supplemented with CTX 40 µg/ml and 0.6%
578 ethanol (control solvent for globomycin). **(C)** ATCC 43300 was grown in MHB 2% NaCl
579 supplemented with CTX 30 µg/ml, MHB 2% NaCl supplemented with CTX 30 µg/ml and
580 globomycin 30 µg/ml or MHB 2% NaCl supplemented with CTX 30 µg/ml and 0.6% ethanol
581 (control solvent for globomycin). The cefotaxime and globomycin concentrations used in these
582 experiments were determined empirically for each strain. The cultures were grown in a Tecan

583 Sunrise incubated microplate reader for 24 h at 35°C OD_{600} was recorded at 15 min intervals
584 and growth curves were plotted in Prism software (GraphPad). The data presented are the
585 average of 3 independent biological replicates, and error bars represent standard deviations.
586

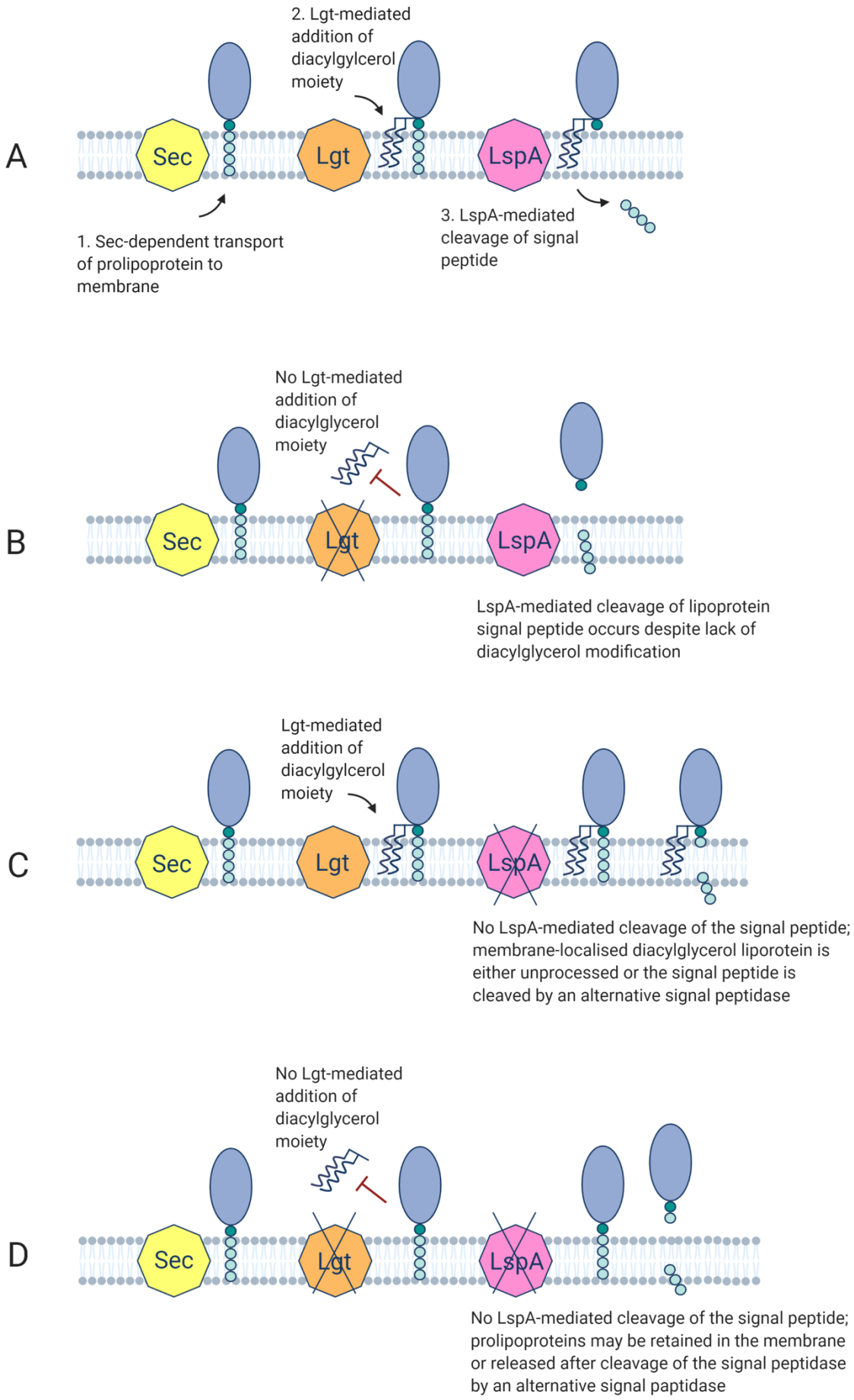


587
588
589 **Fig. 6. Mutation of *lgt* increases susceptibility to Congo red.** Overnight cultures of JE2,
590 NE1757 (*lspA*), NE1905 (*lgt*), NE1757/NE1905 (*lgt*/*lspA*), NE1757 (markerless *lspA* mutation),
591 NE1757 pLI50, NE1757 p*lspA* and JE2 pLI50 were grown in TSB (without antibiotic selection),
592 normalised to an OD_{600} of 1 in PBS, serially diluted and spotted onto TSA plates supplemented
593 with 0.125% Congo red, and the plates were incubated for 24 h at 37°C. A representative
594 image is shown.
595

596


A
MHB
OD600
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
JE2
NE1757
Ne1757 MM
NE1905
NE1757 / NE1905
B
MH 2% NaCl
OD600
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
JE2
NE1757
NE1757 MM
NE1905
NE1905/1757
C
TSB
OD600
2.0
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
JE2
NE1757
NE1757 MM
NE1905
NE1757 / NE1905
D
CDM
OD600
0.8
0.6
0.4
0.2
0.0
0
4
8
12
16
20
24
Time (hrs)
JE2
NE1757
NE1757 MM
NE1905
NE1757 / NE1905

597 **Fig. 7. Mutation of *lspA* or *lgt* impacts growth in nutrient limited media but not in complex**
598 **media.** Growth of JE2 wild type, NE1757 (*lspA*::Tn), NE1757 MM (markerless *lspA*::Tn), NE1905
599 (*lgt*::Tn) and NE1757/NE1905 (markerless *lspA*::Tn / *lgt*::Tn) in Muller Hinton broth **(A)**, Muller
600 Hinton broth with 2% NaCl **(B)**, TSB **(C)**, and chemically-defined medium **(D)**. Growth
601 experiments were performed in 96-well hydrophobic plates in a Tecan Sunrise incubated
602 microplate reader for 24 h at 37ºC. OD_{600} was recorded at 15 min intervals and growth curves
603 were plotted in Prism software (GraphPad). The data presented are the average of 3
604 independent biological replicates, and error bars represent standard deviations.


A
2. Lgt-mediated addition of diacylgylcerol moiety
Sec
Lgt
LspA
1. Sec-dependent transport of prolipoprotein to membrane
3. LspA-mediated cleavage of signal peptide
B
No Lgt-mediated addition of diacylglycerol moiety
Sec
Lgt
LspA
LspA-mediated cleavage of lipoprotein signal peptide occurs despite lack of diacylglycerol modification
C
Lgt-mediated addition of diacylgylcerol moiety
Sec
Lgt
LspA
No LspA-mediated cleavage of the signal peptide; membrane-localised diacylglycerol liporotein is either unprocessed or the signal peptide is cleaved by an alternative signal peptidase
D
No Lgt-mediated addition of diacylglycerol moiety
Sec
Lgt
LspA
No LspA-mediated cleavage of the signal peptide; prolipoproteins may be retained in the membrane or released after cleavage of the signal peptidase by an alternative signal paptidase

605

606 **Fig. 8. Predicted impacts of *lgt*, *lspA* and *lgt/lspA* mutations on lipoprotein processing in *S.***
607 ***aureus*. (A)** Overview of lipoprotein processing in Gram-positive bacteria. **(B)** The *lgt* mutant
608 lacks diacylglyceryl transferase activity and is unable to add the diacylglycerol molecule to the
609 prolipoprotein, thus blocking accumulation of the LspA substrate. The prolipoprotein is likely
610 to be released from the membrane due to LspA-mediated cleavage of the signal peptide signal
611 peptide. **(C)** LspA-mediated cleavage of the signal peptide is absent in the *lspA* mutant, but
612 an alternative signal peptidase may undertake this activity. **(D)** Prolipoproteins are not
613 processed in mutants lacking Lgt and LspA activity, and may be retained in the membrane or
614 released after cleavage of the signal peptide by an alternative signal peptidase.

615

Supplementary Tables and Figures

628
629
630 **Supplementary Table S1.** Genome sequence changes in NE1757 (*lspA*::Tn)

Reference Position[1]	Type[2]	Ref.[3]	Allele[4]	Freq.[5]	Annotation
1192002 - 1192003	INS	TA	T-Tn-A	100	*Bursa aurealis* transposon

631
632 [1]Reference position: Position in the USA300 FPR3757 genome sequence (NC_007793.1).
633 [2]Type of mutation: INS, insertion.
634 [3]Ref: Nucleotide base in the USA300 FPR3757 reference genome.
635 [4]Allele: Nucleotide base at the same position in NE1757.
636 [5]Freq.: % Frequency at which INS was found in NE1757 compared to JE2
637

638 **Table S2.** Bacterial strains and plasmids used in this study

Strain/plasmid	Description and resistance	Source/reference
***Escherichia coli* strains**		
HST08	TaKaRa *E. coli* HST08 Premium Electro-Cells	TaKaRa
HST08 pLI50	*E. coli* HST08 pLI50; Amp^R	This study
HST08 p*lspA*	*E. coli* HST08 p*lspA*; Amp^R	This study
***Staphylococcus aureus* strains**		
JE2	JE2 (plasmid-cured derivative of strain LAC)	(36)
8325-4	NCTC 8325 derivative cured of prophages, methicillin susceptible, CC8.	(67)
RN4220	RN4220 (Restriction-deficient derivative of 8325-4)	(68)
BHICC	MRSA clinical isolate; SCC*mec* type II; CC8	(39)
ATCC 29213	Quality control strain for susceptibility testing; oxacillin sensitive	*S. aureus* ATCC 29213
ATCC 43300	Quality control strain for susceptibility testing; oxacillin resistant, SCC*mec* Type II	*S. aureus* ATCC 43300
USA300	USA300_FPR3757	(69)
NE1757 (*lspA*)	JE2 *lspA*::Tn; Erm^R	(36)
NE1905 (*lgt*)	JE2 *lgt*::Tn; Erm^R	(36)
NE1757 MM	JE2 *lspA*:Tn (truncated, markerless Tn); Erm^S	This study
NE1757/NE1905	JE2 *lspA* MM *lgt*::Tn; Erm^R	This study
RN4220 pTnT	RN4220 pTnT	(36)
RN4220 pLI50	RN4220 pLI50; Cam^R	This study
RN4220 p*lspA*	RN4220 p*lspA*	This study
NE1757 p*lspA*	JE2 *lspA*::Tn p*lspA*; Erm^R Cam^R	This study
NE1757 pLI50	JE2 *lspA*::Tn pLI50; Erm^R Cam^R	This study
JE2 pLI50	JE2 pLI50; Cam^R	This study
JE2 *lspA* MM pLI50	JE2 *lspA* MM pLI50; Cam^R	This study
NE1757 MM p*lspA*	JE2 *lspA* MM p*lspA*; Cam^R	This study
JE2 *lspA*::Tn #2	JE2 *lspA*::Tn; Erm^R	This study
JE2 *lspA*::Tn #4	JE2 *lspA*::Tn; Erm^R	This study
NE788	JE2 *trkA*::Tn; Erm^R	(36)
NE460	JE2 *atl*::Tn; Erm^R	(36)
Plasmids		
pTnT	Plasmid to replace Erm^R marker from transposon mutants with a markerless mutation. Cam^R	(58)
pLI50	*S. aureus-E. coli* shuttle vector	(70)
p*lspA*	pLI50 carrying the *lspA* gene from JE2	This study

639

640

641 **Table S3**. Oligonucleotides used in this study

Target	Name	Primer Sequence (5' – 3')
lspA (infusion primers)	NE1757_INF#3_Fwd	TCGTCTTCAAGAATTTTATGAAGGAGGCTGGGACA
	NE1757_INF#3_Rev	TACCGAGCTCGAATTCAGGCAGCAACTTATCTACACG
Tn-check primers	**Name**	**Primer Sequence (5' – 3')**
lspA	NE1757_fwd	GTTCCAGCCTGCTTTCCTAATT
	NE1757_rev	ACACGCATACCTGTTTGTTCT
lgt	NE1905_fwd	GCATTAACACGGCCGAAGAA
	NE1905_rev	CAACCGTACCAGCTGCAAC

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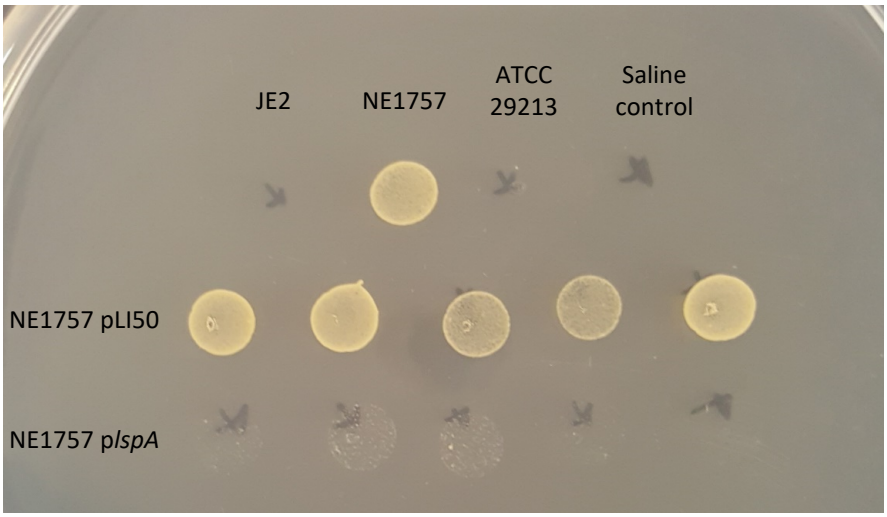
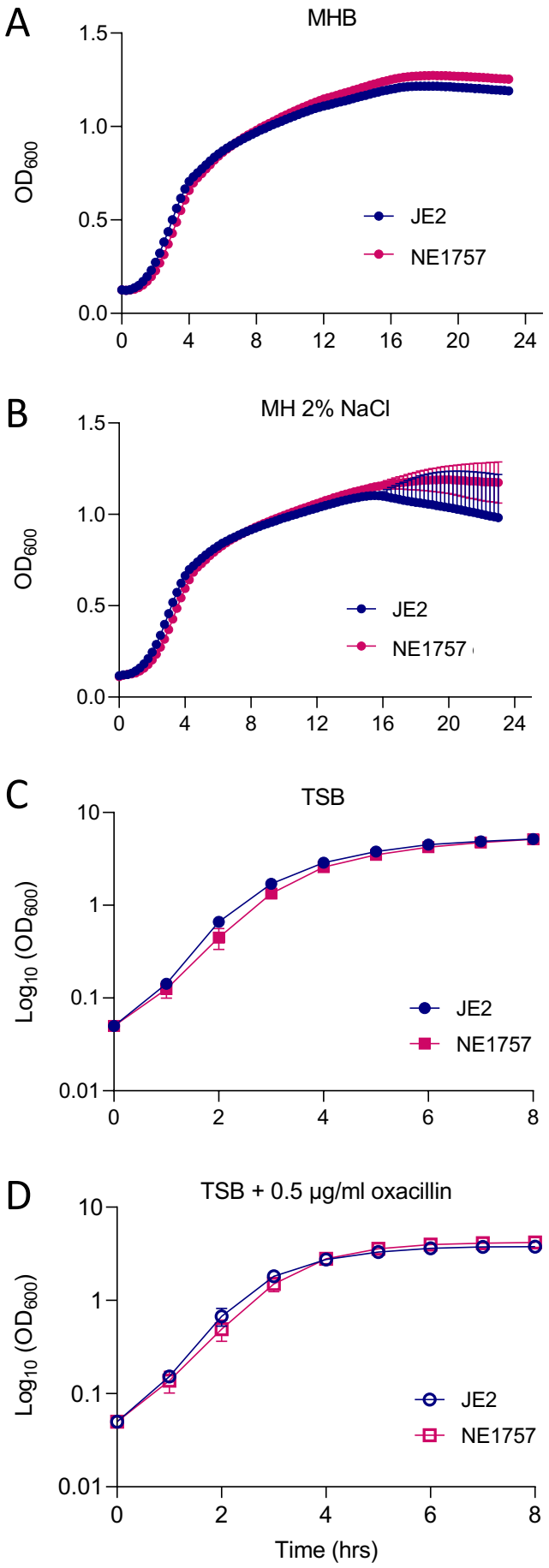
643



644

645

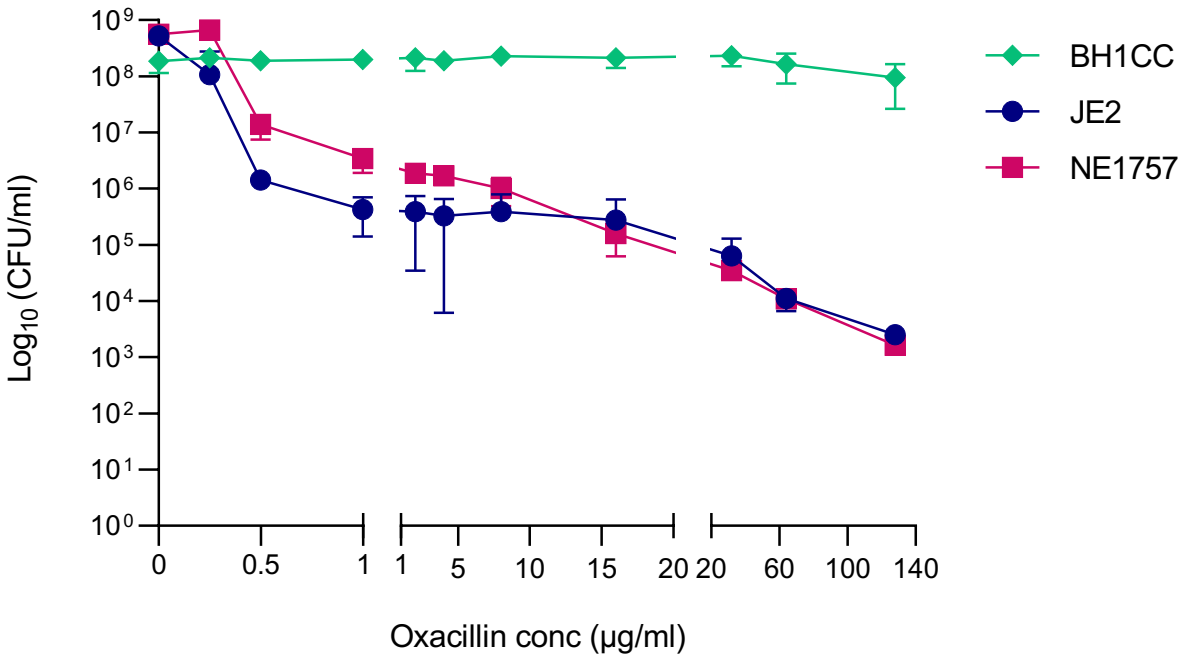
646 **Supplementary Fig. S1. Complementation of the NE1757 mutant with the *lspA* gene is**
647 **accompanied by a wild type oxacillin resistance phenotype.** JE2, NE1757 (*lspA*::Tn), NE1757
648 pLI50 and NE1757 p*lspA* were grown on MHA 2% NaCl supplemented with oxacillin 32 µg/ml.
649 The plate shows 5 biological replicates each for NE1757 pLI50 and NE1757 p*lspA*. ATCC 29213
650 was included as an oxacillin susceptible control).
651


A
MHB
OD600
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
JE2
NE1757
B
MH 2% NaCl
OD600
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
JE2
NE1757
C
TSB
Log10 (OD600)
10
1
0.1
0.01
0
2
4
6
8
JE2
NE1757
D
TSB + 0.5 µg/ml oxacillin
Log10 (OD600)
10
1
0.1
0.01
0
2
4
6
8
JE2
NE1757
Time (hrs)

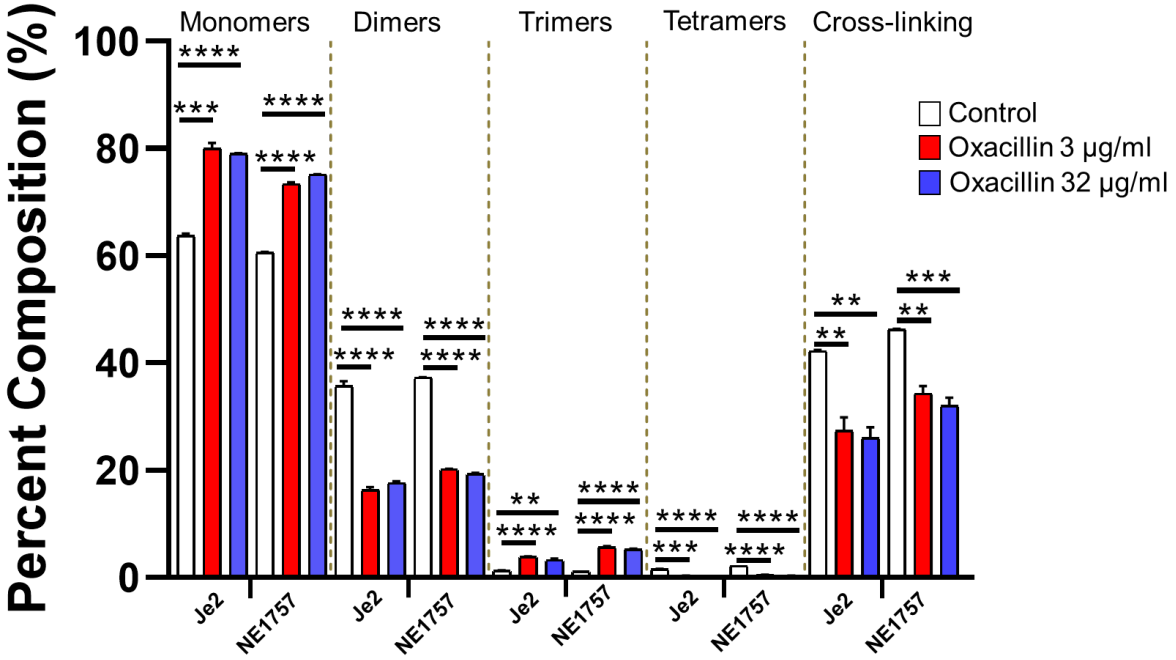
652

653 **Supplementary Fig. S2. Mutation of *lspA* does not impact growth in MHB or TSB media.**
654 Growth of JE2 and NE1757 cultures in **(A)** MHB, **(B)** MHB 2% NaCl, **(C)** TSB and **(D)** TSB
655 0.5mg/ml oxacillin. MHB cultures were grown in 96-well plates in a Tecan Sunrise incubated
656 microplate reader for 24 h at 37°C. The OD_{600} was recorded at 15 min intervals and growth
657 curves were plotted in Prism software (GraphPad). TSB cultures were grown in flasks and
658 the OD_{600} monitored every 2 h. All data presented are the average of 3 independent biological
659 replicates, and error bars represent standard deviations.

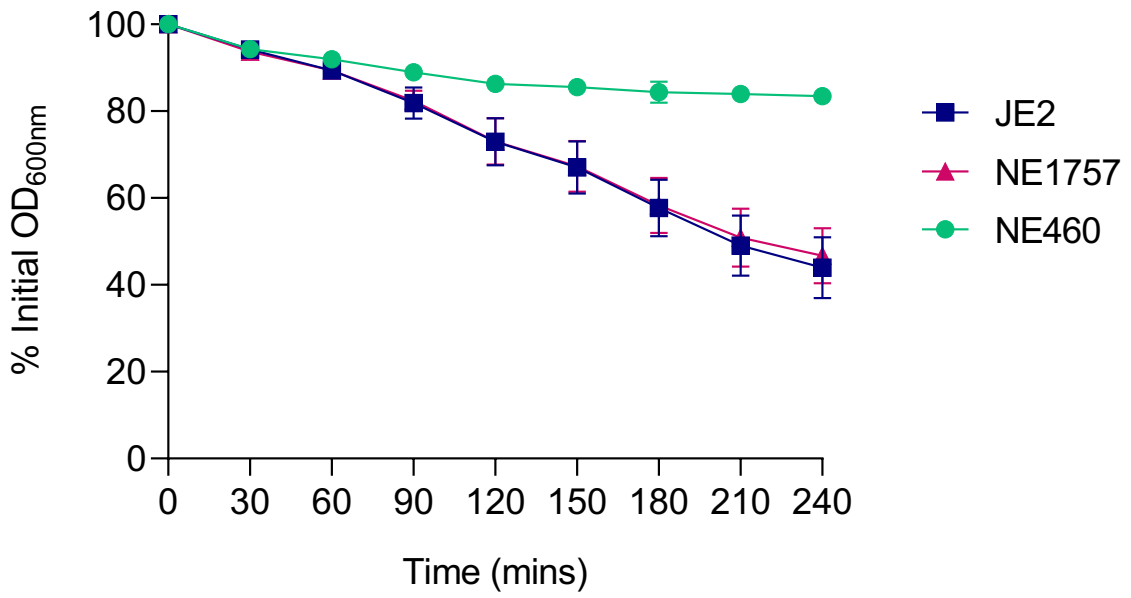
660



661
662 **Supplementary Fig. S3. Population analysis profiling shows that the *lspA* mutant NE1757**
663 **exhibits heterogenous resistance to oxacillin.** Overnight cultures of JE2, NE1757 and BH1CC
664 were grown in TSB, adjusted to OD_{600} of 1, serially diluted and plated onto TSA and TSA
665 supplemented with 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 µg/ml oxacillin. CFUs were
666 enumerated after overnight incubation at 37°C. The data are expressed as CFU/ml at each
667 oxacillin concentration, plotted using Prism software (GraphPad). Three independent
668 experiments were performed, and error bars represent standard deviations. BH1CC, which
669 exhibits homogenous oxacillin resistance, was included as a positive control.
670



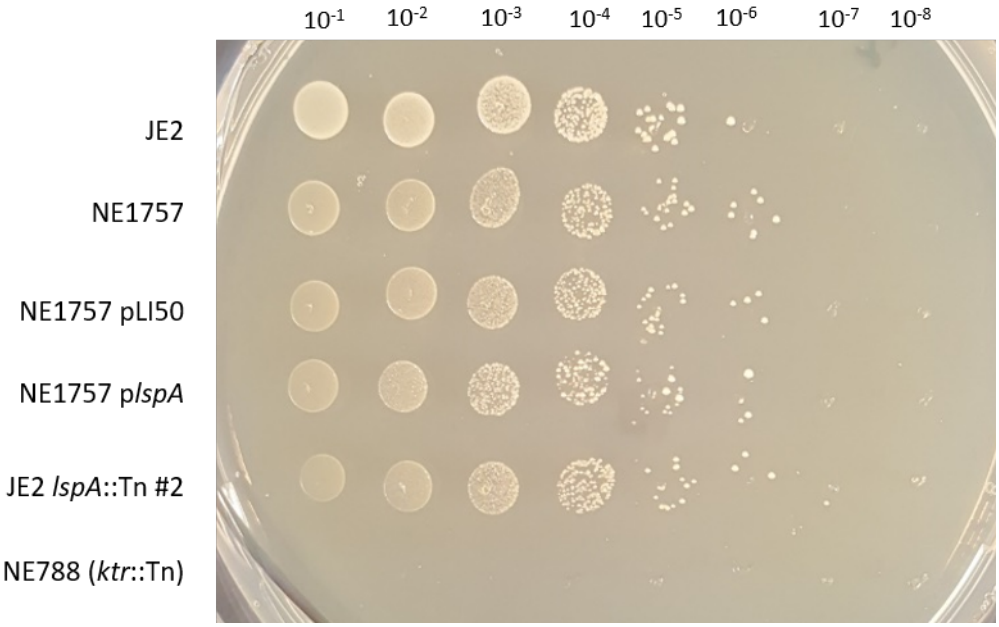
Supplementary Fig. S4. Relative proportions of cell wall muropeptide fractions based on oligomerization and relative cross-linking efficiency of cell wall muropeptide fractions in peptidoglycan extracted from JE2 and NE1757. Cells were collected from cultures grown to exponential phase in MHB or MHB supplemented with oxacillin 3 µg/ml or 32 µg/ml. Each profile shown is a representative of 3 biological replicates. Significant differences determined using Students t-test (**P < .01; ***P < .001; ****P<.0001).



680
681
682 **Supplementary Fig. S5. Autolytic activity is unaffected by the *lspA* mutation.** Triton X-100-
683 induced autolysis of JE2, NE1757 (*lspA::*Tn) and NE406 (*atl*::Tn, negative control). The strains
684 were grown to OD_{600} = 0.5 in MHB medium at 37°C, before being washed in cold PBS and
685 resuspended in 0.1% Triton X-100. The OD_{600} was monitored, and autolysis was expressed as
686 a percentage of the initial OD_{600}. The experiments were repeated 3 independent times,
687 plotted using Prism software (GraphPad) and standard deviations are shown.
688

689

690

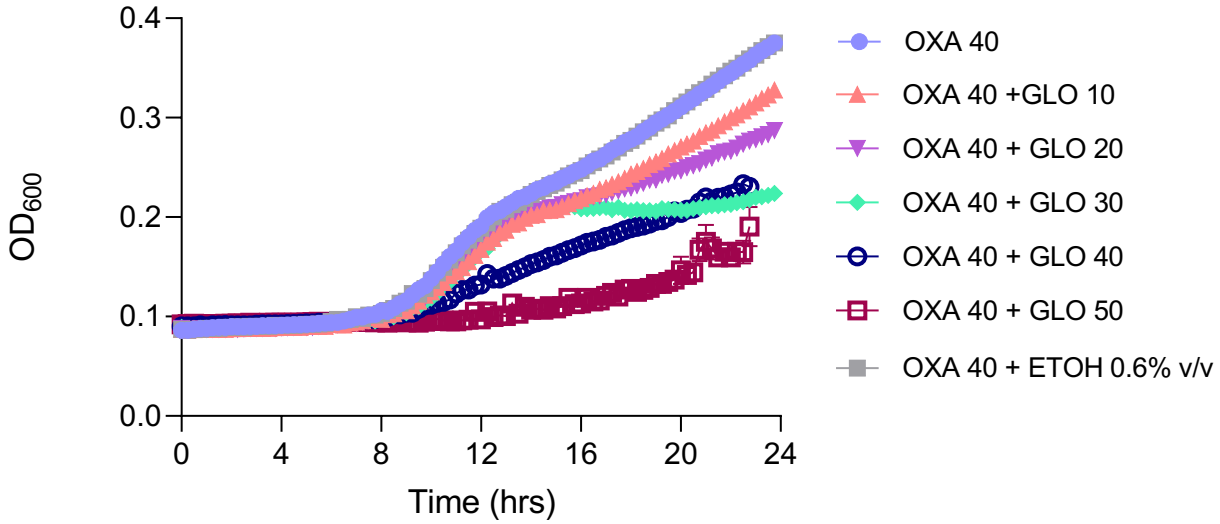


691

692 **Supplementary Fig. S6. Mutation of *lspA* has no significant impact on salt tolerance in JE2.**
693 Overnight cultures of JE2, NE1757, NE1757 pLI50, NE1757 p*lspA*, JE2 *lspA*::Tn #2 transductant
694 and NE788 (JE2 *ktr*::Tn, NaCl-sensitive control) were grown in TSB and cell density was
695 standardised to OD_{600} of 1. Four ml aliquots from 10-fold serial dilutions were spotted onto
696 TSA supplemented with 2.2 M NaCl and the plates incubated overnight at 37°C. Three
697 independent experiments were carried out and a representative image of a plate is shown

698

699



700

701 **Supplementary Fig. S7. Globomycin does not increase oxacillin resistance in the *lspA* mutant**
702 **NE1757.** NE1757 was grown in MHB 2% NaCl supplemented with a sub-inhibitory
703 concentration of oxacillin (40 µg/ml) and a range (10 – 50 µg/ml) of globomycin
704 concentrations. The solvent for globomycin, 0.6% ethanol, was included as a control. The
705 cultures were grown in a Tecan Sunrise incubated microplate reader for 24 h at 35°C. OD_{600}
706 was recorded at 15 min intervals and growth curves were plotted in Prism software
707 (GraphPad). The data presented are the average of 3 independent biological replicates, and
708 error bars represent standard deviations.
709

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