An alanine permease mutation or exposure to D-cycloserine increases the susceptibility of MRSA to β-lactam antibiotics

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Running title: Re-sensitization of AMR pathogens to β-lactams

Abstract. Prolonging the clinical effectiveness of β-lactams, which remain first-line antibiotics for many infections, is an important part of efforts to address antimicrobial resistance. We report here that inactivation of the predicted D-cycloserine (DCS) transporter gene *cycA* re-sensitizes MRSA to β-lactam antibiotics. The *cycA* mutation also resulted in hyper-susceptibility to DCS, an alanine analogue antibiotic that inhibits the alanine racemase and D-alanine ligase enzymes required for D-alanine incorporation into peptidoglycan. Amino acid transport studies showed that CycA functions as an alanine permease, and that impaired alanine transport in the *cycA* mutant correlated with increased susceptibility to oxacillin and DCS. The *cycA* mutation was accompanied by increased accumulation of muropeptides with tripeptide stems lacking the terminal D-ala-D-ala, and reduced peptidoglycan cross linking. Exposure of MRSA to DCS was also associated with a dose-dependent accumulation of muropeptides with tripeptide stems and reduced PG crosslinking. Because impaired alanine transport or DCS-treatment have similar effects on PG structure, synergism between β-lactams and DCS was investigated. DCS re-sensitised MRSA to β-lactam antibiotics *in vitro* and significantly enhanced MRSA eradication by oxacillin in a mouse bacteraemia model. The susceptibility of several other ESKAPE group pathogens to β-lactams was also increased by DCS. These data reveal that impaired uptake of alanine or DCS-meditated inhibition of D-alanine incorporation into peptidoglycan increases the susceptibility of MRSA to β-lactam antibiotics.

Importance. Treatment options for infections caused by ESKAPE group pathogens continue to dwindle with increasing antimicrobial resistance. Particularly important are the β-lactams, which remain first line antibiotics for many infections, and finding new ways to maintain their clinical effectiveness is a priority. Here we report that impaired alanine transport in an MRSA *cycA* mutant was associated with hyper-susceptibility to β-lactam antibiotics and D-cycloserine (DCS). DCS, which is used in the treatment of multi-drug resistant tuberculosis infections, re-sensitised MRSA to oxacillin and other β-lactams *in vitro* and significantly enhanced oxacillin activity in a mouse model of bacteraemia. DCS also sensitised other ESKAPE pathogens to β-lactams. These findings reveal important roles for alanine transport and the pathway for D-alanine incorporation into peptidoglycan in controlling the susceptibility of MRSA to β-lactam antibiotics.

Introduction

Whilst many bacteria can exhibit resistance to select antimicrobials, isolates of the human pathogen *Staphylococcus aureus* can express resistance to all licensed anti-staphylococcal drugs. This results in significant morbidity and mortality, with up to 20% of patients with systemic methicillin resistant *S. aureus* (MRSA) infections dying, despite receiving treatment with anti-staphylococcal drugs (1). Although aggressive hospital infection prevention and control initiatives appear to be having a positive impact on hospital-acquired MRSA rates in developed countries, *S. aureus* infections caused by community-associated MRSA strains and strains that are currently methicillin susceptible remain stubbornly high (2),(3).

As part of our efforts to identify improved therapeutic approaches for MRSA infections, we recently described the novel use of β-lactam antibiotics to attenuate the virulence of MRSA-induced invasive pneumonia and sepsis (4). We demonstrated that oxacillin-induced repression of the Agr quorum-sensing system and altered cell wall architecture resulted in downregulated toxin production and increased MRSA killing by phagocytic cells, respectively (4). Supporting this *in vitro* data, a randomised controlled trial involving 60 patients showed that the β-lactam antibiotic flucloxacillin in combination with vancomycin shortened the duration of MRSA bacteraemia from 3 days to 1.9 days (5, 6).

Because expression of methicillin resistance in *S. aureus* impacts fitness and virulence and is a regulated phenotype, further therapeutic interventions may also be possible. The complexity of the methicillin resistance phenotype is evident among clinical isolates of MRSA, which express either low-level, heterogeneous (HeR) or homogeneous, high-level methicillin resistance (HoR) (7-9). Exposure of HeR isolates to β-lactam antibiotics induces expression of *mecA,* which encodes the alternative penicillin binding protein 2a (PBP2a) and can select for mutations in auxiliary genes resulting in a HoR phenotype, including mutations that affect the stringent response and c-di-AMP signalling (10-14). Because auxiliary genes can influence the expression of methicillin resistance in MRSA, targeting the pathways associated with such genes may identify new ways to increase the susceptibility of MRSA to β-lactams. To pursue this, we performed a forward genetic screen to identify loci that impact the expression of resistance to β-lactam antibiotics in MRSA. Using the Nebraska Transposon Mutant Library, which comprises 1,952 sequence-defined transposon insertion mutants (15), inactivation of an amino acid permease gene was found to reduce resistance to cefoxitin, the β-lactam drug recommended by the Clinical and Laboratory Standards

Institute for measuring *mecA*-mediated methicillin resistance in MRSA isolates. Impaired alanine transport in the permease mutant also correlated with hyper-susceptibility to D-cycloserine (DCS), an alanine analogue antibiotic that interferes with two enzymatic steps in the D-alanine pathway of peptidoglycan biosynthesis. DCS exposure was shown to increase the susceptibility of MRSA and other ESKAPE pathogens to β-lactam antibiotics. Peptidoglycan analysis revealed that the *cycA* mutation or exposure to DCS results in loss of the terminal D-ala-D-ala from the stem peptide and reduced cross-linking of the cell wall. Given that β-lactam antibiotics remain among the most effective, safe and affordable antibiotics, DCS deserves further consideration as a potential agent to restore the therapeutic efficacy of β-lactam antibiotics.

101 **Results**

102 **Mutation of *cycA* increases the susceptibility of MRSA to β-lactam antibiotics.** To identify
103 new ways of controlling expression of methicillin resistance, we sought to identify novel
104 mutations involved in this phenotype. To achieve this, an unbiased screen of the Nebraska
105 Transposon Mutant Library (NTML) was performed using a disk diffusion assay to identify
106 mutants with increased susceptibility to cefoxitin. The parent strain of the library (JE2, a
107 derivative of USA300 LAC cured of plasmids p01 and p03 (15)) was used as a positive
108 control. Phage 80α-mediated backcross of transposon mutations into the parent strain JE2,
109 together with genetic complementation were used to confirm the role of putative genes
110 involved in cefoxitin resistance. Only mutants verified by both transduction and
111 complementation were examined further. Among the mutants identified was NE810
112 (SAUSA300_1642) (Fig. 1A), which exhibited significantly increased susceptibility to cefoxitin
113 as determined by a disk diffusion assay (Fig. S1A) and a >500-fold increase in susceptibility
114 to oxacillin as determined by E-test (Fig. 1B). Consistent with this, NE810 was previously
115 reported to be one of several NTML mutants shown to be more susceptible to amoxicillin
116 (16), but was not investigated further. RT-qPCR analysis revealed that expression of *mecA*
117 was not significantly affected in NE810 compared to JE2 (Fig. S1B), whilst whole genome
118 sequence analysis of NE810 further revealed that the SCC*mec* element was fully intact and
119 that no other mutations were present (data not shown). These findings implied that
120 increased cefoxitin susceptibility was not attributable to a failure of this strain to express a
121 functional *mecA* gene product or disruption/mutation of an auxiliary gene essential for
122 expression of resistance. Transduction of the *cycA* transposon insertion into several MRSA
123 strains from a number of clonal complexes and with different SCC*mec* types (USA300 strains
124 JE2, FPR3757 and LAC-13C, DAR173, DAR22 and DAR169 (17)) was also accompanied by
125 significant increases in cefoxitin and oxacillin susceptibility (Table 1).

126

127 **Mutation of *cycA* increases susceptibility to D-cycloserine.** SAUSA300_1642 is annotated as
128 a D-serine/L- and D-alanine/glycine transporter with homology to CycA in *Mycobacterium*
129 *tuberculosis* (18, 19). Nonsynonymous point mutations in *cycA* contribute, in part, to
130 increased D-cycloserine (DCS) resistance in *Mycobacteria* (18, 19), prompting us to
131 investigate the susceptibility of the *S. aureus cycA* mutant to DCS. In contrast to the
132 observations in *Mycobacteria*, our data showed that NE810 was significantly more

133 susceptible to DCS than the wild type JE2 and was successfully complemented by the wild-
134 type *cycA* gene (Fig. 2A). By broth dilution, the DCS MIC decreased from 32 μg/ml in JE2 to 2
135 μg/ml in NE810 (Table 1). The *cycA* mutants of the MRSA strains USA300, DAR173, DAR22
136 and DAR169 exhibited similar and significant increases in susceptibility to DCS (Table 1).
137 Transduction of the *cycA* allele from NE810 into the MSSA strains 8325-4 and ATCC29213
138 was also associated with a reduction in the DCS MIC from 32 to 4 μg/ml. DCS is a broad-
139 spectrum antibiotic produced by several *Streptomyces* species used as a second line drug in
140 the treatment of TB infections in humans. This drug is a cyclic analogue of alanine and serine
141 that inhibits the alanine racemase (Alr) enzyme that converts L-alanine to D-alanine, as well
142 as the ligase enzyme that links two D-alanine amino acids together (20). These are important
143 enzymes in both peptidoglycan (cell wall cross-linking) and teichoic acid biosynthesis in
144 Gram-positive bacteria. A mutant in the putative D-alanyl:D-alanine ligase (*ddl*,
145 SAUSA300_2039) is not available in the NTML library, suggesting that this gene may be
146 essential. However, the NTML library *alr* mutant NE1713 was associated with significantly
147 increased susceptibility to cefoxitin (Fig. S1A) and hyper-susceptibility to DCS (Fig. S1C),
148 which is consistent with the important role for D-alanine in *S. aureus* peptidoglycan
149 biosynthesis and β-lactam resistance.
150
151 **CycA is an alanine permease that is required for D-ala-D-ala incorporation into the**
152 **peptidoglycan stem peptide.** To investigate the role of CycA as a potential permease, JE2
153 and NE810 were grown for 8 h in chemically defined media containing 14mM glucose
154 (CDMG) and amino acid consumption in spent media was measured. Although no growth
155 rate or yield difference was noted between JE2 and NE810 in CDMG (Fig. 2B), alanine uptake
156 by NE810 was significantly impaired compared to JE2 (Fig. 2C). Utilisation of other amino
157 acids by NE810 and JE2, including serine and glycine, were similar (Figs. 2D and 2E and Fig.
158 S2). Impaired alanine transport in the *cycA* mutant grown in CDMG correlated with
159 increased susceptibility to oxacillin (1 μg/ml) (Fig. 2F) and DCS (1 μg/ml) (Fig. 2G). These
160 data demonstrate for the first time that CycA in *S. aureus* functions as an alanine permease.
161 Scanning and transmission electron microscopy revealed gross differences in whole cell
162 morphology (Fig. 3A and B) and cell wall thickness (Fig. 3C and D) in NE810 compared to JE2.
163 NE810 exhibited a highly irregular cell shape, and many cells appeared collapsed (Fig. 3B).
164 TEM imaging revealed that NE810 cells had a wrinkled cell surface (Fig. 3D), a thinner cell

165 wall (19 ± 2.5nm) compared to JE2 (26 ± 1.8nm) and increased rupturing, although cell size
166 (mean cell diameter) was unaffected (Table S2).
167 Quantitative peptidoglycan compositional analysis was performed using UPLC analysis of
168 muramidase-digested muropeptide fragments extracted from exponential phase cultures of
169 JE2 and NE810 grown for 220 mins in TSB media (Fig. S3). The PG profile of the *cycA* mutant
170 revealed a significant accumulation of tripeptides compared to wild-type JE2 (Fig. 4A-C),
171 which was associated with a significant reduction in crosslinking (Fig. 4D). In NE810, the
172 dimer, trimer and tetramer fractions were decreased, which was accompanied by a
173 concomitant increase in the monomer fraction (Fig. 4E). Consistent with this data, exposure
174 of JE2 to DCS 8μg/ml was also associated with a similar accumulation in muropeptides with
175 tripeptide stems (Fig. 4C), reduced cross-linking (Fig. 4D), increased muropeptide monomers
176 and reduced dimers, trimers and tetramers (Fig. 4E). DCS had a strong dose-dependent
177 effect, and at concentrations of 20 and 32 μg/ml, led to amplified effects on the
178 accumulation of muropeptides with tripeptide stems, reduced cross-linking and
179 accumulation of monomers (Fig. 4 A-E). These data are consistent with the previously
180 reported impacts of sub-inhibitory (0.25× MIC) and 4× MIC DCS concentrations, which were
181 accompanied by incorporation of an incomplete stem peptide (tripeptide) (20) and a 4-fold
182 reduction in D-ala-D-ala levels (21), respectively. Collectively these data indicate that
183 impaired D-ala incorporation into the PG stem peptide in the *cycA* mutant or following
184 exposure to DCS is accompanied by reduced PG cross-linking and increased β-lactam
185 susceptibility.
186
187 **Mutation of *cycA* or exposure to D-cycloserine increases the susceptibility of MRSA to β-**
188 **lactam antibiotics.** Previously reported synergy between DCS and β-lactam antibiotics (20,
189 22) suggests that impaired alanine uptake in the *cycA* mutant may have the same impact on
190 cell wall biosynthesis as DCS-mediated inhibition of Alr and Ddl. To further investigate this,
191 we compared the activity of DCS and β-lactam antibiotics, alone and in combination, against
192 JE2 and NE810. Checkerboard microdilution assay fractional inhibitory concentration indices
193 (ΣFICs ≤0.5) revealed synergy between DCS and several licensed β-lactam antibiotics with
194 different PBP selectivity against JE2 and USA300 FPR3757 (Table 1). Oxacillin and nafcillin
195 were not included in checkerboard assays because measurement of their MICs involves
196 supplementing the media with 2% NaCl, which distorts the MIC of DCS (data not shown).

197 Using the MRSA strains JE2, USA300, DAR173, DAR22, DAR169 and their corresponding *cycA*
198 mutants, the kinetics of killing by DCS, oxacillin and cefoxitin, alone and in combination was
199 measured over 24h using antibiotic concentrations corresponding to 0.125×, 0.25× and 0.5×
200 MICs. Recovery of growth in media supplemented with oxacillin or cefoxitin alone was
201 evident after 8 h (Fig. 5A-E), reflecting the selection and expansion of HoR mutants as
202 described previously (4, 21, 23). Recovery of growth in cultures exposed to DCS alone was
203 also evident (Fig. 5A-E), which may correlate with our observation that mutants resistant to
204 DCS (on BHI agar supplemented with 128 μg/ml DCS) arise at a rate of approximately 5.5 ×
205 10^{-8} per cell per generation (data not shown). Using combinations of DCS and oxacillin or
206 cefoxitin at 0.125× MIC did not achieve a ≥2 $\log^{10}$ reduction in the number of CFU/ml (data
207 not shown). However, at 0.5× MIC for strains JE2, USA300, DAR73 and DAR22, DCS (16
208 μg/ml)/oxacillin (32 μg/ml) and DCS (16 μg/ml)/cefoxitin (32 μg/ml) combinations achieved
209 a ≥5 $\log^{10}$ reduction in the number of CFU/ml compared to oxacillin, cefoxitin or DCS alone
210 (Fig. 5A-D). For strain DAR169, DCS/β-lactam combinations at 0.25× MIC was sufficient to
211 achieve a ≥5 $\log^{10}$ reduction in CFUs recovered compared to the individual antibiotics (Fig.
212 5E). DCS/β-lactam combinations at 0.5× MIC were also able to achieve ≥5 $\log^{10}$ reduction in
213 the number of CFU/ml against the methicillin resistant *S. epidermidis* (MRSE) strain RP62A
214 (24) compared to either antibiotic alone (Fig. S4). Checkerboard experiments with fourteen
215 MRSA strains and MRSE strain RP62A further revealed synergy (ΣFICs ≤0.5) between DCS
216 and a range of β-lactam antibiotics with different penicillin binding protein (PBP) specificity,
217 namely cefoxitin (PBP4), cefaclor (PBP3), cefotaxime (PBP2), piperacillin-tazobactin (PBP3/β-
218 lactamase inhibitor) and imipenem (PBP1) (Table 1).
219 Preliminary studies using disk diffusion assays also revealed synergy between DCS and
220 imipenem (IMP) or piperacillin-tazobactin (Pip/Taz) against several ESKAPE pathogens. Using
221 checkerboard assays synergy (ΣFIC values ≤0.5) was measured between DCS and Pip/Taz or
222 IMP against *Pseudomonas aeruginosa* LES431, *Acinetobacter baumannii* DSM 30007, *K.*
223 *pneumoniae* 511232.1 ($ESBL^+$), and two vancomycin resistant *Enterococcus* strains (Table 2).
224 This synergy appears to be specific to β-lactams and no synergy (ΣFICs > 0.5) was measured
225 between DCS and several antibiotics that are used topically or systemically for the
226 decolonization or treatment of patients colonized/infected with *S. aureus* or MRSA
227 (clindamycin, trimethoprim, mupirocin, ciprofloxacin), or several antibiotics to which *S.*

228 *aureus* isolates commonly exhibit resistance (tobramycin, kanamycin and spectinomycin)
229 (Table S1). Furthermore the *cycA* mutation had no impact on susceptibility to any of these
230 non-β-lactam antibiotics (apart from clindamycin resistance which is encoded by *ermB* on
231 the transposon).
232
233 **Combination therapy with DCS and oxacillin significantly reduces the bacterial burden in**
234 **the kidneys and spleen of mice infected with MRSA**. The virulence of the NE810 mutant
235 and the therapeutic potential of oxacillin in combination with DCS in the treatment of MRSA
236 infections were assessed in mice. Mice infected with NE810 displayed more piloerection
237 than those infected with wild-type JE2 suggestive of more severe infection, and one mouse
238 infected with NE810 required euthanization during the experiment. The average number of
239 CFUs recovered from the kidneys of mice infected with NE810 ($1.5 \times 10^8 \pm 1.49 \times 10^8$) was
240 higher than for mice infected with JE2 ($5.7 \times 10^7 \pm 6.3 \times 10^7$), but did not reach significance.
241 Interestingly, NE810 colonies exhibited visibly increased haemolytic activity on sheep blood
242 agar (Fig. S5A). We previously reported that increased haemolytic activity in MSSA strains
243 was associated with increased expression of the cell-density controlled, global accessory
244 gene regulator (*agr*) system (4, 21, 25). Comparison of *agr* (RNAIII) temporal expression
245 using RT-qPCR revealed a more significant activation of the *agr* system after 6 hrs growth in
246 NE810 compared to JE2 (Fig. S5B). This difference in *agr* activation was not related to any
247 change in growth of NE810 compared to JE2 (Fig. S5C). Taken together these data indicate
248 that increased β-lactam susceptibility in the NE810 *cycA* mutant is accompanied by both
249 increased *agr* expression and haemolytic activity, and a trend towards increased virulence in
250 this model.
251 Treatment with oxacillin or DCS alone significantly reduced the number of CFUs recovered
252 from the kidneys of mice infected with JE2 (Fig. 6). Furthermore the oxacillin/DCS
253 combination was significantly more effective than either antibiotic alone and the
254 combination was equally effective in reducing the bacterial burden in the kidneys of animals
255 infected with JE2 or NE810 when compared to no treatment ($p \leq 0.0001$) (Fig. 6)
256 demonstrating the capacity of DCS to significantly potentiate the activity of β-lactam
257 antibiotics against MRSA under *in vivo* conditions. Unexpectedly, oxacillin- or DCS-mediated
258 eradication of NE810 infections was similar to JE2 (Fig. 6). In the spleen, only oxacillin/DCS

259 combination treatment was associated with a significant reduction in the number of CFUs
260 recovered from mice infected with JE2 or NE810 (Fig. S6).
261
262 **CycA-independent alanine transport increases oxacillin and DCS resistance in the *cycA***
263 **mutant.** The failure of oxacillin or DCS treatment to enhance the eradication of NE810
264 infections in the mouse bacteraemia model prompted us to further characterise the growth
265 conditions used for the *in vitro* antibiotic susceptibility assays. Specifically we investigated
266 the role of glucose, which we previously reported to increase the growth requirement for
267 amino acids (26), and which we reasoned may be important for CycA alanine permease
268 activity. Growth of JE2 and NE810 was similar in CDM lacking glucose (Fig. 7A) and uptake of
269 alanine (Fig. 7B) and other amino acids (Fig. S7) was unchanged in NE810 compared to JE2.
270 Furthermore NE810 and JE2 grew equally well in CDM supplemented with oxacillin and DSC
271 (Fig. 7C and D). These data reveal a strong correlation between CycA-dependent alanine
272 transport and susceptibility to oxacillin and DCS, and may explain in part why the *cycA*
273 mutant does not exhibit increased β-lactam and DCS susceptibility in the mouse
274 bacteraemia model.

275 **Discussion**

276 The exploitation of antibiotic re-purposing as part of concerted efforts to address the
277 antimicrobial resistance crisis has been hampered by a lack of mechanistic data to explain
278 demonstrated therapeutic potential and the perception that studies attempting to identify
279 new uses for existing drugs are not hypothesis-driven. However, basic research discoveries
280 that advance our understanding of bacterial virulence and antibiotic resistance mechanisms
281 can also be used to inform targeted antibiotic re-purposing, in which the mechanism of
282 action is understood. In this study, we identified an alanine permease (CycA) required for
283 full expression of resistance to β-lactam antibiotics and DCS. Loss of function of this alanine
284 transporter significantly increased the susceptibility of MRSA to β-lactam antibiotics, an
285 outcome that could be reproduced through exposure to DCS. DCS targets the early steps in
286 cell wall biosynthesis by inhibiting the activity of Alr and Ddl.

287 The potential of β-lactam / DCS combinations in the treatment of recalcitrant *S. aureus*
288 infections is particularly important given that *S. aureus* isolates resistant to all licensed anti-
289 staphylococcal drugs have been reported, and in light of the regulatory barriers to the
290 introduction of new antimicrobial drugs. The recent report that DCS can potentiate the
291 activity of vancomycin against a laboratory-generated vancomycin highly-resistant *S. aureus*
292 (VRSA) strain *in vitro* and in a silkworm infection model (27) is also important. For MRSA
293 infections, the excellent safety profile of β-lactam antibiotics makes these drugs particularly
294 attractive as components of combination antimicrobial therapies. When used in the
295 treatment of tuberculosis DCS (trade name Seromycin, The Chao Centre) is typically
296 administered orally in 250 mg tablets twice daily for up to two years. At this dosage, the DCS
297 concentration in blood serum is generally 25-30 μg/ml, which is similar to the
298 concentrations used in our *in vitro* and *in vivo* experiments. The known neurological side
299 effects associated with DCS therapy mean that this antibiotic is unlikely to be considered for
300 the treatment of MRSA infections unless alternative therapeutic approaches have been
301 exhausted. DCS is a co-agonist of the *N*-methyl-D-aspartic acid receptor in the central
302 nervous system can include seizures and peripheral neuropathy (28, 29), and the use of
303 this antibiotic is limited to the treatment of recalcitrant, multi-drug resistant tuberculosis
304 infections, for which long-term therapy is required (30). Oxacillin/DCS combination therapy
305 was significantly more effective than DCS or oxacillin alone over a 5-day therapeutic window

306 suggesting that further studies on using DCS to augment β-lactams as a treatment option for
307 recalcitrant staphylococcal infections is merited.
308 The alanine permease identified in this study, CycA, shares 46% and 53% identity with CycA
309 in *M. tuberculosis* and *E. coli*, respectively. Mutation of *cycA* increases the susceptibility of
310 MRSA to β-lactam antibiotics and results in hyper-susceptibility to D-cycloserine, whereas a
311 *cycA* point mutation in *M. bovis* contributes, in part, to increased DCS resistance presumably
312 by interfering with DCS transport into the cell (19). In *E. coli*, *cycA* null mutations can also
313 result in increased resistance or have no effect in DCS susceptibility depending on the
314 growth media (31-35), suggesting that CycA is primarily important for DCS resistance under
315 conditions when its contribution to amino acid transport is also important. The opposite
316 effects of *cycA* mutation on DCS susceptibility in *S. aureus* and *E. coli* or *Mycobacteria*
317 indicates that DCS uptake is not significantly blocked by the *cycA* mutation in *S. aureus*.
318 Under growth conditions where CycA is required for alanine transport (in nutrient/glucose-
319 replete media), mutation of *cycA* or DCS-exposure have similar effects on the structure of *S.*
320 *aureus* peptidoglycan (Fig. 8). Consistent with previous studies in *S. aureus* (20) and in *M.*
321 *tuberculosis* (36), our studies showed a dose-dependent accumulation of muropeptides with
322 a tripeptide stem in MRSA exposed to DCS. The *cycA* mutation was also associated with the
323 increased accumulation of muropeptides with a tripeptide stem. These data indicate that a
324 reduced intracellular alanine pool or inhibition of Alr and Ddl is associated with reduced D-
325 ala-D-ala incorporation into the PG stem peptide. The increased accumulation of tripeptides
326 in turn interferes with normal PBP transpeptidase activity and offers a plausible explanation
327 for increased susceptibility to β-lactam antibiotics. The importance of the terminal stem
328 peptide D-ala-D-ala for β-lactam resistance has previously been reported. Mutation of the
329 *murF*-encoded ligase, which catalyses of the D-ala-D-ala into the stem peptide also increased
330 β-lactam (but not DCS) susceptibility (37, 38). Similarly growth of a HoR MRSA strain in
331 media supplemented with high concentrations of glycine was accompanied by replacement
332 of the D-ala-D-ala with D-ala-gly and decreased methicillin resistance (39).
333 Impaired uptake of alanine in CDMG correlated with increased susceptibility to oxacillin and
334 DCS, suggesting that alanine utilisation via CycA is important to make D-alanine available for
335 cell wall biosynthesis and consequently resistance to β-lactam antibiotics. Consistent with
336 this, NE810 also exhibited increased oxacillin susceptibility in BHI, TSB and MH media.

337 However no change in alanine transport or susceptibility to oxacillin and DCS was measured
338 in CDM lacking glucose, which was consistent with the failure of oxacillin and DCS to more
339 efficiently eradicate NE810 infections in the mouse bacteraemia model. The availability of
340 nutrients such as glucose and amino acids varies in different niches colonised by *S. aureus*
341 during infection ranging from glucose-rich in organs such as the liver (40), to glucose-
342 depleted in established abscesses (41), which in turn impacts the role of amino acids as
343 carbon sources (26, 42), and potentially the activity of CycA in alanine transport and β-
344 lactam susceptibility. Furthermore, alanine transport was unaffected in the *cycA* mutant
345 grown in CDM indicating that an alternative alanine transport mechanism(s) may be active
346 under these growth conditions (Fig. 8). Identification of this alternative alanine permease
347 may be important in the development of therapeutic strategies targeting alanine transport
348 to increase β-lactam susceptibility in MRSA, while elucidation of the role of glucose in the
349 control of alanine transport should provide new insights into β-lactam resistance.

350 **Materials and Methods**

351 **Bacterial strains, growth conditions and antimicrobial susceptibility testing.** Bacterial
352 strains used in this study (Table S3) were grown in Luria Bertoni (LB) (Sigma), brain heart
353 infusion (BHI) (Oxoid), Mueller Hinton (MH) (Oxoid), nutrient (Oxoid), sheep blood BHI or
354 chemically defined media (CDM).

355 The minimum inhibitory concentrations (MICs) of oxacillin, cefaclor, cefataxime, nafcillin,
356 cefoxitin, imipenem, piperacillin-tazobactam (Pip/Taz), clindamycin, tobramycin,
357 trimethoprim, mupirocin, ciprofloxacin, spectinomycin, kanamycin and D-cycloserine were
358 determined in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines
359 using plate and broth dilution assays in MH or MH containing 2% NaCl for oxacillin and
360 nafcillin. Oxacillin MICs were also measured using E-tests strips from Oxoid on MH agar
361 containing 2% NaCl. Quality control strains ATCC29213 and ATCC25923 were used for broth
362 dilution and disk diffusion oxacillin and cefoxitin MIC assays, respectively.

363 **Identification of cefoxitin susceptible MRSA mutant NE810.** The Nebraska transposon
364 mutant library (NTML) comprising 1,952 strains harbouring transposon insertions in all non-
365 essential genes was constructed in the USA300 derivative JE2, which has been cured of
366 plasmids p01 and p03 (15). Cefoxitin susceptibility of individual mutants was determined
367 using the disk diffusion method in accordance with the CLSI guidelines using cefoxitin (30μg)
368 disks (Oxoid) on Mueller Hinton agar (Oxoid). Briefly, each library mutant was revived from
369 the freezer on BHI agar supplemented with 10μg/ml erythromycin and grown overnight for
370 18 h at 37°C. The wild-type parent strain of the library JE2 and the library mutant containing
371 a transposon insertion in *mecA* (NE1868) were used as positive (cefoxitin resistant) and
372 negative (cefoxitin susceptible) controls, respectively. The cefoxitin susceptible *S. aureus*
373 strain ATCC25923 was also used as a negative control. Several colonies were used to
374 prepare a bacterial suspension in sterile phosphate-buffered saline (PBS), which was
375 standardised to 0.5 McFarland and used to inoculate a MH agar plate before the application
376 of the cefoxitin-impregnated disc. The MH agar plates were then incubated at 35°C for 18 h
377 and the zone sizes measured. The zone diameter for JE2 was 18mm and >35mm for NE1868
378 (*mecA*). The cefoxitin susceptible mutant NE810 exhibited a zone diameter = 22mm. The
379 entire library was screened in duplicate and putative cefoxitin susceptible mutants were

380 tested at least five times. This susceptibility of cefoxitin sensitive mutants to oxacillin was
381 also determined by E-test, as well as broth and plate dilution assays.
382 PCR was used to verify the presence of the transposon insertion NE810 using the primers
383 NE810_Fwd and NE810_Rev (Table S4). Phage 80α was used to transduce the transposon
384 mutation from NE810 back into JE2 and USA300 to ensure that secondary mutations were
385 not involved in increased cefoxitin susceptibility phenotype. DNA extracted from
386 transductants selected on BHI agar supplemented with erythromycin 10μg/ml were verified
387 by PCR using primers NE810_Fwd and NE810_Rev. Comparative whole genome sequencing
388 of JE2 and NE810 (MicrobesNG, UK) using the USA300_FPR3757 genome as a reference
389 revealed the absence of other mutations in NE810.
390 To complement NE810, the *cycA* gene was amplified from JE2 on a 1608 bp PCR product
391 using primers NE810F1_Fwd and NE810F1_Rev (Table S4) and cloned into the *E. coli-*
392 *Staphylococcus* shuttle plasmid pLI50 using the Clontech In-fusion HD cloning kit. The
393 primers were designed using the CloneAmp In-fusion tool with 15 bp extensions that were
394 complementary to the ends of the linearized vector. The recombinant plasmid generated
395 was transformed into *E. coli* HST08 and verified by Sanger sequencing (Source Biosciences)
396 before being transformed by electroporation into the restriction-deficient laboratory strain
397 RN4220, and subsequently NE810. All plasmid-harbouring strains were cultured in medium
398 supplemented with 100 μg/ml ampicillin (*E. coli*) or 10 μg/ml chloramphenicol (*S. aureus*) to
399 maintain plasmid selection.

400 **Growth measurements of JE2 strains in chemically defined media and amino acid analysis.**
401 Overnight cultures of JE2 and NE810 were grown at 37°C in TSB, washed in PBS and
402 inoculated at a starting cell density of A_{600} = 0.05 into chemically defined medium (CDM) or
403 CDM supplemented with 14mM glucose (CDMG) (43). The bacteria were grown aerobically
404 (250 rpm; 10:1 flask to volume ratio, 37°C) and the A_{600} was determined every two hours for
405 8-12 hours. One ml of the bacterial culture was collected every two hours and pelleted by
406 centrifugation at 14,000x g for 3 minutes. The spent media was filtered using an Amicon
407 Ultra centrifugal filter (Millipore; 3,000 molecular weight cutoff [MWCO]). Amino acid
408 analysis was performed by the Protein Structure Core Facility, University of Nebraska
409 Medical Center, using a Hitachi L-8800 amino acid analyzer as described previously (26).

410 ***mecA* gene expression analysis.** Quantitative reverse transcription PCR (RT-qPCR) was used
411 to measure *mecA* transcription on the Roche LightCycler 480 instrument using the
412 LightCycler 480 Sybr Green Kit (Roche) with primers mecA1_Fwd and mecA1_Rev (Table S4).
413 The following cycling conditions were used: 95°C for 5 minutes and followed by 45 cycles of
414 95°C for 10 seconds, 58°C for 20 seconds and 72°C for 20 seconds. Melt curve analysis was
415 performed at 95°C for 5 seconds followed by 65°C for one minute up to 97 C at a ramp rate
416 of 0.11c/sec with five readings taken for every degree of temperature increase. The *gyrB*
417 gene amplified with primers gyrB_Fwd and gyrB_Rev (Table S4) served as an internal
418 standard for all reactions. For each reaction, the ratio of *mecA* and *gyrB* transcript number
419 was calculated as follows: $2^{(Ct\ gyrB\ -\ Ct\ mecA)}$. Each RT-qPCR experiment was performed three
420 times and presented as average data with standard errors.

421 **Scanning Electron Microscopy (SEM).** Cell shape and cell size analysis were carried out using
422 SEM imaging. Overnight BHI cultures were diluted 1:200 in BHI broth supplemented with 1%
423 glucose and incubated for 24h at 37°C. Subsequently, the slides were rinsed three times
424 with distilled water, dried at 60°C for 1h, before being rinsed with 0.1M phosphate buffer,
425 fixed in 2.5% glutaraldehyde for 2h, rinsed again with 0.1M phosphate buffer, dehydrated in
426 ethanol (20%, 30%, and 50% for 10 min each step; 70% ethanol with 0.5% uranyl acetate for
427 30 min; and then 90%, 96%, and 100% ethanol), soaked in hexamethyldisilazane (HMDS) for
428 30 minutes and finally dried overnight. The slides were fixed to metal stubs before being
429 coated in gold and imaged using a Hitachi S2600N Variable Pressure Scanning Electron
430 Microscope.

431 **Transmission Electron Microscopy (TEM).** Overnight cultures were diluted 1:200 in 10ml
432 BHI media and grown at 37°C to A_{600} = 1. The cells were pelleted at 8,000 × *g* before being
433 re-suspended in fixation solution (2.5% glutaraldehyde in 0.1 M cacodylate buffer [pH 7.4])
434 and incubated overnight at 4°C. The fixed cells were further treated with 2% osmium
435 tetroxide, followed by 0.25% uranyl acetate for contrast enhancement. The cell pellets were
436 then dehydrated in increasing concentrations of ethanol as described above for the SEM cell
437 preparation, followed by pure propylene oxide, and transferred to a series of resin and
438 propylene oxide mixtures (50:50, 75:25, pure resin) before being embedded in Epon resin.
439 Thin sections were cut on an ultramicrotome. Images were analysed using a Hitachi H7000

440 instrument. At least 3 to 5 measurements of cell wall thickness were performed on each cell
441 and approximately 30 cells were measured for each sample.

442 **Analysis of peptidoglycan composition in NE810 and JE2 treated with D-cycloserine**. Using
443 overnight cultures of JE2 and NE810, independent quadruplicate 50ml broth cultures were
444 set up at a starting cell density of A_{600}=0.05 and grown for approximately 2 hours to
445 ≈A_{600}=0.5, before being left untreated or dosed with DCS at a final concentration of 8, 20 or
446 32 μg/ml for a further 100 mins before the cells in the entire 50 ml culture were harvested
447 by centrifugation and resuspended in 5ml PBS (Fig. S3). The cell samples were slowly
448 dropped into an equal volume of boiling 10% (wt/vol) SDS, vigorously stirred for 2h in a
449 boiling water bath and left stirring overnight at room temperature. The insoluble fractions
450 were recovered by high speed centrifugation (150,000 x g, 15 min, 25°C) and washed until
451 the fractions were free from SDS. The insoluble fractions were purified for peptidoglycan
452 according to the protocol described previously (44). Purified peptidoglycan was re-
453 suspended in 50 mM $NaPO_4$ buffer, pH 4.9 and treated with 80 µg/mL muramidase (Cellosyl)
454 for 16 h at 37°C. Muramidase digestion was stopped by incubation in a boiling water bath,
455 and coagulated proteins were removed by centrifugation (21,000 x g, 10 min). The
456 supernatants were adjusted to pH 9.0 with sodium borate and reduced with sodium
457 borohydride for 30 min at room temperature. Finally, samples were adjusted to pH 3.5 with
458 orthophosphoric acid and filtered prior to UPLC analysis with a Waters Acquity UPLC H-class
459 system (Waters) equipped with a Waters Xevo G2-XS QTof MS (Waters MS
460 Technologies). Chromatographic separation was achieved on acquity UPLC® BEH C18, 1.7
461 µm, 150 x 2.1 mm id (Waters) column. The mobile consisted of solvent A: 0.1% formic acid
462 in Milli-Q water and B: 0.1% formic acid in acetonitrile. The gradient was set as follows: 0-3
463 min 2-5% B, 3-6 min 5-6.8% B, 6-7.5 min 6.8-9% B, 7.5-9 min 9-14% B, 9-11 min 14-20% B,
464 11-12 min hold at 20% B keeping the constant flow of 0.250µl/min, 12-12.1 min 20-90% B
465 with a flow rate ramp of 0.250µl/min to 0.300µl/min further holding the same flow rate and
466 % B till 13.5 min, 13.5-13.6 min 90-2% B, 13.6-16 min hold at 2% B. The flow rate was
467 ramped back again to 0.250µl/min at 16.1 min and column was equilibrated form next
468 analysis till 18min. The chromatographic separation was recorded at 204nm.
469 Mass spectrometry was performed on a Xevo G2-XS QTof quadrupole time-of-flight mass
470 spectrometer (Waters MS Technologies). The scan range was from 10 to 2000 m/z with a

471 scan rate of 0.25s. Instrument was operated in positive electro-spray ionization mode. The
472 capillary and sample cone voltages were 3kV and 40 V, respectively. Gas flows were set at
473 100 and 500 l/hr for cone gas and desolvation gas, respectively. The source temperature
474 was 120°C and desolvation temperature was 350°C. For MS^E low collision energy was set at
475 6 eV while ramping the high collision energy from 15-40 eV. Leucine-enkephalin reference
476 was used as the lockmass at a concentration of 200 pg/mL with a continuous flow of
477 5µL/min and 0.25s scan time, to maintain the accuracy of the analysis. All the acquisition
478 and analysis of data were controlled by Waters UNIFI software. Structural characterization
479 of muropeptides was performed based on their MS data and MS/MS fragmentation pattern,
480 which matched with the PG composition and structure reported in previous publications
481 (39, 45-47).

482 **Antibiotic synergy analysis using the microdilution checkerboard assay.** The synergistic
483 activity of D-cycloserine and other antibiotics was measured using the checkerboard
484 microdilution method in 96-well plates. The final inoculum in each well was 5×10^5 CFU/ml,
485 and the results (growth or no growth) were determined after 24 h incubation at 37°C. Un-
486 inoculated MH broth supplemented with the antibiotics was used as a negative control.
487 Inoculated MH broth with no antibiotics was used as a positive control. The fractional
488 inhibitory concentration index (ΣFIC) was calculated for each drug combination. An FIC index
489 of ≤0.5 was considered synergistic, one of >0.5 to <2 was considered indifferent, and one of
490 >2 was considered antagonistic. All experiments were performed in triplicate.

491 **Kill curve assays.** Suspensions of bacterial cells from overnight cultures adjusted to 10^7
492 CFU/ml were exposed to increasing concentrations (0.125×, 0.25×, and 0.5× MIC) of
493 oxacillin, cefoxitin and DCS alone or in combination, and the number of colony forming units
494 (CFU)/ml enumerated at 0, 2, 4, 8 and 24 h. Data is presented at the antibiotic
495 concentrations where synergy was measured i.e. 0.5× MIC for JE2, USA300, DAR173, DAR22,
496 DAR113, BH1CC, and RP62A, and 0.25× MIC for DAR169. Antibiotic synergism was defined
497 as a ≥2 $\log^{10}$ decrease in the number of CFU/ml in cell suspensions exposed to DCS/β-lactam
498 combinations compared to the most effective individual drug after 8 h.

499 **Mouse infection experiments.** 6- to 8-week-old, age matched, outbred CD1 female mice
500 (Charles River, UK) were used in a non-lethal model of bacteremia. JE2 and NE810 cultures
501 were grown to A_{600}=0.5 in BHI, washed in PBS, adjusted to 1×10^8 CFU/ml. Mice were

502 infected intravenously (via the tail vein) with 5 × 10^6 CFU ($n = 10$ mice per group). The
503 infections were left untreated (PBS control) or treated with either 75 mg oxacillin/Kg/12
504 hours, 30 mg DCS/Kg/12 hours or a combination of both (first antibiotic dose administered
505 16 hours post infection), before being sacrificed after 5 days. Bacteria present in
506 homogenised spleens and kidneys recovered from the mice were enumerated on blood
507 agar. Statistical significance was assessed using one-way ANOVA with Kruskal-Wallis test
508 followed by Dunn's multiple comparisons test in the GraphPad Prism application.

509 **Ethics Statement.** Mouse experiments were approved by the United Kingdom Home Office
510 (Home Office Project License Number 40/3602) and the University of Liverpool Animal
511 Welfare and Ethics Committee. This study was carried out in strict accordance with the
512 United Kingdom Animals (Scientific Procedures) Act 1986. All efforts were made to minimize
513 suffering.

514 **Statistical analysis**. Two-tailed Student's t-Tests and one-way ANOVA with Kruskal-Wallis
515 test followed by Dunn's multiple comparisons test (*in vivo* experiments) were used to
516 determine statistically significant differences in assays performed during this study. A p
517 value <0.05 was deemed significant.

Acknowledgements:

We thank Craig Winstanley and Kate Reddington for generously providing bacterial strains.

520 **Figure Legends**

521 **Figure 1. Mutation of *cycA* increases the susceptibility of MRSA to β-lactam antibiotics. A.**
522 Chromosomal location of *cycA* and neighbouring genes *icd* (isocitrate dehydrogenase), *gltA*
523 (citrate synthase), *pyk* (pyruvate kinase) and *pfkA* (6-phosphofructokinase). The locations of
524 transposon insertions in NE810, NE491, NE594 and NE1497 mutants from the Nebraska
525 library are indicated. **B.** E-test measurement of oxacillin minimum inhibitory concentrations
526 (MICs) in JE2 (wild type), NE810 (*cycA*::Tn), NE810 carrying pLI50 (control) and p*cycA*.

527 **Figure 2. Mutation of *cycA* increases susceptibility to D-cycloserine in MRSA and impairs**
528 **alanine uptake. A.** Comparison of zones of inhibition around D-cycloserine 30µg disks on
529 lawns of JE2, NE810 (*cycA*::Tn), NE810 pLI50 (control) and NE810 p*cycA* grown on Mueller
530 Hinton (MH) agar. **B.** Growth of JE2 and NE810 in chemically defined media supplemented
531 with glucose (CDMG). Cell density was measured at A_{600}. **C, D and E.** Alanine (C), Serine (D)
532 and Glycine (E) consumption by JE2 and NE810 grown aerobically in CDMG. Residual amino
533 acid was measured in spent media after 0, 4, 6 and 8 h growth. **F and G.** Growth of JE2 and
534 NE810 cultures for 12 h in CDMG supplemented with 1 µg/ml oxacillin (F) or 1 µg/ml D-
535 cycloserine (G). Cell density was measured at A_{600}.

536 **Figure 3. Mutation of *cycA* reduces cell wall thickness in MRSA. A and B.** Comparison of JE2
537 (A) and NE810 (B) cell morphology by scanning electron microscopy (SEM). **C and D.**
538 Comparison of JE2 (C) and NE810 (D) cell wall thickness by transmission electron microscopy
539 (TEM). TEM magnifications are ×15,000 (top) and ×70,000 (bottom) and cell wall thickness
540 measurements are provided in Table S2.

541 **Figure 4. Mutation of *cycA* or D-cycloserine (DCS) treatment impacts peptidoglycan**
542 **peptide stem length and reduces cell wall crosslinking. A.** Representative UV
543 chromatograms of peptidoglycan from wild-type JE2, NE810 and JE2 treated with increasing
544 concentrations of DCS (8, 20 and 32 µg/ml). Muropeptides with tripeptide stems are
545 numbered 1-3. **B.** Proposed structures of the three muropeptides with tripeptide stems
546 identified in NE810 and DCS-treated JE2 cells. NAG, N-acetylglucosamine; NAM, N-
547 acetylmuramic acid; L-Ala, L-alanine; D-Gln, D-glutamine; D-Glu, D-glutamic; L-Lys, L-lysine.
548 The theoretical and observed neutral masses determined by MS are indicated. **C.** Relative
549 abundance of muropeptides with tripeptides in the stem. **D.** Relative crosslinking efficiency.
550 **E.** Relative proportions of cell wall muropeptide fractions based on oligomerization. All
551 errors bars represent 95% confidence interval (n = 4). Significant differences determined
552 using Students t-test are denoted using asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

553 **Figure 5.** *In vitro* kill curves for D-cycloserine (DCS), oxacillin and cefoxitin with JE2 (A),
554 USA300 FPR3757 (B), DAR173 (C), DAR22 (D), DAR169 (E) and their isogenic *cycA* mutants.
555 Antibiotics at the concentrations indicated (µg/ml) were added to suspensions of overnight
556 bacterial cultures adjusted to 10^7 CFU/ml in BHI (A_{600}=0.05), incubated at 37°C and the
557 number of CFU/ml enumerated at 0, 2, 4, 8 h. The data presented are the mean of three
558 independent experiments, and standard error of the mean is shown. Antibiotic synergism

559 was defined as a ≥2 $\log^{10}$ decrease in the number of CFU/ml in cell suspensions exposed to
560 DCS/β-lactam combinations compared to the most effective individual antibiotic alone.

561 **Figure 6. Combination therapy with D-cycloserine and oxacillin significantly reduces the**
562 **bacterial burden in the kidneys of mice infected with MRSA.** The number of colony-forming
563 units (CFU) recovered from the kidneys of mice infected by tail vein injection with 5×10^6 JE2
564 or NE810 (CycA) and left untreated or treated with 75mg of oxacillin (Ox)/kg, 30mg of
565 DCS/kg or a combination of both Ox and DCS delivered subcutaneously every 12 hours for 5
566 days. The first antibiotic dose was given 16 hours after infection. Significant differences
567 determined using one-way ANOVA with Kruskal-Wallis test followed by Dunn's multiple
568 comparisons test are denoted using asterisks (*p≤0.05, **p≤0.01, ****p≤0.0001). The limit
569 of detection (50 colonies) is indicated with a hashed red line.

570 **Figure 7. CycA-independent alanine transport increases oxacillin and D-cycloserine**
571 **resistance in the *cycA* mutant**. **A.** Growth of JE2 and NE810 in chemically defined medium
572 lacking glucose (CDM). Cell density was measured at A_{600}. **B.** Alanine consumption by JE2
573 and NE810 grown aerobically in CDM. Residual amino acid was measured in spent media
574 after 0, 4, 6 and 8 h growth. **C and D.** Growth (cell density at A_{600}) of JE2 and NE810 cultures
575 for 12 h in CDM supplemented with 1 μg/ml oxacillin (C) or 1 μg/ml DCS (D).

576 **Figure 8.** Proposed model depicting how impaired alanine transport associated with
577 mutation of CycA or exposure to DCS can inhibit the D-alanine pathway for peptidoglycan
578 biosynthesis leading to increased susceptibility to β-lactam antibiotics.

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741

Table 1. Antibacterial activity (minimum inhibitory concentrations, MIC) and drug synergy (fractional inhibitory concentration indices, ΣFIC) of D-cycloserine (DCS) and several β-lactam antibiotics with different PBP specificity, namely oxacillin (OX; PBP1, 2, 3), nafcillin (NAF; PBP1), cefoxitin (FOX; PBP4), cefaclor (CEC; PBP3), cefotaxime (CTX; PBP2), piperacillin-tazobactin (TZP; PBP3/β-lactamase inhibitor) and imipenem (IMP; PBP1), alone and in β-lactam combinations, against fourteen *S. aureus* strains and *S. epidermidis* RP62A.

Antibiotic → / ↓ Strain	OX*	NAF*	DCS*	FOX*	DCS/FOX** (ΣFIC)***	CEC*	DCS/CEC** (ΣFIC)***	CTX*	DCS/CTX** (ΣFIC)***	TZP*	DCS/TZP** (ΣFIC)***	IPM*	DCS/IPM** (ΣFIC)***
JE2	64	32	32	64	8/8 (0.37)	64	8/2 (0.28)	64	8/4 (0.31)	32	8/0.5 (0.26)	1	ND
NE810 (*cycA*)	0.25	0.5	2	8	ND	4	ND	8	ND	2	ND	0.125	ND
USA300	64	32	32	64	8/8 (0.37)	64	8/8 (0.37)	64	8/8 (0.37)	64	8/2 (0.28)	1	ND
USA300 *cycA*	0.25	1	2	8	ND	4	ND	8	ND	4	ND	0.125	ND
DAR173	128	128	32	256	4/32 (0.25)	128	8/4 (0.28)	512	8/4 (0.25)	128	4/4 (0.15)	64	4/2 (0.15)
DAR173 *cycA*	0.5	8	4	32	ND	16	ND	16	ND	4	ND	1	ND
DAR22	128	128	32	128	8/8 (0.31)	128	8/8 (0.31)	512	8/4 (0.25)	128	4/4 (0.15)	128	4/1 (0.13)
DAR22 *cycA*	0.5	8	0.5	16	ND	16	ND	16	ND	4	ND	0.5	ND
DAR169	32	32	32	32	8/2 (0.31)	128	4/32 (0.37)	64	4/8 (0.25)	8	4/1 (0.25)	2	ND
DAR169 *cycA*	16	4	0.5	16	ND	64	ND	16	ND	2	ND	0.5	ND
COL	512	256	64	512	8/128 (0.37)	128	16/32 (0.5)	>2048	ND	128	32/0.5 (0.5)	256	8/64 (0.37)
DAR113	128	64	32	128	8/8 (0.3)	64	8/4 (0.3)	256	8/8 (0.2)	128	8/4 (0.2)	16	8/0.5 (0.2)
BH1CC	256	512	32	256	8/32 (0.3)	128	8/8 (0.3)	1028	8/16 (0.2)	256	8/4 (0.2)	64	8/1 (0.2)
BH14B(04)	128	64	16	128	4/32 (0.5)	128	4/32 (0.5)	512	4/32 (0.31)	64	4/1 (0.26)	64	2/8 (0.25)
BH8(03)	128	128	32	128	8/32 (0.5)	256	8/64 (0.5)	128	8/16 (0.25)	128	8/8 (0.3)	64	8/0.5 (0.1)
BH6	128	128	32	128	8/16 (0.37)	256	8/64 (0.5)	512	8/32 (0.31)	128	8/4 (0.28)	32	8/0.5 (0.26)
DAR202	64	64	32	128	4/32 (0.37)	128	4/32 (0.37)	64	8/16 (0.5)	64	8/2 (0.28)	4	8/1 (0.5)
DAR45	2	0.5	32	4	ND	32	8/2 (0.31)	4	ND	2	ND	0.5	ND
DAR13	128	32	32	128	4/32 (0.37)	128	8/4 (0.28)	128	4/32 (0.37)	32	8/1 (0.28)	8	4/2 (0.37)
RP62A	128	2	32	64	6/16 (0.5)	64	6/16 (0.5)	32	8/8 (0.5)	8	8/2 (0.5)	32	8/1 (0.2)

* MIC values for each antibiotic when measured individually; μg/ml

** MIC values for each antibiotic when measured in combination, also known as the fractional inhibitory concentration (FIC); μg/ml

*** FIC indices (ΣFIC) for the antibiotic combination. ΣFIC = FIC A + FIC B, where FIC A is the MIC of DCS in combination with the β-lactam/MIC of DCS alone, and FIC B is the MIC of the β-lactam in combination with DCS/MIC of the β-lactam alone. The combination is considered synergistic when the ΣFIC is ≤0.5, indifferent when the ΣFIC is >0.5 to <2.

‡ND. Not determined if strain is susceptible (or hyper-resistant) to β-lactam antibiotic or for *cycA* mutants with reduced DCS & β-lactam MICs.

Table 2. Antibacterial activity (minimum inhibitory concentrations, MIC) and drug synergy (fractional inhibitory concentration indices, ΣFIC) of D-cycloserine (DCS) and the β-lactam antibiotics piperacillin-tazobactin (TZP) and imipenem (IMP), alone and in β-lactam/DCS combinations, against selected strains of *Pseudomonas aeruginosa*, vancomycin resistant *Enterococcus, Klebsiella pneumoniae and Acinetobacter baumanii*

Strain	DCS*	TZP*	DCS/TZP** (ΣFIC)***	IPM*	DCS/IPM** (ΣFIC)***
***Pseudomonas aeruginosa* LES431**	256	128	64/8 (0.312)	16	32/4 (0.375)
Vancomycin resistant *Enterococcus* CIP104105	64	512	32/1 (0.502)	256	16/16 (0.5)
Vancomycin resistant *Enterococcus* DSM17050	64	256	16/64 (0.5)	128	16/1 (0.257)
***Klebsiella pneumoniae* 511232.1 (ESBL^{+})**	128	32	8/32 (0.5)	1	ND
***Acinetobacter baumanii* DSM30007**	128	32	32/8 (0.5)	1	ND

* MIC values for each antibiotic when measured individually; μg/ml

** MIC values for each antibiotic when measured in combination, also known as the fractional inhibitory concentration (FIC); μg/ml

*** FIC indices (ΣFIC) for the antibiotic combination. ΣFIC = FIC A + FIC B, where FIC A is the MIC of DCS in combination with the β-lactam/MIC of DCS alone, and FIC B is the MIC of the β-lactam in combination with DCS/MIC of the β-lactam alone. The combination is considered synergistic when the ΣFIC is ≤0.5 and indifferent when the ΣFIC is >0.5 to <2. Green shading indicates synergy, orange shading indicates no synergy.

[‡]ND. Not determined where the bacterial strain is susceptible to the β-lactam antibiotic.

Supplementary Tables

Table S1. Antibacterial activity (minimum inhibitory concentrations, MIC) and drug synergy (fractional inhibitory concentration indices, ΣFIC) of D-cycloserine (DCS), and clindamycin (CLI), tobramycin (TOB), trimethoprim (TMP), mupirocin (MUP), ciprofloxacin (CIP), kanamycin (KAN) and spectinomycin (SPT) alone and in DCS/antibiotic combinations, against fourteen *S. aureus* strains and *S. epidermidis* RP62A.

Antibiotic → / **↓ Strain**	DCS*	CLI*	DCS/CLI** (ΣFIC)***	TOB	DCS/TOB (ΣFIC)	TMP	DCS/TMP (ΣFIC)	CIP	DCS/CIP (ΣFIC)	KAN	DCS/KAN (ΣFIC)	SPT	DCS/SPT (ΣFIC)	MUP	DCS/MUP (ΣFIC)
JE2	32	0.25	ND	1	ND	1	ND	32	16/16 (1)	2	ND	64	16/32 (1)	0.25	ND
USA300	32	2048	32/2048 (2)	0.5	ND	1	ND	32	16/16 (1)	2	ND	64	16/32 (1)	2048	32/2048 (2)
DAR173	32	0.5	ND	512	32/512 (2)	0.5	ND	256	16/128 (1)	512	16/256 (1)	64	16/32 (1)	0.5	ND
DAR22	32	0.5	ND	512	16/256 (1)	0.5	ND	256	16/128 (1)	256	16/128 (1)	64	16/32 (1)	0.5	ND
DAR169	32	0.5	ND	0.5	ND	0.5	4/8 (0.25)	1	ND	2	ND	64	16/32 (1)	0.5	ND
COL	64	0.5	ND	0.5	ND	0.5	ND	1	ND	0.5	ND	64	16/32 (1)	0.5	ND
DAR113	32	0.25	ND	0.5	ND	1	ND	0.5	ND	2	ND	64	16/32 (1)	0.25	ND
BH1CC	32	1024	32/1024 (2)	32	32/32 (2)	256	32/256 (2)	32	16/16 (1)	>1024	ND	>1024	ND	32	16/8 (0.75)
BH14B(04)	16	0.5	ND	0.5	ND	0.5	ND	128	16/128 (1.5)	2	ND	64	8/32 (1)	0.5	ND
BH8(03)	32	1024	32/1024 (2)	0.5	ND	0.5	ND	256	16/128 (1)	8	32/4 (2)	128	16/64 (1)	4	32/4 (2)
BH6	32	0.5	ND	0.5	ND	0.5	ND	>512	16/256 (1)	1	ND	128	16/64 (1)	0.5	ND
DAR202	32	0.5	ND	128	16/64 (1)	0.5	ND	2	ND	>1024	ND	>1024	ND	0.5	ND
DAR45	32	1024	32/1024 (2)	512	16/256 (1)	0.5	ND	128	16/64 (1)	128	16/64 (1)	512	32/1024 (2)	0.5	ND
DAR13	32	0.5	ND	0.5	ND	32	16/16 (1)	32	16/16 (1)	2048	ND	>1024	ND	0.5	ND
RP62A	32	2048	32/2048 (2)	16	16/8 (1)	256	32/256 (2)	0.25	ND	>1024	ND	>1024	ND	0.25	ND

* MIC values for each antibiotic when measured individually; μg/ml

** MIC values for each antibiotic when measured in combination, also known as the fractional inhibitory concentration (FIC); μg/ml

*** FIC indices (ΣFIC) for the antibiotic combination. ΣFIC = FIC A + FIC B, where FIC A is the MIC of DCS in combination with the antibiotic/MIC of DCS alone, and FIC B is the MIC of the antibiotic in combination with DCS/MIC of the antibiotic alone. The combination is considered synergistic when the ΣFIC is ≤0.5, indifferent when the ΣFIC is >0.5 to <2.

‡ND. Not determined if strain is susceptible (or hyper-resistant) to the antibiotic or for *cycA* mutants with reduced DCS & antibiotic MICs.

Table S2. Cell diameter and cell wall thickness of JE2 and NE810 (*cycA* mutant).

	Mean cell diameter (µm) ± SD	Mean cell wall thickness (nm) ± SD
JE2	0.89 ± 0.075	25.92 ± 1.79
NE810	0.88 ± 0.05	18.95 ± 2.5

Table S3. Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant Details
RN4220	Restriction-deficient laboratory *S. aureus*
USA300 FPR3757	Community associated MRSA isolate of the USA300 lineage (48). SCCmec type IV. CC8.
JE2	USA300 cured of p01 & p03. Parent of Nebraska Transposon Mutant Library (NTML).
13C	USA300 derivative cured of plasmid p03. Erm^s.
NE810	JE2 NTML *cycA* (SAUSA300_1642) mutation. Erm^r.
USA300 *cycA*	USA300 FPR3757 *cycA*. Constructed by transduction of *cycA*::Tn allele from NE810.
13C *cycA*	13C *cycA* mutant (*cycA*::Tn allele from NE810).
NE1868	JE2 NTML *mecA* mutation.
NE1713	JE2 NTML *alr* (SAUSA300_2027) mutation.
NE799	JE2 NTML *alr2* (SAUSA300_1292) mutation.
NE923	JE2 NTML *ynfA* (SAUSA300_2286) mutation.
NE1025	JE2 NTML *thrE* (SAUSA300_0729) mutation.
BH1CC	MRSA clinical isolate; SCC*mec* type II; CC8 (17)
COL	MRSA reference strain; SCC*mec* type I; CC8 (49)
BH14(04)	MRSA clinical isolate; SCC*mec* type IV; CC22 (17)
BH8(03)	MRSA clinical isolate; SCC*mec* type IV; CC22 (17)
BH6(03)	MRSA clinical isolate; SCC*mec* type II; CC8 (17)
DAR113	MRSA reference isolate; SCC*mec* type IV; CC22 (17, 50)
DAR13	MRSA reference isolate; SCC*mec* type IV; CC8 (17, 50)
DAR45	MRSA reference isolate; SCC*mec* type II; CC30 (17, 50)
DAR202	MRSA reference isolate; SCC*mec* type III; CC239 (17, 50)
DAR173	MRSA reference isolate; SCC*mec* type IV; CC5 (17, 50)
DAR173 *cycA*	DAR173 *cycA* mutant (*cycA*::Tn allele from NE810)
DAR22	MRSA reference isolate; SCC*mec* type III; CC5 (17, 50)
DAR22 *cycA*	DAR22 *cycA* mutant (*cycA*::Tn allele from NE810)
DAR169	MRSA Reference strain; SCCmec type I; CC8 (17, 50)
DAR169 *cycA*	DAR169 *cycA* mutant (*cycA*::Tn allele from NE810)
8325-4	NCTC 8325 derivative cured of prophages (51), methicillin susceptible, CC8.
8325-4 *cycA*	8325-4 *cycA* mutant (*cycA*::Tn allele from NE810).
ATCC 29213	Methicillin susceptible *S. aureus* strain for antibiotic susceptibility testing.
ATCC 29213 *cycA*	ATCC 29213 *cycA* mutant (*cycA*::Tn allele from NE810).
ATCC 25923	Methicillin susceptible *S. aureus* strain for antibiotic susceptibility testing.
S. epidermidis RP62A	ATCC 35984. Methicillin resistant, biofilm positive. (24)
E. coli	*E. coli* HST08
Vancomycin resistant *Enterococcus* CIP104105	Reference strain
Vancomycin resistant *Enterococcus* DSM17050	Reference strain
Acinetobacter baumanii DSM 30007	Reference strain
Klebsiella pneumoniae 511232.1	Clinical Isolate (Dublin Hospital).
Pseudomonas aeruginosa LES431	Liverpool epidemic strain (LES) of *Pseudomonas aeruginosa* (52).
Plasmids	
pLI50	*E. coli-Staphylococcus* shuttle vector. Ap^r (*E. coli*), Cm^r. (*Staphylococcus*)
p*cycA*	pLI50 carrying *cycA* from JE2

Table S4. Oligonucleotide primers used in this study

Target Gene	Primer Name	Primer Sequence (5'-3')
cycA	NE810_Fwd	ACAGAATAGCCACAAATAGCACC
	NE810_Rev	ACAGAATAGCCACAAATAGCACC
cycA	NE810F1_Fwd	GTCTTCAAGAATTCGGCCACAAATAGCACCATTAA
	NE810F1_Rev	CGACTCTAGAGGATCATGTCCCAAGCCCTAAAAC
mecA	mecA1_Fwd	TGCTCAATATAAAATTAAAACAAACTACGGTAAC
	mecA1_Rev	GAATAATGACGCTATGATCCCAA
gyrB	gyrB_Fwd	CCAGGTAAATTAGCCGATTGC
	gyrB_Rev	AAATCGCCTGCGTTCTAGAG
	RNAIII FWD	GAAGGAGTGATTTCAATGGCACAAG
RNAIII	RNAIII REV	GAAAGTAATTAATTATTCATCTTATTTTTTAGTGAATTTG

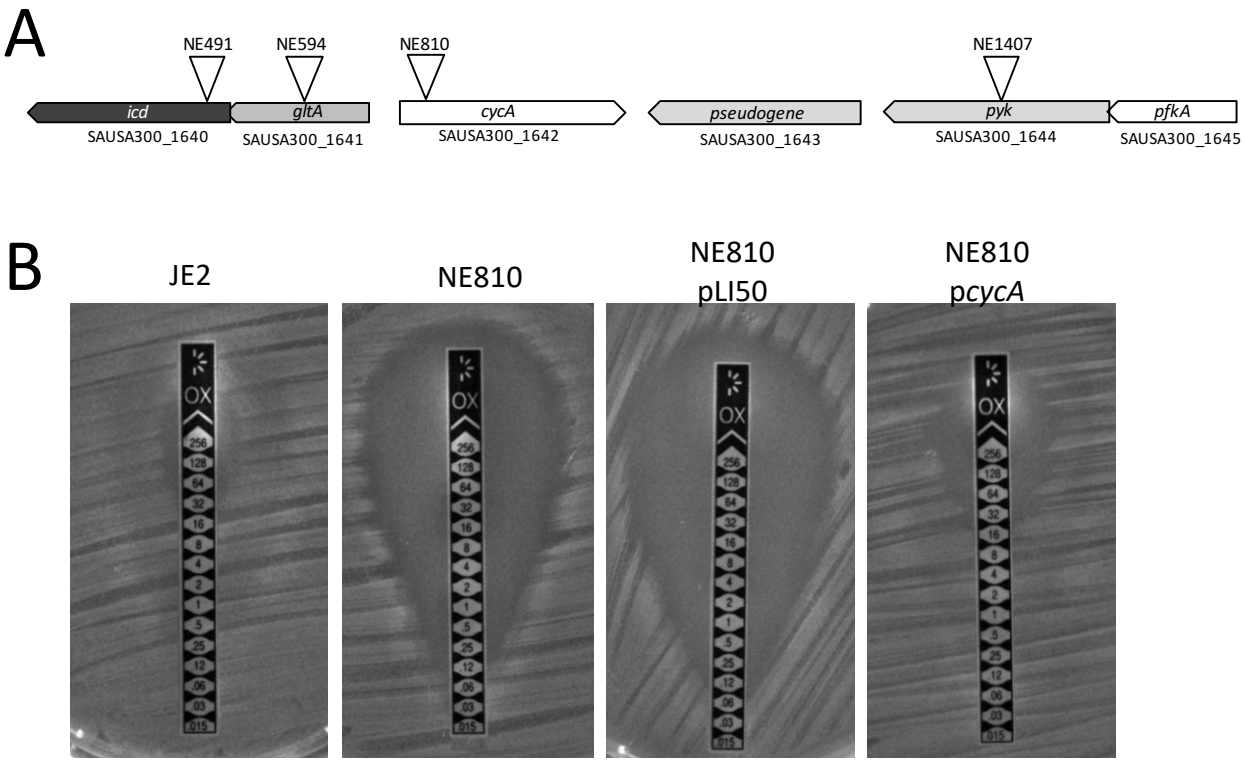
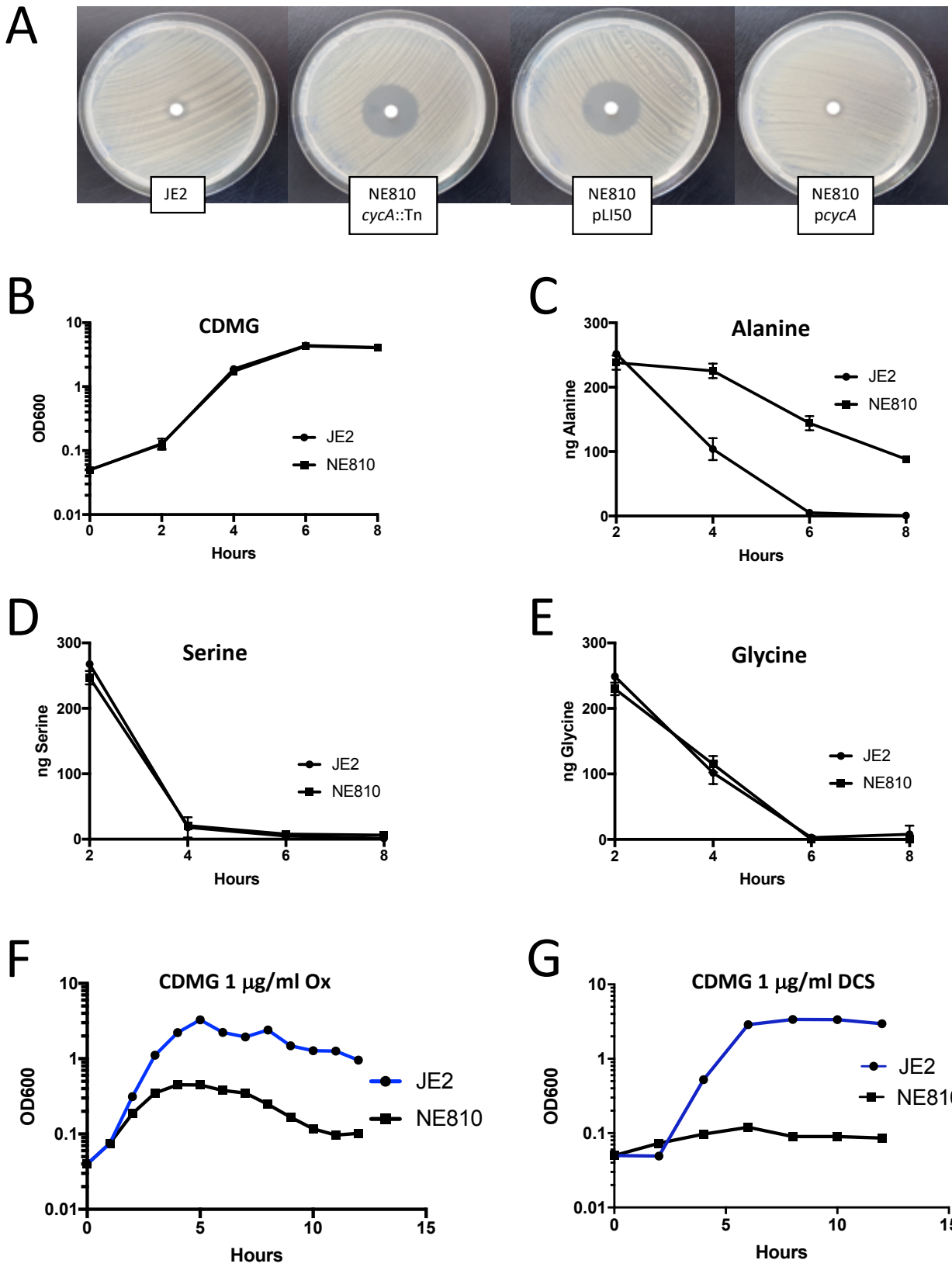

A
NE491
NE594
NE810
NE1407
icd
gltA
cycA
pseudogene
pyk
pfkA
SAUSA300_1640
SAUSA300_1641
SAUSA300_1642
SAUSA300_1643
SAUSA300_1644
SAUSA300_1645
B
JE2
NE810
NE810 pLI50
NE810 pcycA
OX
256
128
64
32
16
8
4
2
1
.5
.25
.12
.06
.03
.015

Fig. 1


A
JE2
NE810
cycA::Tn
NE810
pLI50
NE810
pcycA
B
CDMG
OD600
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JE2
NE810
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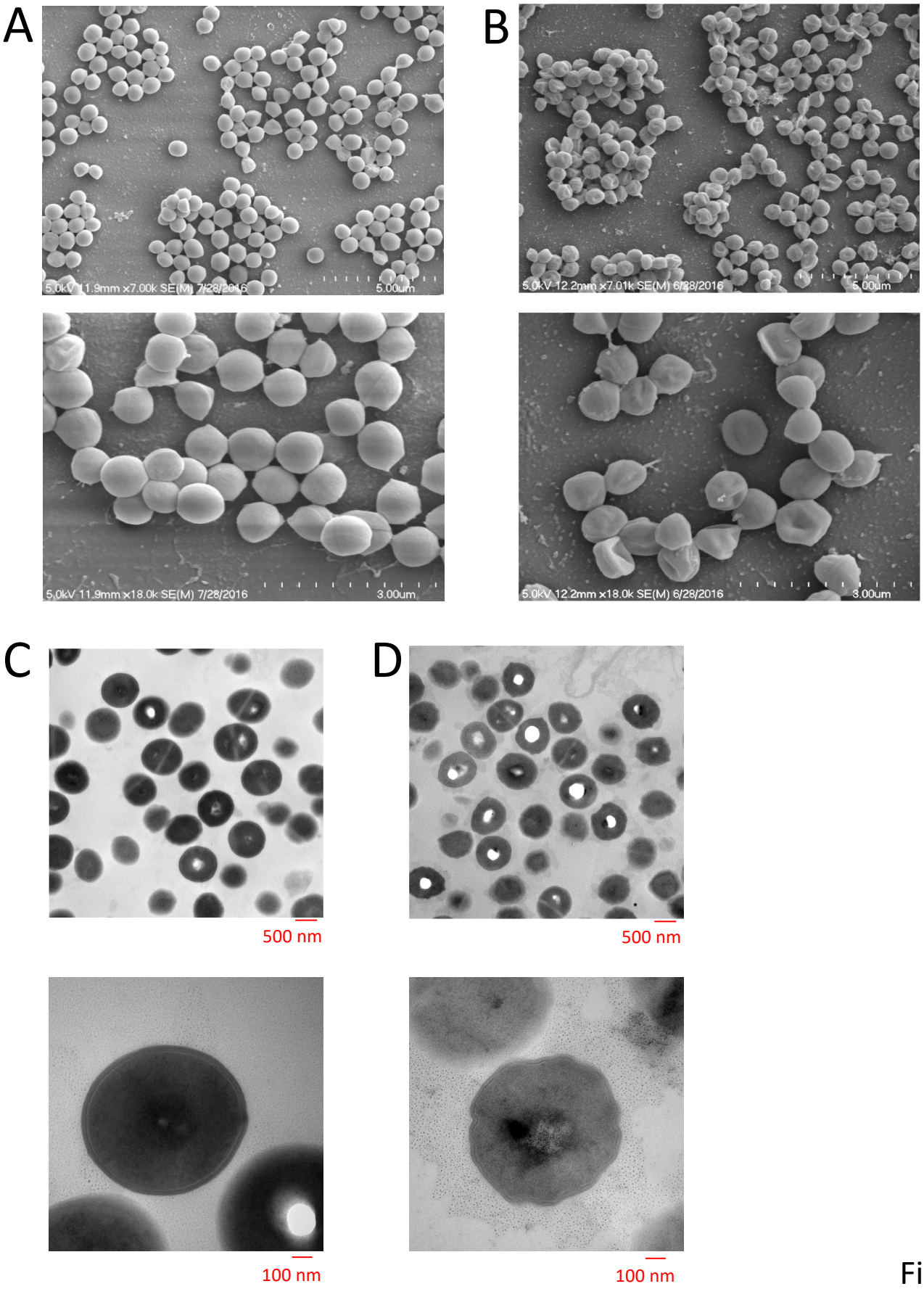
Alanine
ng Alanine
Hours
JE2
NE810
D
Serine
ng Serine
Hours
JE2
NE810
E
Glycine
ng Glycine
Hours
JE2
NE810
F
CDMG 1 µg/ml Ox
OD600
Hours
JE2
NE810
G
CDMG 1 µg/ml DCS
OD600
Hours
JE2
NE810

Fig. 2


A
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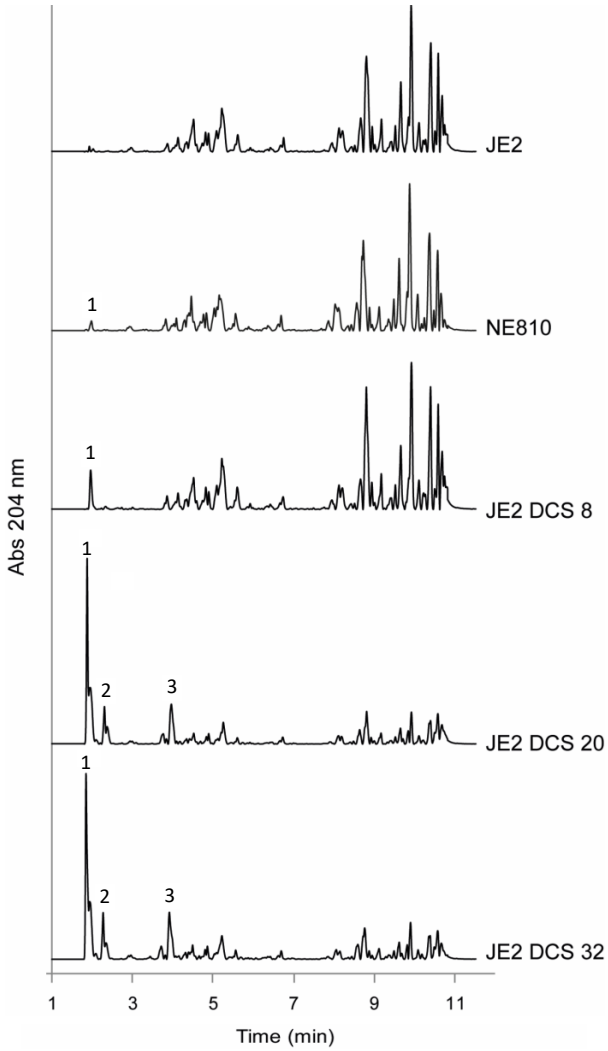
5.00um
B
5.0kV 12.2mm x7.01k SE(M) 6/28/2016
5.00um
5.0kV 11.9mm x18.0k SE(M) 7/28/2016
3.00um
5.0kV 12.2mm x18.0k SE(M) 6/28/2016
3.00um
C
500 nm
100 nm
D
500 nm
100 nm

Fig. 3

Fig. 4

A



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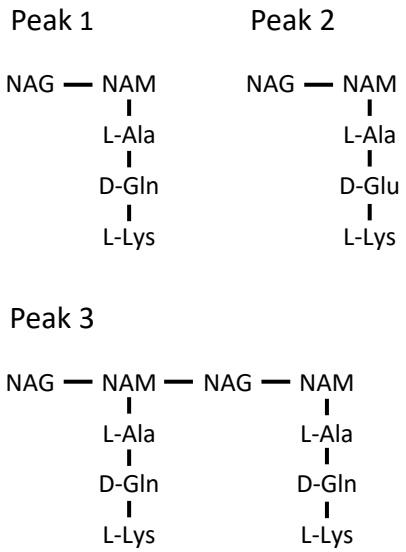
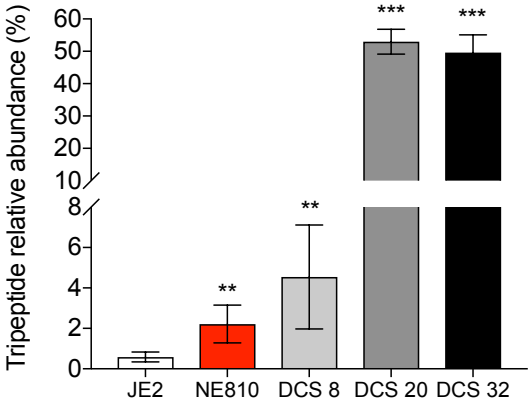


Peak	Muropeptide	Neutral mass (Da)	
		Theoretical	Observed
1	Monomer tripeptide (Gln)	825.3967	825.3976
2	Monomer tripeptide (Glu)	826.3815	826.3818
3	Chain of two monomers tripeptide (Gln)	1630.7673	1630.7627

C



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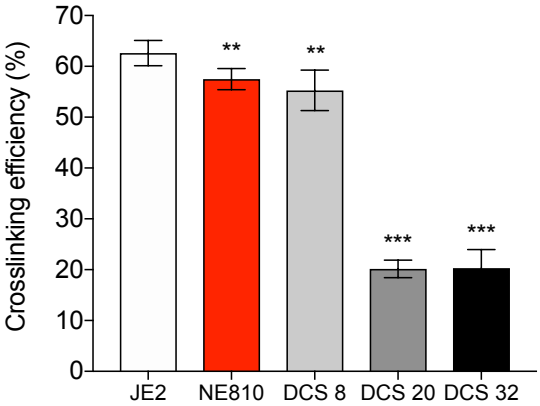

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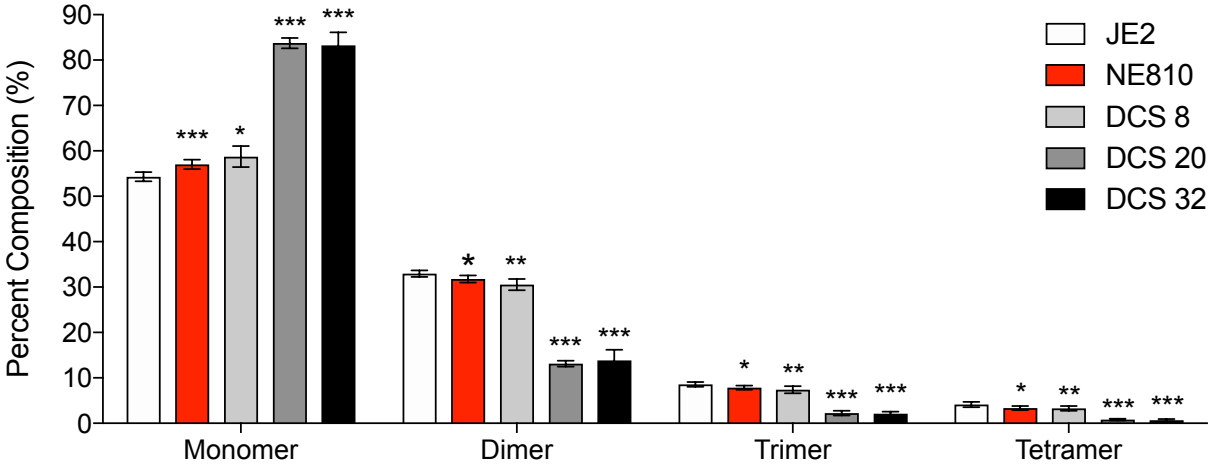


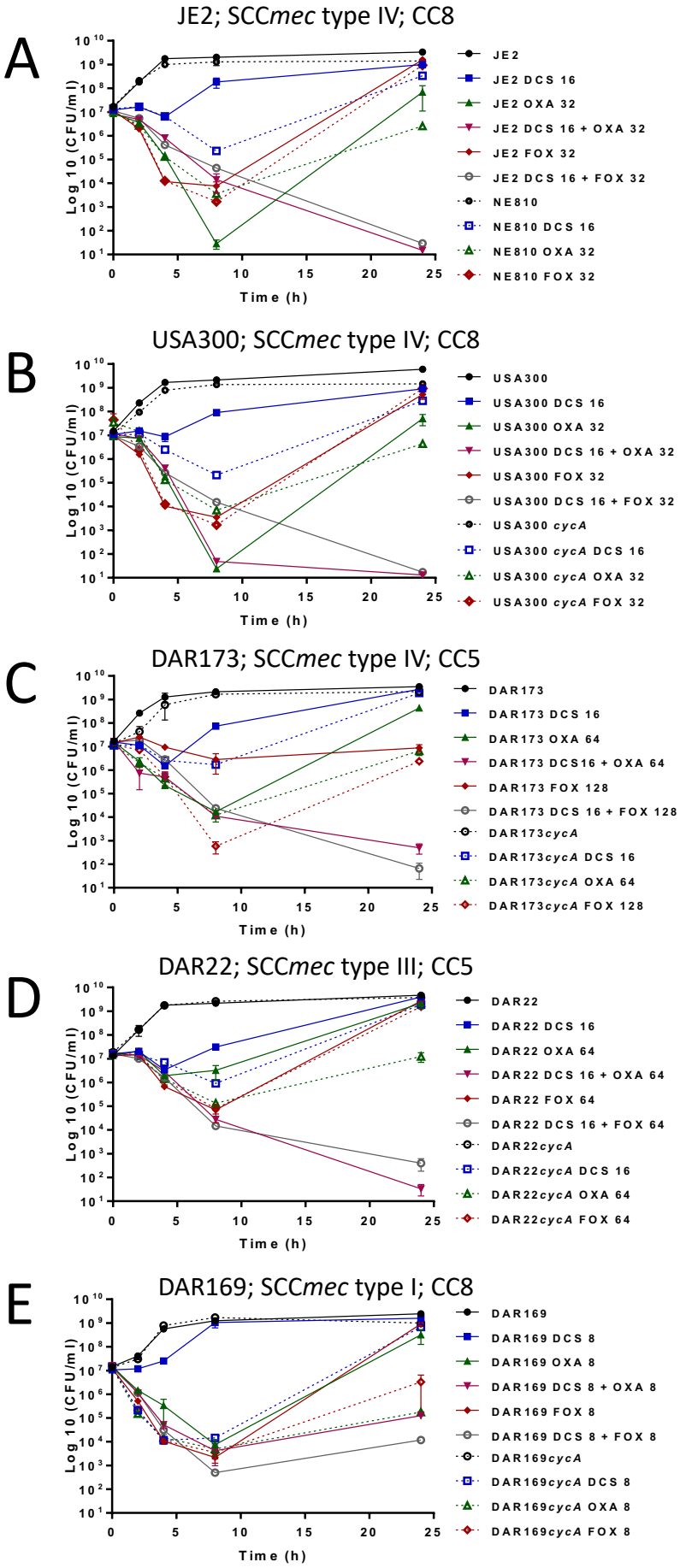

A
JE2; SCCmec type IV; CC8
Log 10 (CFU/ml)
Time (h)
JE2
JE2 DCS 16
JE2 OXA 32
JE2 DCS 16 + OXA 32
JE2 FOX 32
JE2 DCS 16 + FOX 32
NE810
NE810 DCS 16
NE810 OXA 32
NE810 FOX 32
B
USA300; SCCmec type IV; CC8
Log 10 (CFU/ml)
Time (h)
USA300
USA300 DCS 16
USA300 OXA 32
USA300 DCS 16 + OXA 32
USA300 FOX 32
USA300 DCS 16 + FOX 32
USA300 cycA
USA300 cycA DCS 16
USA300 cycA OXA 32
USA300 cycA FOX 32
C
DAR173; SCCmec type IV; CC5
Log 10 (CFU/ml)
Time (h)
DAR173
DAR173 DCS 16
DAR173 OXA 64
DAR173 DCS16 + OXA 64
DAR173 FOX 128
DAR173 DCS 16 + FOX 128
DAR173cycA
DAR173cycA DCS 16
DAR173cycA OXA 64
DAR173cycA FOX 128
D
DAR22; SCCmec type III; CC5
Log 10 (CFU/ml)
Time (h)
DAR22
DAR22 DCS 16
DAR22 OXA 64
DAR22 DCS 16 + OXA 64
DAR22 FOX 64
DAR22 DCS 16 + FOX 64
DAR22cycA
DAR22cycA DCS 16
DAR22cycA OXA 64
DAR22cycA FOX 64
E
DAR169; SCCmec type I; CC8
Log 10 (CFU/ml)
Time (h)
DAR169
DAR169 DCS 8
DAR169 OXA 8
DAR169 DCS 8 + OXA 8
DAR169 FOX 8
DAR169 DCS 8 + FOX 8
DAR169cycA
DAR169cycA DCS 8
DAR169cycA OXA 8
DAR169cycA FOX 8

Fig. 5

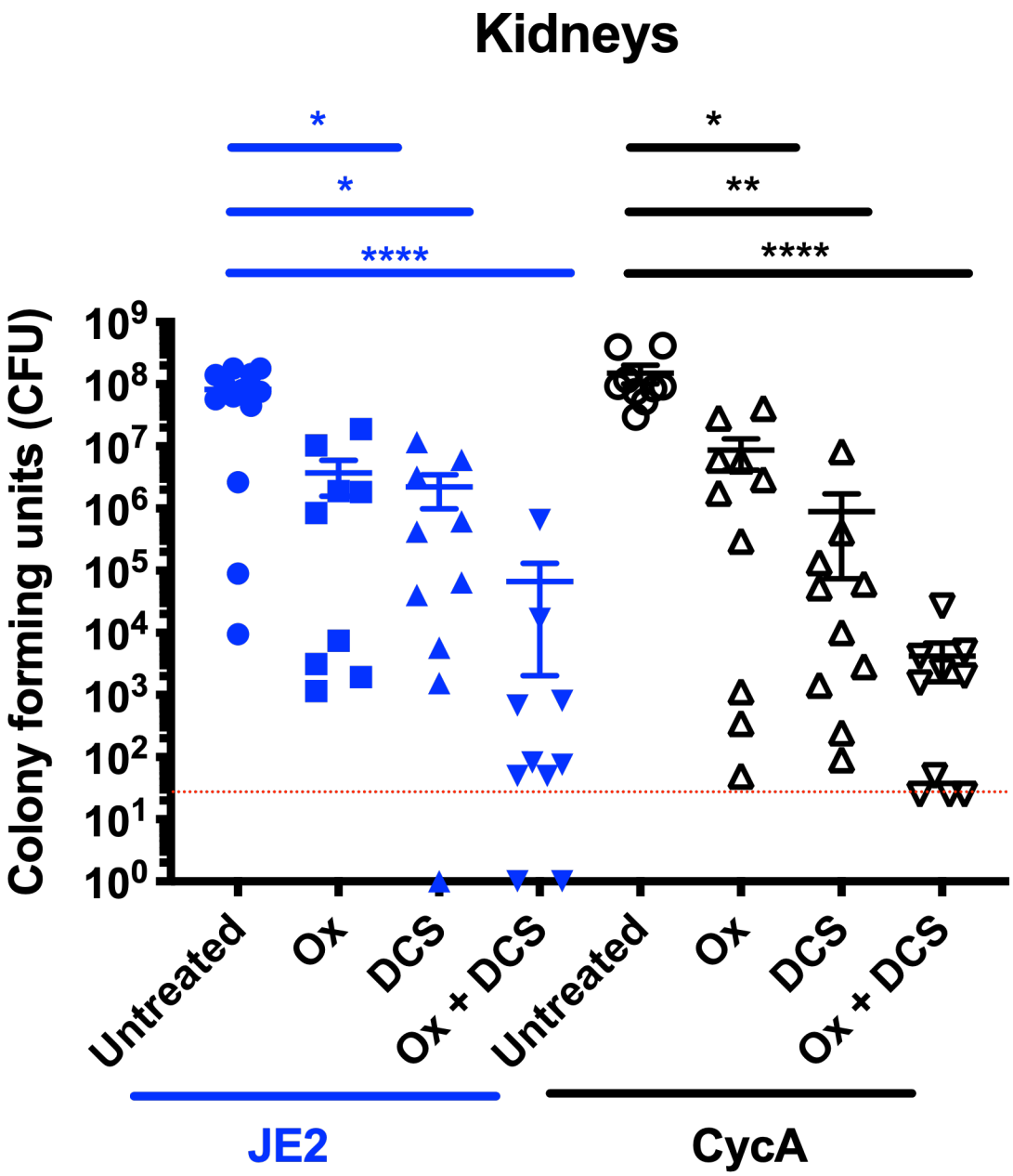

Kidneys
*
*
****
*
**
****
Colony forming units (CFU)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Untreated
Ox
DCS
Ox + DCS
Untreated
Ox
DCS
Ox + DCS
JE2
CycA

Fig. 6

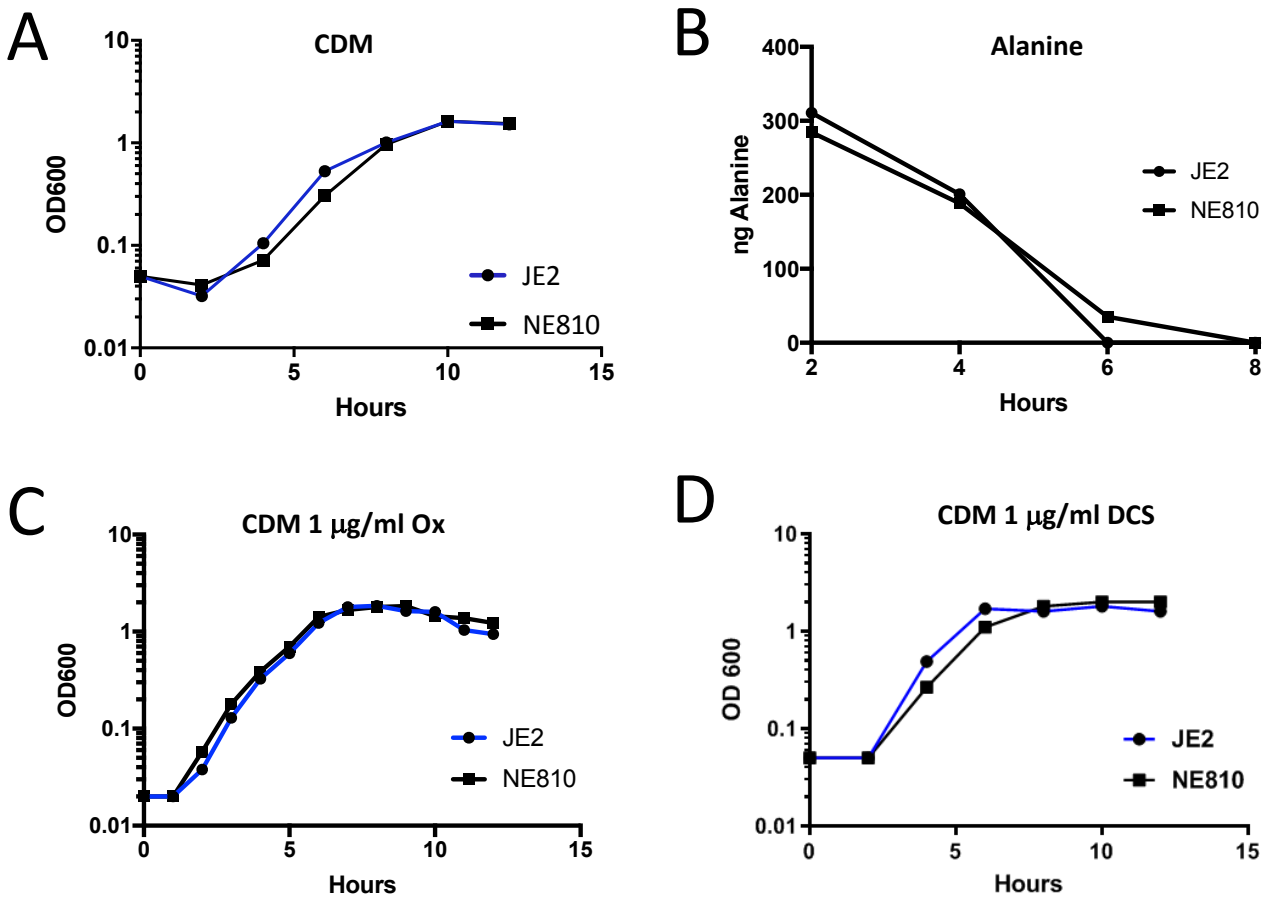

A
CDM
OD600
10
1
0.1
0.01
0
5
10
15
Hours
JE2
NE810
B
Alanine
ng Alanine
400
300
200
100
0
2
4
6
8
Hours
JE2
NE810
C
CDM 1 µg/ml Ox
OD600
10
1
0.1
0.01
0
5
10
15
Hours
JE2
NE810
D
CDM 1 µg/ml DCS
OD 600
10
1
0.1
0.01
0
5
10
15
Hours
JE2
NE810

Fig. 7

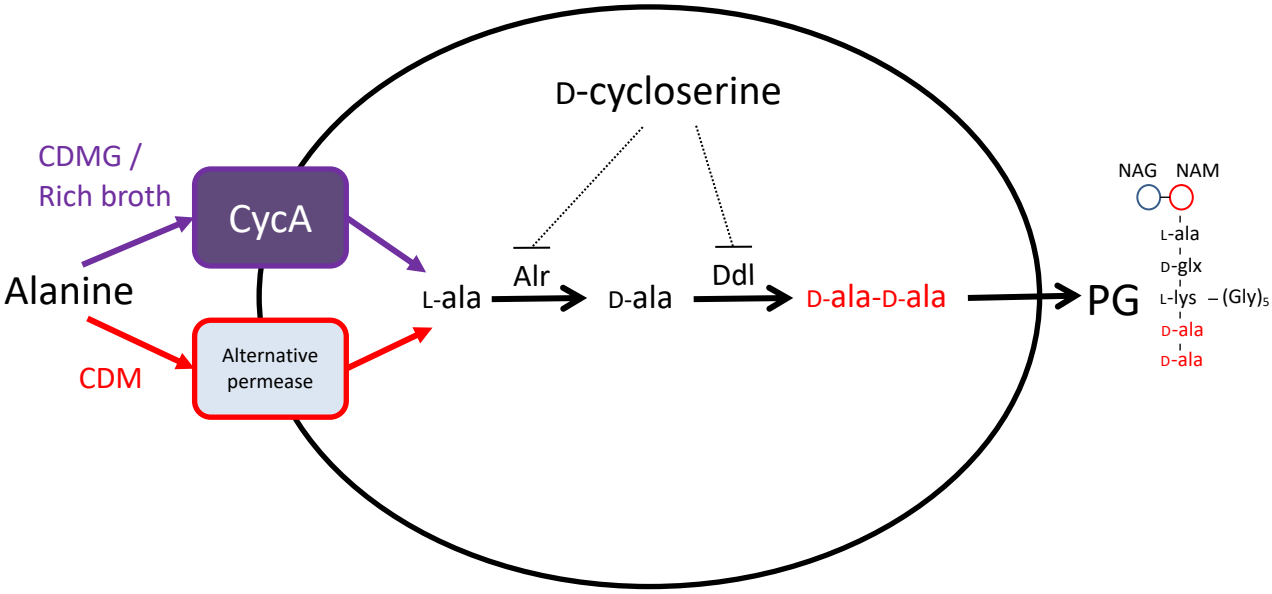

D-cycloserine
CDMG /
Rich broth
CycA
Alanine
CDM
Alternative
permease
L-ala
Alr
D-ala
Ddl
D-ala-D-ala
PG
NAG
NAM
L-ala
D-glx
L-lys
– (Gly)5
D-ala
D-ala

Fig. 8

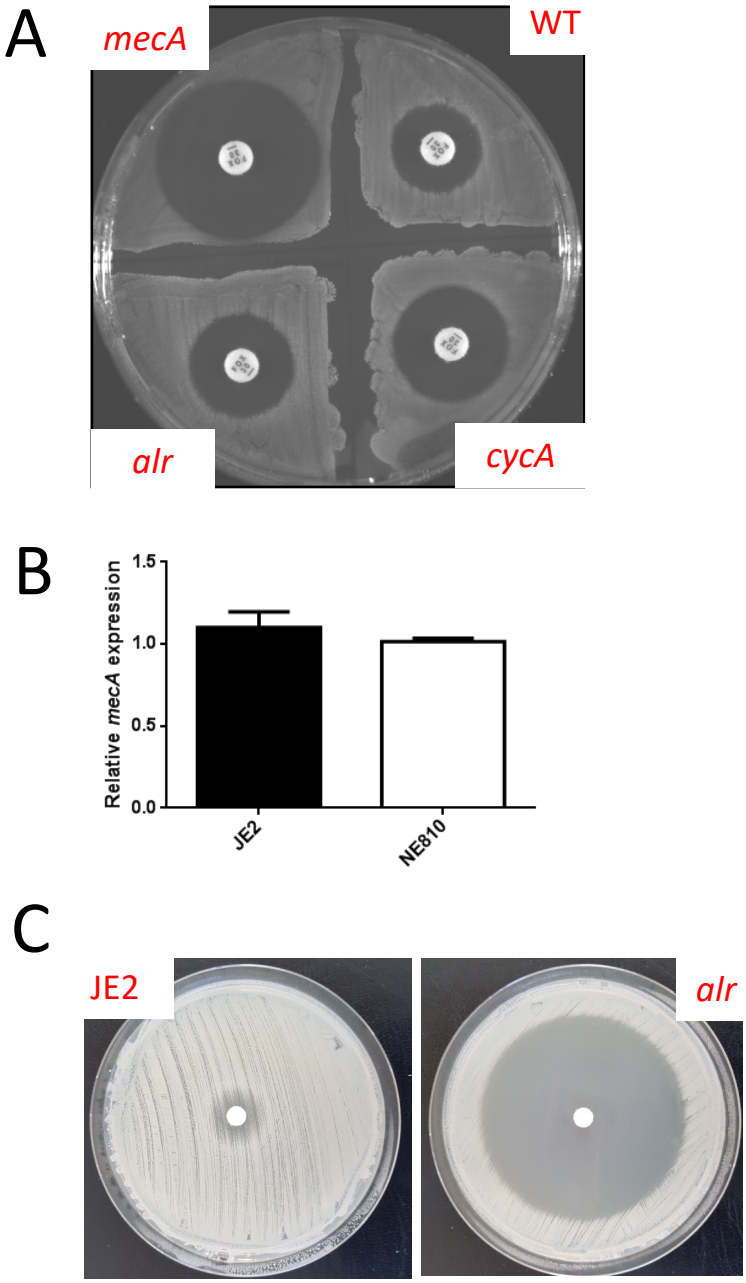


Figure S1. A. Susceptibility of JE2 (wild type), NE1868 (*mecA*::Tn), NE810 (*cycA*::Tn), and NE1713 (*alr*::Tn) grown on MH agar to cefoxitin (FOX, 30μg disks). **B.** Comparison of relative *mecA* gene expression by LightCycler RT-PCR in JE2 and NE810 grown to A_{600}=3 in BHI media. The data are the average of three independent experiments and standard deviations are shown. **C.** Susceptibility of JE2 (wild type) and NE1713 (*alr*::Tn) grown on MH agar to D-cycloserine (DCS, 30μg disks).

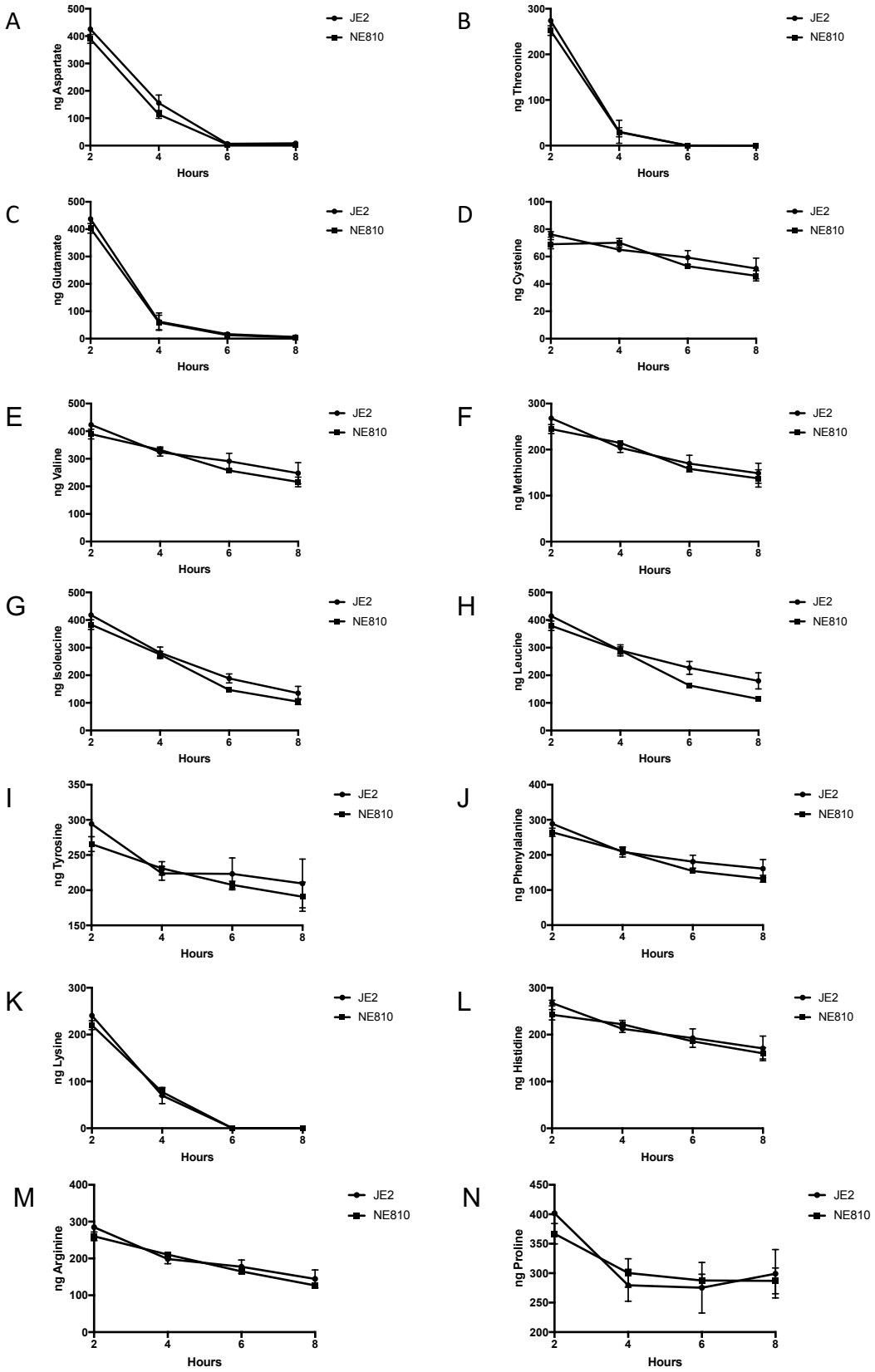


Figure S2. Amino acid consumption by JE2 and NE810 grown aerobically in chemically defined media containing 14mM of glucose (CDMG). Residual amino acids were measured in spent media after 0, 4, 6 and 8 h growth.

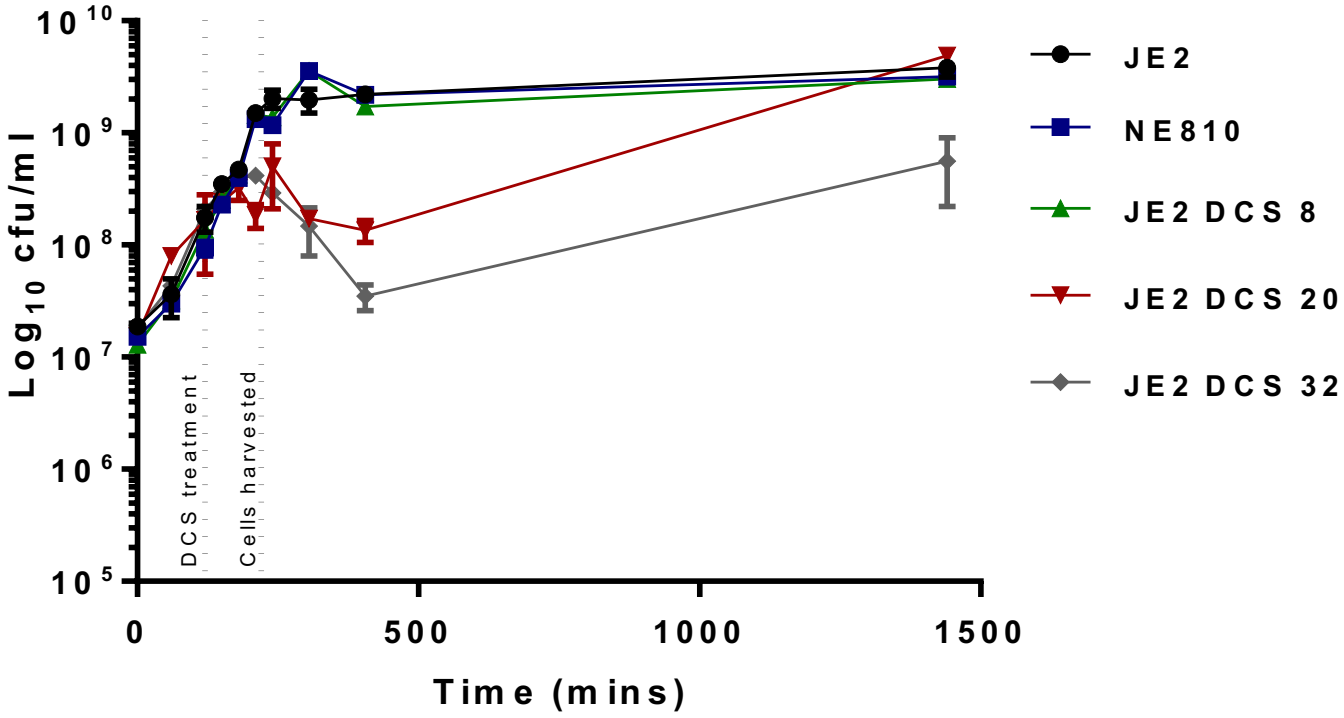


Figure S3. Preparation of cell suspensions for peptidoglycan extraction and structural analysis by UPLC-MS. 50 ml flask cultures were inoculated into fresh BHI media from overnight cultures at a starting cell density of A_{600}=0.05 and incubated at 37°C. The number of CFU/ml was enumerated every 1-2 h for 6 h and again after 24 hours. For JE2 cultures being dosed with DCS, the antibiotic was added after approximated 2 h (A_{600}≈0.5) and the cells collected after a further 100 mins. Cells from untreated JE2 and NE810 control cultures were collected at the same time point.

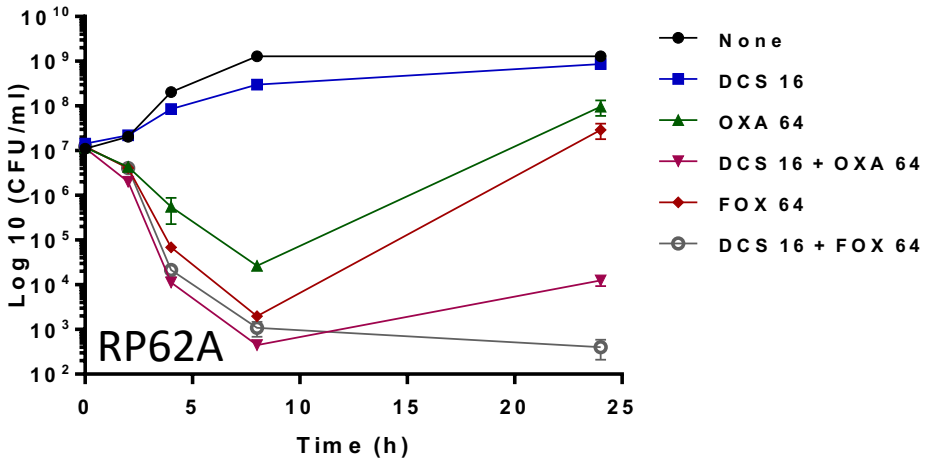
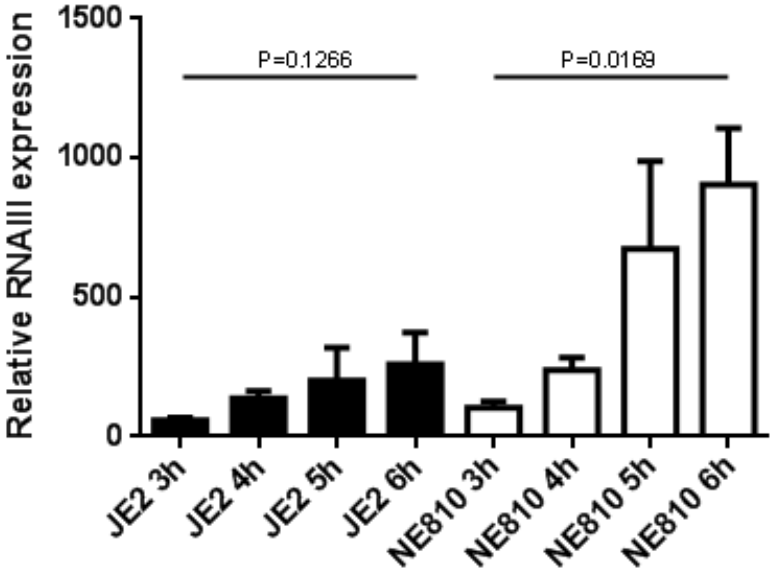


Figure S4. *In vitro* kill curves for D-cycloserine (DCS), oxacillin and cefoxitin with methicillin resistant *S. epidermidis strain* RP62A. Antibiotics at the concentrations indicated (equivalent to 0.5× MIC) were added to suspensions of overnight bacterial cultures adjusted to 10^7 CFU/ml in BHI, incubated at 37°C and the number of CFU/ml enumerated at 0, 2, 4, 8 and 24 h. The data presented are the mean of three independent experiments. Antibiotic synergism was defined as a ≥2 $\log^{10}$ decrease in the number of CFU/ml in cell suspensions exposed to DCS/β-lactam combinations compared to the most effective individual antibiotic alone.

A

B



C

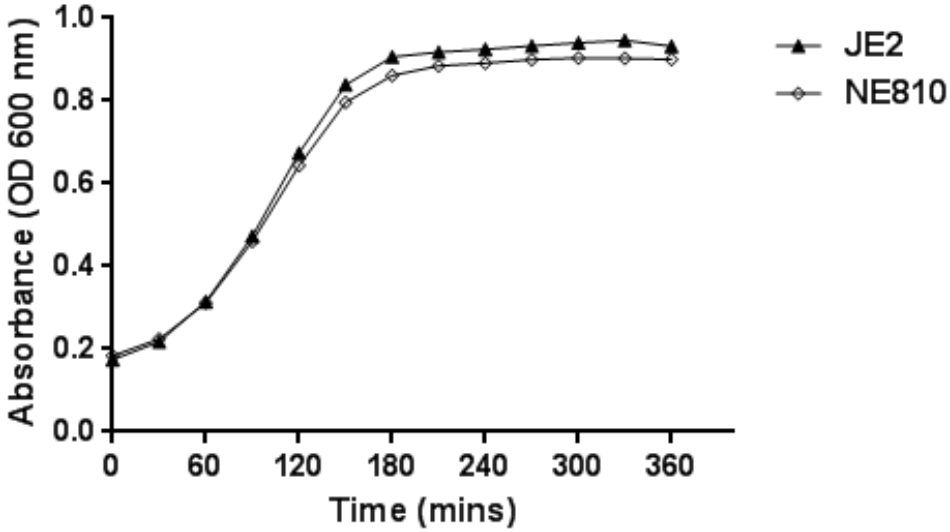


Figure S5. A. Zones of haemolysis of JE2 and NE810 grown on sheep blood agar for 24 hours. **B**. Comparison of relative *agr (RNAIII)* gene expression by LightCycler RT-PCR in JE2 and NE810 grown for 3, 4, 5 and 6 h in BHI media. The data are the average of at least three independent experiments and standard deviations are shown. **C.** Growth of JE2 and NE810 cultures for 6 h in BHI. Cell density was measured at A_{600}.

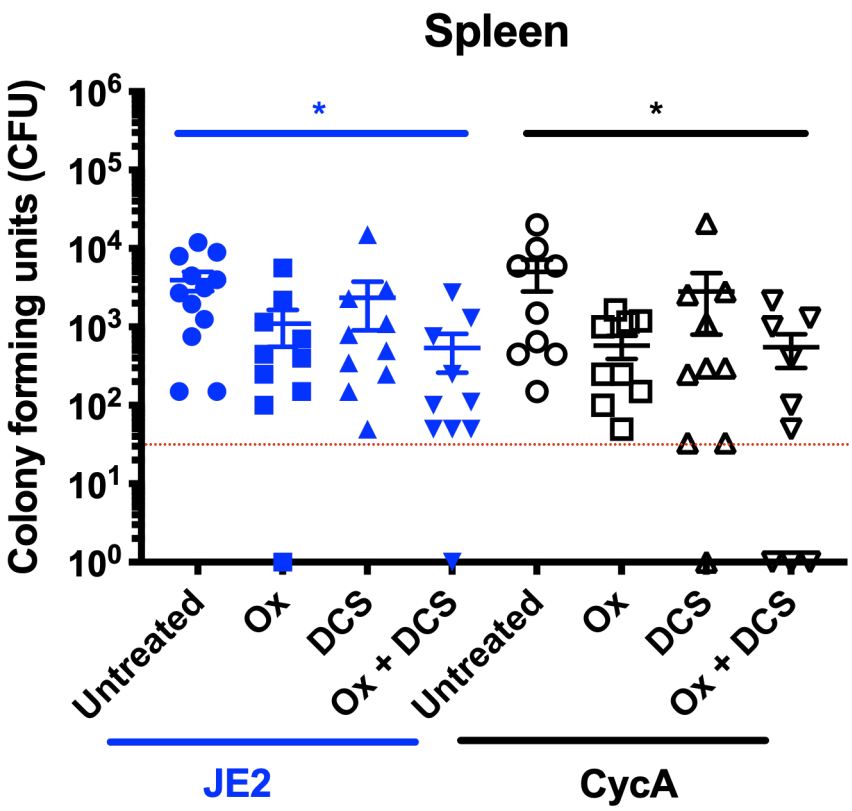


Figure S6. Combination therapy with D-cycloserine and oxacillin significantly reduces the bacterial burden in the spleen of mice infected with MRSA. The number of colony-forming units (CFU) recovered from the spleens of mice infected by tail vein injection with 5×10^6 JE2 or NE810 (CycA) and left untreated or treated with 75mg of oxacillin (Ox)/kg, 30mg of DCS/kg or a combination of both Ox and DCS delivered subcutaneously every 12 hours for 5 days. The first antibiotic dose was given 16 hours after infection. Significant differences determined using one-way ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparisons test are denoted using asterisks (*$p \leq 0.05$). The limit of detection (50 colonies) is indicated with a hashed red line.

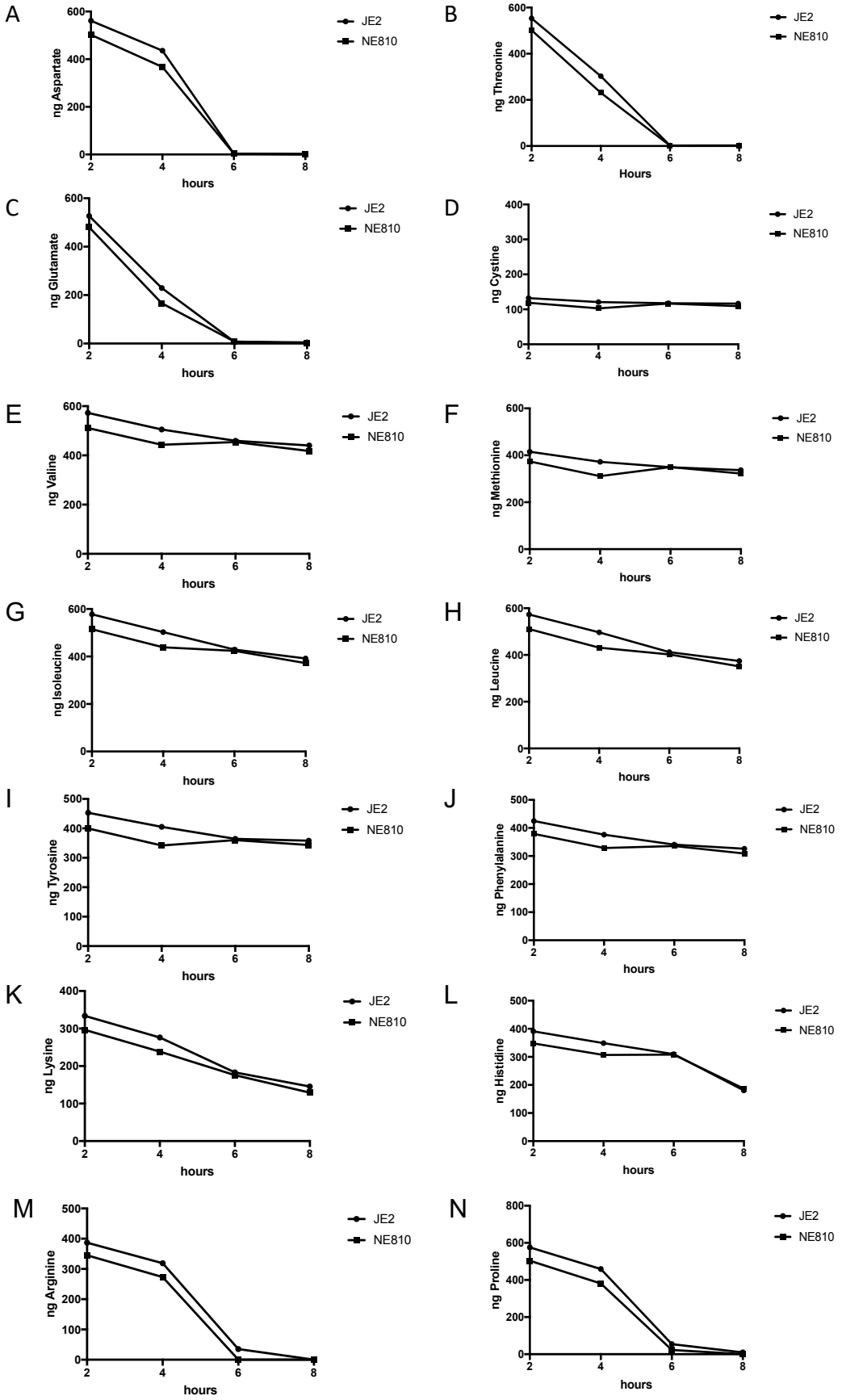


Figure S7. Amino acid consumption by JE2 and NE810 grown aerobically in chemically defined media lacking glucose(CDM). Residual amino acids were measured in spent media after 0, 4, 6 and 8 h growth.