An alanine permease mutation or exposure to D-cycloserine re-sensitizes MRSA to β-lactam antibiotics

Laura A. Gallagher[1], Rebecca Shears[2], Claire Fingleton[1], Laura Alvarez[3], Elaine M. Waters[1,2], Jenny Clarke[2], Laura Bricio-Moreno[2], Christopher Campbell[1], Akhilesh K. Yadav[3], Fareha Razvi[4], Eoghan O'Neill[5], Alex J. O'Neill[6], Felipe Cava[3], Paul D. Fey[4], Aras Kadioglu[2] and James P. O'Gara[1]*

[1]Department of Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland.

[2]Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, UK.

[3]MIMS-Molecular Infection Medicine Sweden, Molecular Biology Department, Umeå University, Umeå, Sweden.

[4]Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska, USA.

[5]Department of Clinical Microbiology, Royal College of Surgeons in Ireland, Connolly Hospital, Dublin 15, Ireland.

[6]Antimicrobial Research Centre, School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, UK.

***Correspondence:** jamesp.ogara@nuigalway.ie

Running title: An amino acid analogue re-sensitizes AMR pathogens to beta-lactam antibiotics

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* gene 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 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. Therapeutically achievable concentrations of DCS re-sensitised MRSA to β-lactam antibiotics *in vitro* and significantly enhanced MRSA eradication in a mouse bacteraemia model. The susceptibility of several other ESKAPE group pathogens to β-lactams was also increased by DCS. These data reveal the potential of DCS, or new drugs targeting CycA, to facilitate the reintroduction of β-lactam antibiotics for the treatment of infections caused by MRSA.

Author Summary. 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 mutation of *cycA* in MRSA, which was found to encode an alanine permease, was associated with hyper-susceptibility to β-lactam antibiotics and D-cycloserine (DCS), used in the treatment of multi-drug resistant tuberculosis infections. DCS 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 revealing the therapeutic potential of DCS as an adjunct drug for these important 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 accessory mutations resulting in a HoR phenotype, including mutations that affect the stringent response and c-di-AMP signalling [10-14]. Because accessory genes can influence the expression of methicillin resistance in MRSA, targeting the pathways associated with the accessory 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. Disruption of this permease also resulted in hyper-susceptibility to D-cycloserine (DCS), an alanine analogue antibiotic that interferes with two enzymatic steps in the D-Alanine pathway of peptidoglycan biosynthesis. At therapeutically achievable concentrations, DCS 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, these data identify CycA as a new drug target to enhance the activity of β-lactam antibiotics.

98 **Results**

99 **Mutation of *cycA* increases the susceptibility of MRSA to β-lactam antibiotics.** To identify
100 new ways of controlling expression of methicillin resistance, we sought to identify novel
101 mutations involved in this phenotype. To achieve this, an unbiased screen of the Nebraska
102 Transposon Mutant Library (NTML) was performed using a disk diffusion assay to identify
103 mutants with increased susceptibility to cefoxitin. The parent strain of the library (JE2, a
104 derivative of USA300 LAC cured of plasmids p01 and p03 [15]) was used as a positive control.
105 Phage 80α-mediated backcross of candidate transposon mutations into the parent strain JE2,
106 together with genetic complementation were used to confirm the role of putative genes
107 involved in cefoxitin resistance. Only mutants verified by both transduction and
108 complementation were examined further. Among the mutants identified was NE810
109 (SAUSA300_1642) (Fig. 1A), which exhibited significantly increased susceptibility to cefoxitin
110 as determined by a disk diffusion assay (Fig. S1A) and a >500-fold increase in susceptibility to
111 oxacillin as determined by E-test (Fig. 1B). Consistent with this, NE810 was recently reported
112 to be more susceptible to amoxicillin [16]. RT-qPCR analysis revealed that expression of *mecA*
113 was not significantly affected in NE810 compared to JE2 (Fig. S1B), whilst whole genome
114 sequence analysis of NE810 further revealed that the SCC*mec* element was fully intact and
115 that no other mutations were present (data not shown). These findings implied that increased
116 cefoxitin susceptibility was not attributable to a failure of this strain to express a functional
117 *mecA* gene product. Transduction of the *cycA* transposon insertion into the USA300 strains
118 JE2, FPR3757 and LAC-13C (all SSC*mec* type IV, CC8), as well as DAR173 (SSC*mec* type IV, CC5)
119 [17], DAR22 (SSC*mec* type III, CC5) [17] and DAR169 (SSC*mec* type I, CC8)[17] was also
120 accompanied by significant increases in cefoxitin and oxacillin susceptibility (Table 1).

121

122 **Mutation of *cycA* increases susceptibility to D-cycloserine.** SAUSA300_1642 is annotated as
123 a D-serine/L- and D-Alanine/glycine transporter with homology to CycA in *Mycobacterium*
124 *tuberculosis* [18, 19]. Nonsynonymous point mutations in *cycA* contribute, in part, to
125 increased D-cycloserine (DCS) resistance in *Mycobacteria* [18, 19], prompting us to investigate
126 the susceptibility of the *S. aureus cycA* mutant to DCS. In contrast to the observations in
127 *Mycobacteria*, our data showed that NE810 was significantly more susceptible to DCS than
128 the wild type JE2 and was successfully complemented by the wild-type *cycA* gene (Fig. 2A). By
129 broth dilution, the DCS MIC decreased from 32 μg/ml in JE2 to 2 μg/ml in NE810 (Table 1).

130 The *cycA* mutants of the MRSA strains LAC-13C (data not shown), USA300, DAR173, DAR22
131 and DAR169 exhibited similar and significant increases in susceptibility to DCS (Table 1).
132 Transduction of the *cycA* allele from NE810 into the MSSA strains 8325-4 and ATCC29213 was
133 also associated with a reduction in the DCS MIC from 32 to 4 μg/ml. DCS (a cyclic analogue of
134 alanine and serine) is a broad-spectrum antibiotic produced by several *Streptomyces* species
135 used as a second line drug in the treatment of TB infections in humans. This drug inhibits the
136 alanine racemase enzyme that converts L-Alanine to D-Alanine, as well as the ligase enzyme
137 that links two D-Alanine amino acids together [20]. These are important enzymes in both
138 peptidoglycan (cell wall cross-linking) and teichoic acid biosynthesis in Gram-positive bacteria.
139 A mutant in the putative D-Alanyl:D-Alanine ligase (*ddl*, SAUSA300_2039) is not available in
140 the NTML library, suggesting that this gene may be essential. However, inactivation of the
141 alanine racemase (*alr*) gene in the NTML library mutant NE1713 was associated with
142 significantly increased susceptibility to cefoxitin (Fig. S1A) and hyper-susceptibility to DCS (Fig.
143 S1C), which was consistent with the important role for D-Alanine in *S. aureus* peptidoglycan
144 biosynthesis and β-lactam resistance.
145 Screening a subset of the NTML library containing known/predicted membrane transporters,
146 failed to identify any mutation(s) associated with markedly increased DCS resistance. Two
147 mutants, NE923 (YnfA) and NE1025 (ThrE), which were identified only exhibited a DCS MIC of
148 64 μg/ml, up from 32 μg/ml (data not shown) suggesting that DCS may be transported
149 inefficiently by multiple transport systems in *S. aureus*.
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 and
153 NE810 were grown for 8 h in chemically defined media containing 14mM glucose (CDMG) and
154 amino acid consumption in spent media was measured. Although no growth rate or yield
155 difference was noted between JE2 and NE810 in CDMG (Fig. 2B), alanine uptake by NE810
156 was significantly impaired compared to JE2 (Fig. 2C). Utilisation of other amino acids by NE810
157 and JE2, including serine and glycine, were similar (Figs. 2D and 2E and Fig. S2). Impaired
158 alanine transport in the *cycA* mutant grown in CDMG correlated with increased susceptibility
159 to oxacillin (1 mg/l) (Fig. 2F). These data demonstrate for the first time that CycA in *S. aureus*
160 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), while cell wall
165 measurements revealed that the NE810 cell wall (19 ± 2.5nm) was thinner than the JE2 cell
166 wall (26 ± 1.8nm), although cell size (mean cell diameter) was unaffected (Table S2).
167 Quantitative peptidoglycan compositional analysis was performed using UPLC analysis of
168 muraminidase-digested muropeptide fragments extracted from exponential phase cultures
169 of 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, B),
171 which was associated with a significant reduction in crosslinking (Fig. 4C). In NE810, the dimer,
172 trimer and tetramer fractions were decreased, which was accompanied by a concomitant
173 increase in the monomer fraction (Fig. 4D). Consistent with this data, exposure of JE2 to DCS
174 8μg/ml was also associated with a similar accumulation in muropeptides with tripeptide
175 stems (Fig. 4A, B), reduced cross-linking (Fig. 4C), increased muropeptide monomers and
176 reduced dimers, trimers and tetramers (Fig. 4D). DCS had a strong dose-dependent effect,
177 and at concentrations of 20 and 32 μg/ml, led to amplified effects on the accumulation of
178 muropeptides with tripeptide stems, reduced cross-linking and accumulation of monomers
179 (Fig. 4 A-D). These data are consistent with the previously reported impacts of sub-inhibitory
180 (0.25× MIC) and 4× MIC DCS concentrations, which were accompanied by incorporation of an
181 incomplete stem peptide (tripeptide) [20] and a 4-fold reduction in D-Ala-D-Ala levels [21],
182 respectively. Collectively these data indicate that impaired D-Ala incorporation into the PG
183 stem peptide in the *cycA* mutant or following exposure to DCS is accompanied by reduced PG
184 cross-linking and increased β-lactam susceptibility.
185
186 **Mutation of *cycA* or exposure to D-cycloserine increases the susceptibility of MRSA to β-**
187 **lactam antibiotics.** Previously reported synergy between DCS and β-lactam antibiotics [20,
188 22] suggests that impaired alanine uptake in the *cycA* mutant may have the same impact on
189 cell wall biosynthesis as DCS-mediated inhibition of alanine racemase and D-Alanine ligase
190 activity. To further investigate this, we compared the activity of DCS and β-lactam antibiotics,
191 alone and in combination, against JE2 and NE810. Checkerboard microdilution assay
192 fractional inhibitory concentration indices (ΣFICs ≤0.5) revealed synergy between DCS and

193 several licensed β-lactam antibiotics with different PBP selectivity against JE2 and USA300
194 FPR3757 (Table 1). Oxacillin and nafcillin were not included in checkerboard assays because
195 measurement of their MICs involves supplementing the media with 2% NaCl, which distorts
196 the MIC of DCS.
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. Overnight cultures were adjusted to approximately 10^7 CFU/ml before being exposed
201 to oxacillin, cefoxitin and DCS alone and in combination. Recovery of growth in media
202 supplemented with oxacillin or cefoxitin alone was evident after 8 h (Fig. 5A-E), reflecting the
203 selection and expansion of HoR mutants as described previously [4, 21, 23]. Using
204 combinations of DCS and oxacillin or cefoxitin at 0.125× MIC did not achieve a ≥2 $\log^{10}$
205 reduction in the number of CFU/ml (data not shown). However, at 0.5× MIC for strains JE2,
206 USA300, DAR73 and DAR22, DCS (16 μg/ml)/oxacillin (32 μg/ml) and DCS (16 μg/ml)/cefoxitin
207 (32 μg/ml) combinations achieved a ≥5 $\log^{10}$ reduction in the number of CFU/ml compared to
208 oxacillin, cefoxitin or DCS alone (Fig. 5A-D). For strain DAR169, DCS/β-lactam combinations at
209 0.25× MIC was sufficient to achieve a ≥5 $\log^{10}$ reduction in CFUs recovered compared to the
210 individual antibiotics (Fig. 5E). DCS/β-lactam combinations at 0.5× MIC were also able to
211 achieve ≥5 $\log^{10}$ reduction in the number of CFU/ml against the methicillin resistant *S.*
212 *epidermidis* (MRSE) strain RP62A [24] compared to either antibiotic alone (Fig. S4).
213 Checkerboard experiments with fourteen MRSA strains and MRSE strain RP62A further
214 revealed synergy (ΣFICs ≤0.5) between DCS and a range of β-lactam antibiotics with different
215 penicillin binding protein (PBP) specificity, namely cefoxitin (PBP4), cefaclor (PBP3),
216 cefotaxime (PBP2), piperacillin-tazobactin (PBP3/β-lactamase inhibitor) and imipenem (PBP1)
217 (Table 1).
218 Preliminary studies using disk diffusion assays also revealed synergy between DCS and
219 imipenem (IMP) or piperacillin-tazobactin (Pip/Taz) against several ESKAPE pathogens. Using
220 checkerboard assays synergy (ΣFIC values ≤0.5) was measured between DCS and Pip/Taz or
221 IMP against *Pseudomonas aeruginosa* LES431, *Acinetobacter baumannii* DSM 30007, *K.*
222 *pneumoniae* 511232.1 ($ESBL^+$), and two vancomycin resistant *Enterococcus* strains (Table 2).

223 In MRSA, these data suggest that mutation of *cycA* has similar effects to DCS exposure,
224 identifying the CycA permease as a new therapeutic target to potentially increase the
225 susceptibility of these pathogens to β-lactam antibiotics. The DCS concentrations used in
226 these experiments with wild-type MRSA strains were within the therapeutic range
227 administered orally to patients (10-15 mg/kg/day to maintain a blood concentration of 25-
228 30μg/ml), and significantly lower for *cycA* mutants. The therapeutic concentrations of DCS
229 could be further reduced if used in combination with an agent inhibiting CycA activity. This
230 synergy appears to be specific to β-lactams and no synergy (ΣFICs > 0.5) was measured
231 between DCS and several antibiotics that are used topically or systemically for the
232 decolonization or treatment of patients colonized/infected with *S. aureus* or MRSA
233 (clindamycin, trimethoprim, mupirocin, ciprofloxacin), or several antibiotics to which *S.*
234 *aureus* isolates commonly exhibit resistance (tobramycin, kanamycin and spectinomycin)
235 (Table S1). Furthermore the NE810 *cycA* transposon mutation had no impact on susceptibility
236 to any of these non-β-lactam antibiotics (apart from clindamycin resistance which is encoded
237 by the *ermB* gene on the transposon).
238
239 **Combination therapy with DCS and oxacillin significantly reduces the bacterial burden in**
240 **the kidneys and spleen of mice infected with MRSA**. The virulence of the NE810 mutant and
241 the therapeutic potential of oxacillin in combination with DCS in the treatment of MRSA
242 infections were assessed in mice. Bacteraemia was established via tail vein injection with $5 \times$
243 10^6 CFU of JE2 or NE810, and the infection allowed to proceed for 16 hours. Mice were treated
244 with 75 mg of oxacillin/kg/12 h, 30 mg of DCS/kg/12 h, a combination of both oxacillin and
245 DCS, or left untreated (sham). The antibiotics were administered subcutaneously every 12
246 hours and the infection terminated after 5 days. Mice infected with NE810 displayed more
247 piloerection than those infected with wild-type JE2 suggestive of more severe infection, and
248 one mouse infected with NE810 required euthanization during the experiment.
249 The average number of CFUs recovered from the kidneys of mice infected with NE810 ($1.5 \times$
250 $10^8 \pm 1.49 \times 10^8$) was higher than for mice infected with JE2 ($5.7 \times 10^7 \pm 6.3 \times 10^7$), but did
251 not reach significance (p=0.07). Interestingly, NE810 colonies exhibited visibly increased
252 haemolytic activity on sheep blood agar (Fig. S5A). We previously reported that increased
253 haemolytic activity in MSSA strains was associated with increased expression of the cell-
254 density controlled, global accessory gene regulator (*agr*) system [4, 21, 25]. Comparison of

255 *agr* (RNAIII) temporal expression using RT-qPCR revealed a more significant activation of the
256 *agr* system after 6 hrs growth in NE810 compared to JE2 (Fig. S5B). This difference in *agr*
257 activation was not related to any change in growth of NE810 compared to JE2 (Fig. S5C). Taken
258 together these data indicate that increased β-lactam susceptibility in the NE810 *cycA* mutant
259 is accompanied by both increased *agr* expression and haemolytic activity, and a trend towards
260 increased virulence in this model.
261 Treatment with oxacillin or DCS alone significantly reduced the number of CFUs recovered
262 from the kidneys of mice infected with JE2 (Fig. 6A) or NE810 (Fig. 6B). The therapeutic
263 effectiveness of oxacillin or DCS was significantly better for NE810 infections ($p \leq 0.0001$) (Fig.
264 6B) than infections caused by wild type JE2 ($p \leq 0.01$) (Fig. 6A), which is consistent with the
265 increased susceptibility of the *cycA* mutant to both antibiotics *in vitro*. The oxacillin/DCS
266 combination was significantly more effective than either antibiotic alone and the combination
267 was equally effective in reducing the bacterial burden in the kidneys of animals infected with
268 JE2 or NE810 when compared to no treatment ($p \leq 0.0001$) (Fig. 6A and B). These data support
269 the *in vitro* observations by demonstrating *i)* the increased susceptibility of NE810 to oxacillin
270 or DCS, *ii)* the anti-MRSA activity of DCS and *iii)* the capacity of DCS to significantly potentiate
271 the activity of β-lactam antibiotics against MRSA under *in vivo* conditions.

272 **Discussion**

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

284 The alanine permease identified in this study, CycA, shares 46% and 53% identity with CycA
285 in *M. tuberculosis* and *E. coli*, respectively. Mutation of *cycA* increases the susceptibility of
286 MRSA to β-lactam antibiotics and results in hyper-susceptibility to D-cycloserine, whereas a
287 *cycA* point mutation in *M. bovis* contributes, in part, to increased DCS resistance presumably
288 by interfering with DCS transport into the cell [19]. In *E. coli*, *cycA* null mutations can also
289 result in increased resistance or have no effect in DCS susceptibility depending on the growth
290 media [26-30], suggesting that CycA is primarily important for DCS resistance under
291 conditions when its contribution to amino acid transport is also important. The opposite
292 effects of *cycA* mutation on DCS susceptibility in *S. aureus* and *E. coli* or *Mycobacteria*
293 indicates that DCS uptake is not significantly blocked by the *cycA* mutation in *S. aureus*. We
294 have identified several predicted membrane-associated transport proteins involved in DCS
295 susceptibility. However, mutations in these genes, alone or in combination only resulted in a
296 2-fold increase in DCS resistance, suggesting that DCS is transported into *S. aureus* cells via
297 multiple routes.

298 Using chemically defined media supplemented with glucose, transport of alanine was
299 impaired in the *cycA* mutant, which correlated with increased susceptibility to β-lactam
300 antibiotics, suggesting that alanine utilisation via CycA is important for cell wall integrity and
301 consequently resistance to β-lactam antibiotics. Mutation of *cycA* or DCS-exposure have
302 similar effects on the structure of *S. aureus* peptidoglycan. Consistent with previous studies

303 in *S. aureus* [20] and in *M. tuberculosis* [31], our studies showed a dose-dependent
304 accumulation of muropeptides with a tripeptide stem in MRSA exposed to DCS. The *cycA*
305 mutation was also associated with the increased accumulation of muropeptides with a
306 tripeptide stem. These data indicate that a reduced intracellular alanine pool or inhibition of
307 the D-Alanine pathway is associated with reduced D-Ala-D-Ala incorporation into the PG stem
308 peptide. The increased accumulation of tripeptides in turn interferes with normal PBP
309 transpeptidase activity and offers a plausible explanation for increased susceptibility to β-
310 lactam antibiotics. This conclusion is reminiscent of a previous study, which demonstrated
311 that growth of a HoR MRSA strain in media supplemented with high concentrations of glycine
312 resulted in decreased methicillin resistance [32]. Peptidoglycan composition analysis revealed
313 that under these growth conditions the D-Ala-D-Ala in the stem peptide was replaced by D-
314 Ala-Gly indicating a central role of the terminal D-Ala-D-Ala in β-lactam resistance [32].
315 The similar effects of a *cycA* mutation and DCS treatment on the structure of peptidoglycan
316 and β-lactam susceptibility identify the CycA permease as a new therapeutic target. New drug
317 leads that interfere with CycA activity, perhaps based on analogues of substrates transported
318 by the permease, should increase susceptibility of MRSA to both β-lactams antibiotics and
319 DCS. Because β-lactam resistance in *S. aureus* is linked mainly to changes in the cell wall and
320 because production of the D-Ala-D-Ala terminus of the peptidoglycan stem peptide is
321 dependent on alanine transport and Alr/Ddl activity, we propose that combination of DCS, β-
322 lactams and/or new drugs targeting CycA have therapeutic potential for the treatment of
323 infections caused by MRSA and potentially other ESKAPE pathogens (Fig. 7).
324 The therapeutic potential of β-lactam / DCS combinations in the treatment of infections
325 caused by MRSA is particularly important given that *S. aureus* isolates resistant to all licensed
326 anti-staphylococcal drugs have been reported, and in light of the regulatory barriers to the
327 introduction of new antimicrobial drugs. The excellent safety profile of β-lactam antibiotics
328 makes these drugs particularly attractive as components of combination antimicrobial
329 therapies. When used in the treatment of tuberculosis DCS is typically administered orally in
330 250 mg tablets twice daily for up to two years. At this dosage, the DCS concentration in blood
331 serum is 25-30 μg/ml. The known neurological side effects associated with DCS therapy mean
332 that this antibiotic is unlikely to be considered for the treatment of MRSA infections unless
333 alternative therapeutic approaches have been exhausted. DCS is a co-agonist of the *N*-

methyl-D-aspartic acid receptor in the central nervous system can include seizures and peripheral neuropathy [33, 34], and the use of this antibiotic is limited to the treatment of recalcitrant, multi-drug resistant tuberculosis infections, for which long-term therapy is required [35]. However in the context of MRSA infections, our *in vivo* data with a mouse bacteraemia model demonstrated the significant effectiveness of DCS over a 5-day therapeutic window. Administration of DCS alone at a therapeutically relevant dose significantly enhanced the eradication of MRSA infections. Infections caused by the *cycA* mutant were eradicated more effectively by oxacillin or DCS alone than wild type infections further suggesting that the CycA permease may also represent a novel drug target as part of strategies to overcome β-lactam resistance. Oxacillin/DCS combination therapy was significantly more effective than DCS or oxacillin alone against wild type and *cycA* mutant infections. The anti-MRSA activity of DCS and its ability to significantly potentiate the active of oxacillin under *in vivo* conditions, together with its therapeutic effectiveness over a very short time frame may outweigh concerns about DCS toxicity and suggest that DCS/β-lactam combination therapy as a treatment option for recalcitrant staphylococcal infections merits further consideration. DCS may also serve as a scaffold from which to synthesise more active and less toxic derivatives, while DCS concentrations may be further reduced if used in combination with an agent that inhibits CycA activity.

352 **Materials and Methods**

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

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

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

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

402 **Growth measurements of JE2 strains in chemically defined media and amino acid analysis.**
403 Overnight cultures of JE2 and NE810 were grown at 37°C in TSB, washed in PBS and inoculated
404 at a starting cell density of A_{600} = 0.05 into chemically defined medium supplemented with
405 14mM glucose (CDMG) [36]. The bacteria were grown aerobically (250 rpm; 10:1 flask to
406 volume ratio, 37°C) and the A_{600} was determined every two hours for 8 hours total. One ml of
407 the bacterial culture was collected every two hours and pelleted by centrifugation at 14,000x
408 g for 3 minutes. The spent media was filtered using an Amicon Ultra centrifugal filter
409 (Millipore; 3,000 molecular weight cutoff [MWCO]). Amino acid analysis was performed by
410 the Protein Structure Core Facility, University of Nebraska Medical Center, using a Hitachi L-
411 8800 amino acid analyzer as described previously [37].

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

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

432 **Transmission Electron Microscopy (TEM).** Overnight cultures were diluted 1:200 in 10ml BHI
433 media and grown at 37°C to A_{600} = 1. The cells were pelleted at 8,000 × *g* before being re-
434 suspended in fixation solution (2.5% glutaraldehyde in 0.1 M cacodylate buffer [pH 7.4]) and
435 incubated overnight at 4°C. The fixed cells were further treated with 2% osmium tetroxide,
436 followed by 0.25% uranyl acetate for contrast enhancement. The cell pellets were then
437 dehydrated in increasing concentrations of ethanol as described above for the SEM cell
438 preparation, followed by pure propylene oxide, and transferred to a series of resin and
439 propylene oxide mixtures (50:50, 75:25, pure resin) before being embedded in Epon resin.
440 Thin sections were cut on an ultramicrotome. Images were analysed using a Hitachi H7000
441 instrument. At least 3 to 5 measurements of cell wall thickness were performed on each cell
442 and approximately 30 cells were measured for each sample.

443 **Analysis of peptidoglycan composition in NE810 and JE2 treated with D-cycloserine**. Using
444 overnight cultures of JE2 and NE810, independent quadruplicate 50ml broth cultures were
445 set up at a starting cell density of A_{600}=0.05 and grown for approximately 2 hours to ≈A_{600}=0.5,
446 before being left untreated or dosed with DCS at a final concentration of 8, 20 or 32 μg/ml
447 for a further 100 mins before the cells in the entire 50 ml culture were harvested by
448 centrifugation and resuspended in 5ml PBS (Fig. S3). The cell samples were slowly dropped
449 into an equal volume of boiling 10% (wt/vol) SDS, vigorously stirred for 2h in a boiling water
450 bath and left stirring overnight at room temperature. The insoluble fractions were recovered
451 by high speed centrifugation (150,000 x g, 15 min, 25°C) and washed until the fractions were
452 free from SDS. The insoluble fractions were purified for peptidoglycan according to the
453 protocol described previously [38]. Purified peptidoglycan was re-suspended in 50 mM $NaPO_4$
454 buffer, pH 4.9 and treated with 80 μg/mL muramidase (Cellosyl) for 16 h at 37°C. Muramidase
455 digestion was stopped by incubation in a boiling water bath, and coagulated proteins were
456 removed by centrifugation (21,000 x g, 10 min). The supernatants were adjusted to pH 9.0
457 with sodium borate and reduced with sodium borohydride for 30 min at room temperature.
458 Finally, samples were adjusted to pH 3.5 with orthophosphoric acid and filtered prior to UPLC
459 analysis with a Waters Acquity UPLC H-class system (Waters) equipped with a Waters Xevo
460 G2-XS QTof MS (Waters MS Technologies). Chromatographic separation was achieved on
461 acquity UPLC® BEH C18, 1.7 µm, 150 x 2.1 mm id (Waters) column. The mobile consisted of
462 solvent A: 0.1% formic acid in Milli-Q water and B: 0.1% formic acid in acetonitrile. The
463 gradient was set as follows: 0-3 min 2-5% B, 3-6 min 5-6.8% B, 6-7.5 min 6.8-9% B, 7.5-9 min
464 9-14% B, 9-11 min 14-20% B, 11-12 min hold at 20% B keeping the constant flow of
465 0.250µl/min, 12-12.1 min 20-90% B with a flow rate ramp of 0.250µl/min to 0.300µl/min
466 further holding the same flow rate and % B till 13.5 min, 13.5-13.6 min 90-2% B, 13.6-16 min
467 hold at 2% B. The flow rate was ramped back again to 0.250µl/min at 16.1 min and column
468 was equilibrated form next analysis till 18min. The chromatographic separation was recorded
469 at 204nm.
470 Mass spectrometry was performed on a Xevo G2-XS QTof quadrupole time-of-flight mass
471 spectrometer (Waters MS Technologies). The scan range was from 10 to 2000 m/z with a scan
472 rate of 0.25s. Instrument was operated in positive electro-spray ionization mode. The
473 capillary and sample cone voltages were 3kV and 40 V, respectively. Gas flows were set at 100
474 and 500 l/hr for cone gas and desolvation gas, respectively. The source temperature was

475 120°C and desolvation temperature was 350°C. For MS^E low collision energy was set at 6 eV
476 while ramping the high collision energy from 15-40 eV. Leucine-enkephalin reference was
477 used as the lockmass at a concentration of 200 pg/mL with a continuous flow of 5µL/min and
478 0.25s scan time, to maintain the accuracy of the analysis. All the acquisition and analysis of
479 data were controlled by Waters UNIFI software. Structural characterization of muropeptides
480 was performed based on their MS data and MS/MS fragmentation pattern, which matched
481 with the PG composition and structure reported in previous publications [32, 39-41].

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 oxacillin,
493 cefoxitin and DCS alone or in combination, and the number of colony forming units (CFU)/ml
494 enumerated at 0, 2, 4, 8 and 24 h. Data is presented at the antibiotic concentrations where
495 synergy was measured i.e. 0.5× MIC for JE2, USA300, DAR173, DAR22, DAR113, BH1CC, and
496 RP62A, and 0.25× MIC for DAR169. Antibiotic synergism was defined as a ≥2 $\log^{10}$ decrease in
497 the number of CFU/ml in cell suspensions exposed to DCS/β-lactam combinations compared
498 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 infected
502 intravenously (via the tail vein) with 5×10^6 CFU ($n = 10$ mice per group). The infections were
503 left untreated (PBS control) or treated with either 75 mg oxacillin/Kg/12 hours, 30 mg
504 DCS/Kg/12 hours or a combination of both (first antibiotic dose administered 16 hours post
505 infection), before being sacrificed after 5 days. Bacteria present in homogenised spleens and

506 kidneys recovered from the mice were enumerated on blood agar. Statistical significance was
507 assessed using Mann-Whitney U tests (unpaired).

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

513 **Statistical analysis**. Two-tailed Student's t-Tests and Mann-Whitney U tests were used to
514 determine statistically significant differences in assays performed during this study. A p value
515 <0.05 was deemed significant.

Acknowledgements:

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

518 **Figure Legends**

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

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

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

538 **Figure 4. Mutation of *cycA* or D-cycloserine (DCS) treatment impacts peptidoglycan peptide**
539 **stem length and reduces cell wall crosslinking. A.** Representative UV chromatograms of
540 peptidoglycan from wild-type JE2, NE810 and JE2 treated with increasing concentrations of
541 DCS (8, 20 and 32 µg/ml). Accumulation of muropeptides with tripeptide stems are indicated
542 by asterisks. **B.** Relative abundance of muropeptides with tripeptides in the stem. **C.** Relative
543 crosslinking efficiency. **D.** Relative proportions of cell wall muropeptide fractions based on
544 oligomerization. All errors bars represent 95% confidence interval (n = 4). Significant
545 differences determined using Students t-test are denoted using asterisks (* $p<0.05$; **
546 $p<0.01$; *** $p<0.001$).

547 **Figure 5.** *In vitro* kill curves for D-cycloserine (DCS), oxacillin and cefoxitin with JE2 (A), USA300
548 FPR3757 (B), DAR173 (C), DAR22 (D), DAR169 (E) and their isogenic *cycA* mutants. Antibiotics
549 at the concentrations indicated (µg/ml) were added to suspensions of overnight bacterial
550 cultures adjusted to 10^7 CFU/ml in BHI (A_{600}=0.05), incubated at 37°C and the number of
551 CFU/ml enumerated at 0, 2, 4, 8 h. The data presented are the mean of three independent
552 experiments, and standard error of the mean is shown. Antibiotic synergism was defined as a
553 ≥2 $\log^{10}$ decrease in the number of CFU/ml in cell suspensions exposed to DCS/β-lactam
554 combinations compared to the most effective individual antibiotic alone.

555 **Figure 6. Combination therapy with D-cycloserine and oxacillin significantly reduces the**
556 **bacterial burden in the kidneys of mice infected with MRSA.** The number of colony-forming

557 units (CFU) recovered from the kidneys of mice infected by tail vein injection with 5×10^6 JE2
558 (A) or NE810 (*cycA*::Tn) (B) and left untreated (control) or treated with 75mg of oxacillin
559 (Oxa)/kg, 30mg of DCS/kg or a combination of both oxacillin and DCS delivered
560 subcutaneously every 12 hours for 5 days. The first antibiotic dose was given 16 hours after
561 infection. Significant differences determined using the Mann-Whitney U test are denoted
562 using asterisks (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001). The limit of detection (50
563 colonies) is indicated with a hashed line.

564 **Figure 7.** Proposed model depicting how mutation of CycA or exposure to DCS can inhibit the
565 D-Alanine pathway for peptidoglycan biosynthesis leading to increased susceptibility to β-
566 lactam antibiotics. Mutation of the CycA alanine permease impairs alanine uptake and
567 transfer into the D-Alanine pathway, whereas the alanine analogue antibiotic DCS the D-
568 Alanine pathway enzymes alanine racemase and D-Alanine ligase.

569

570 **References**

571

572 1. Clatworthy AE, Pierson E, Hung DT. Targeting virulence: a new paradigm for antimicrobial
573 therapy. Nat Chem Biol. 2007;3(9):541-8.
574 2. Jones M, Jernigan JA, Evans ME, Roselle GA, Hatfield KM, Samore MH. Vital Signs: Trends in
575 Staphylococcus aureus Infections in Veterans Affairs Medical Centers - United States, 2005-2017.
576 MMWR Morb Mortal Wkly Rep. 2019;68(9):220-4.
577 3. Kourtis AP, Hatfield K, Baggs J, Mu Y, See I, Epson E, et al. Vital Signs: Epidemiology and Recent
578 Trends in Methicillin-Resistant and in Methicillin-Susceptible Staphylococcus aureus Bloodstream
579 Infections - United States. MMWR Morb Mortal Wkly Rep. 2019;68(9):214-9.
580 4. Waters EM, Rudkin JK, Coughlan S, Clair GC, Adkins JN, Gore S, et al. Redeploying beta-lactam
581 antibiotics as a novel antivirulence strategy for the treatment of methicillin-resistant Staphylococcus
582 aureus infections. J Infect Dis. 2017;215(1):80-7.
583 5. Davis JS, Sud A, O'Sullivan MV, Robinson JO, Ferguson PE, Foo H, et al. Combination of
584 Vancomycin and beta-Lactam Therapy for Methicillin-Resistant Staphylococcus aureus Bacteremia: A
585 Pilot Multicenter Randomized Controlled Trial. Clin Infect Dis. 2016;62(2):173-80.
586 6. Tong SY, Nelson J, Paterson DL, Fowler VG, Jr., Howden BP, Cheng AC, et al. CAMERA2 -
587 combination antibiotic therapy for methicillin-resistant Staphylococcus aureus infection: study
588 protocol for a randomised controlled trial. Trials. 2016;17(1):170.
589 7. Chambers HF. Methicillin resistance in staphylococci: molecular and biochemical basis and
590 clinical implications. Clin Microbiol Rev. 1997;10(4):781-91.
591 8. Chambers HF, Hackbarth CJ. Effect of NaCl and nafcillin on penicillin-binding protein 2a and
592 heterogeneous expression of methicillin resistance in Staphylococcus aureus. Antimicrob Agents
593 Chemother. 1987;31(12):1982-8.
594 9. Sabath LD, Wallace SJ. The problems of drug-resistant pathogenic bacteria. Factors influencing
595 methicillin resistance in staphylococci. Annals of the New York Academy of Sciences. 1971;182:258-
596 66.
597 10. Griffiths JM, O'Neill AJ. Loss of function of the gdpP protein leads to joint beta-
598 lactam/glycopeptide tolerance in Staphylococcus aureus. Antimicrobial agents and chemotherapy.
599 2012;56(1):579-81.
600 11. Dengler V, McCallum N, Kiefer P, Christen P, Patrignani A, Vorholt JA, et al. Mutation in the C-
601 di-AMP cyclase dacA affects fitness and resistance of methicillin resistant Staphylococcus aureus. PloS
602 one. 2013;8(8):e73512.
603 12. Geiger T, Goerke C, Fritz M, Schafer T, Ohlsen K, Liebeke M, et al. Role of the (p)ppGpp
604 synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of Staphylococcus aureus.
605 Infection and immunity. 2010;78(5):1873-83.
606 13. Geiger T, Kastle B, Gratani FL, Goerke C, Wolz C. Two small (p)ppGpp synthases in
607 Staphylococcus aureus mediate tolerance against cell envelope stress conditions. Journal of
608 bacteriology. 2014;196(4):894-902.
609 14. Mwangi MM, Kim C, Chung M, Tsai J, Vijayadamodar G, Benitez M, et al. Whole-genome
610 sequencing reveals a link between beta-lactam resistance and synthetases of the alarmone (p)ppGpp
611 in Staphylococcus aureus. Microbial drug resistance (Larchmont, NY). 2013;19(3):153-9.
612 15. Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, Bose JL, et al. A genetic resource for rapid
613 and comprehensive phenotype screening of nonessential Staphylococcus aureus genes. MBio.
614 2013;4(1):e00537-12.
615 16. Mlynek KD, Callahan MT, Shimkevitch AV, Farmer JT, Endres JL, Marchand M, et al. Effects of
616 Low-Dose Amoxicillin on Staphylococcus aureus USA300 Biofilms. Antimicrobial agents and
617 chemotherapy. 2016;60(5):2639-51.
618 17. O'Neill E, Pozzi C, Houston P, Smyth D, Humphreys H, Robinson DA, et al. Association between
619 methicillin susceptibility and biofilm regulation in Staphylococcus aureus isolates from device-related
620 infections. J Clin Microbiol. 2007;45(5):1379-88.

621 18. Desjardins CA, Cohen KA, Munsamy V, Abeel T, Maharaj K, Walker BJ, et al. Genomic and
622 functional analyses of Mycobacterium tuberculosis strains implicate ald in D-cycloserine resistance.
623 Nat Genet. 2016;48(5):544-51.
624 19. Chen JM, Uplekar S, Gordon SV, Cole ST. A point mutation in cycA partially contributes to the
625 D-cycloserine resistance trait of Mycobacterium bovis BCG vaccine strains. PLoS One.
626 2012;7(8):e43467.
627 20. Sieradzki K, Tomasz A. Suppression of beta-lactam antibiotic resistance in a methicillin-
628 resistant Staphylococcus aureus through synergic action of early cell wall inhibitors and some other
629 antibiotics. J Antimicrob Chemother. 1997;39 Suppl A:47-51.
630 21. Pozzi C, Waters EM, Rudkin JK, Schaeffer CR, Lohan AJ, Tong P, et al. Methicillin resistance
631 alters the biofilm phenotype and attenuates virulence in Staphylococcus aureus device-associated
632 infections. PLoS Pathog. 2012;8(4):e1002626.
633 22. Soper TS, Manning JM. Synergy in the antimicrobial action of penicillin and beta-chloro-D-
634 alanine in vitro. Antimicrobial agents and chemotherapy. 1976;9(2):347-9.
635 23. Plata KB, Riosa S, Singh CR, Rosato RR, Rosato AE. Targeting of PBP1 by beta-lactams
636 determines recA/SOS response activation in heterogeneous MRSA clinical strains. PLoS One.
637 2013;8(4):e61083.
638 24. Conlon KM, Humphreys H, O'Gara JP. icaR encodes a transcriptional repressor involved in
639 environmental regulation of ica operon expression and biofilm formation in Staphylococcus
640 epidermidis. J Bacteriol. 2002;184(16):4400-8.
641 25. Rudkin JK, Edwards AM, Bowden MG, Brown EL, Pozzi C, Waters EM, et al. Methicillin
642 resistance reduces the virulence of healthcare-associated methicillin-resistant Staphylococcus aureus
643 by interfering with the agr quorum sensing system. J Infect Dis. 2012;205(5):798-806.
644 26. Baisa G, Stabo NJ, Welch RA. Characterization of Escherichia coli D-cycloserine transport and
645 resistant mutants. J Bacteriol. 2013;195(7):1389-99.
646 27. Curtiss R, 3rd, Charamella LJ, Berg CM, Harris PE. Kinetic and genetic analyses of D-cycloserine
647 inhibition and resistance in Escherichia coli. Journal of bacteriology. 1965;90(5):1238-50.
648 28. Russell RR. Mapping of a D-cycloserine resistance locus in escherichia coli K-12. Journal of
649 bacteriology. 1972;111(2):622-4.
650 29. Wargel RJ, Hadur CA, Neuhaus FC. Mechanism of D-cycloserine action: transport mutants for
651 D-alanine, D-cycloserine, and glycine. Journal of bacteriology. 1971;105(3):1028-35.
652 30. Feher T, Cseh B, Umenhoffer K, Karcagi I, Posfai G. Characterization of cycA mutants of
653 Escherichia coli. An assay for measuring in vivo mutation rates. Mutat Res. 2006;595(1-2):184-90.
654 31. Prosser GA, Rodenburg A, Khoury H, de Chiara C, Howell S, Snijders AP, et al. Glutamate
655 Racemase Is the Primary Target of beta-Chloro-d-Alanine in Mycobacterium tuberculosis. Antimicrob
656 Agents Chemother. 2016;60(10):6091-9.
657 32. de Jonge BL, Chang YS, Gage D, Tomasz A. Peptidoglycan composition of a highly methicillin-
658 resistant Staphylococcus aureus strain. The role of penicillin binding protein 2A. J Biol Chem.
659 1992;267(16):11248-54.
660 33. Fujihira T, Kanematsu S, Umino A, Yamamoto N, Nishikawa T. Selective increase in the
661 extracellular D-serine contents by D-cycloserine in the rat medial frontal cortex. Neurochem Int.
662 2007;51(2-4):233-6.
663 34. Batson S, de Chiara C, Majce V, Lloyd AJ, Gobec S, Rea D, et al. Inhibition of D-Ala:D-Ala ligase
664 through a phosphorylated form of the antibiotic D-cycloserine. Nat Commun. 2017;8(1):1939.
665 35. Chen J, Zhang S, Cui P, Shi W, Zhang W, Zhang Y. Identification of novel mutations associated
666 with cycloserine resistance in Mycobacterium tuberculosis. J Antimicrob Chemother. 2017.
667 36. Hussain M, Hastings JG, White PJ. A chemically defined medium for slime production by
668 coagulase-negative staphylococci. Journal of medical microbiology. 1991;34(3):143-7.
669 37. Halsey CR, Lei S, Wax JK, Lehman MK, Nuxoll AS, Steinke L, et al. Amino Acid Catabolism in
670 Staphylococcus aureus and the Function of Carbon Catabolite Repression. MBio. 2017;8(1).

671 38. Alvarez L, Hernandez SB, de Pedro MA, Cava F. Ultra-Sensitive, High-Resolution Liquid
672 Chromatography Methods for the High-Throughput Quantitative Analysis of Bacterial Cell Wall
673 Chemistry and Structure. Methods Mol Biol. 2016;1440:11-27.
674 39. De Jonge BL, Gage D, Xu N. The carboxyl terminus of peptidoglycan stem peptides is a
675 determinant for methicillin resistance in Staphylococcus aureus. Antimicrob Agents Chemother.
676 2002;46(10):3151-5.
677 40. Kuhner D, Stahl M, Demircioglu DD, Bertsche U. From cells to muropeptide structures in 24 h:
678 peptidoglycan mapping by UPLC-MS. Sci Rep. 2014;4:7494.
679 41. Boneca IG, Xu N, Gage DA, de Jonge BL, Tomasz A. Structural characterization of an abnormally
680 cross-linked muropeptide dimer that is accumulated in the peptidoglycan of methicillin- and
681 cefotaxime-resistant mutants of Staphylococcus aureus. J Biol Chem. 1997;272(46):29053-9.
682 42. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, et al. Complete genome sequence
683 of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus.
684 Lancet. 2006;367(9512):731-9.
685 43. Gill SR, Fouts DE, Archer GL, Mongodin EF, Deboy RT, Ravel J, et al. Insights on evolution of
686 virulence and resistance from the complete genome analysis of an early methicillin-resistant
687 Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus
688 epidermidis strain. J Bacteriol. 2005;187(7):2426-38.
689 44. Robinson DA, Enright MC. Evolutionary models of the emergence of methicillin-resistant
690 Staphylococcus aureus. Antimicrob Agents Chemother. 2003;47(12):3926-34.
691 45. Horsburgh MJ, Foster TJ, Barth PT, Coggins JR. Chorismate synthase from Staphylococcus
692 aureus. Microbiol-Uk. 1996;142:2943-50.
693 46. Salunkhe P, Smart CH, Morgan JA, Panagea S, Walshaw MJ, Hart CA, et al. A cystic fibrosis
694 epidemic strain of Pseudomonas aeruginosa displays enhanced virulence and antimicrobial resistance.
695 J Bacteriol. 2005;187(14):4908-20.

696

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 [42]. 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 [43]
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, 44]
DAR13	MRSA reference isolate; SCC*mec* type IV; CC8 [17, 44]
DAR45	MRSA reference isolate; SCC*mec* type II; CC30 [17, 44]
DAR202	MRSA reference isolate; SCC*mec* type III; CC239 [17, 44]
DAR173	MRSA reference isolate; SCC*mec* type IV; CC5 [17, 44]
DAR173 *cycA*	DAR173 *cycA* mutant (*cycA*::Tn allele from NE810)
DAR22	MRSA reference isolate; SCC*mec* type III; CC5 [17, 44]
DAR22 *cycA*	DAR22 *cycA* mutant (*cycA*::Tn allele from NE810)
DAR169	MRSA Reference strain; SCCmec type I; CC8 [17, 44]
DAR169 *cycA*	DAR169 *cycA* mutant (*cycA*::Tn allele from NE810)
8325-4	NCTC 8325 derivative cured of prophages [45], 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* [46].
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

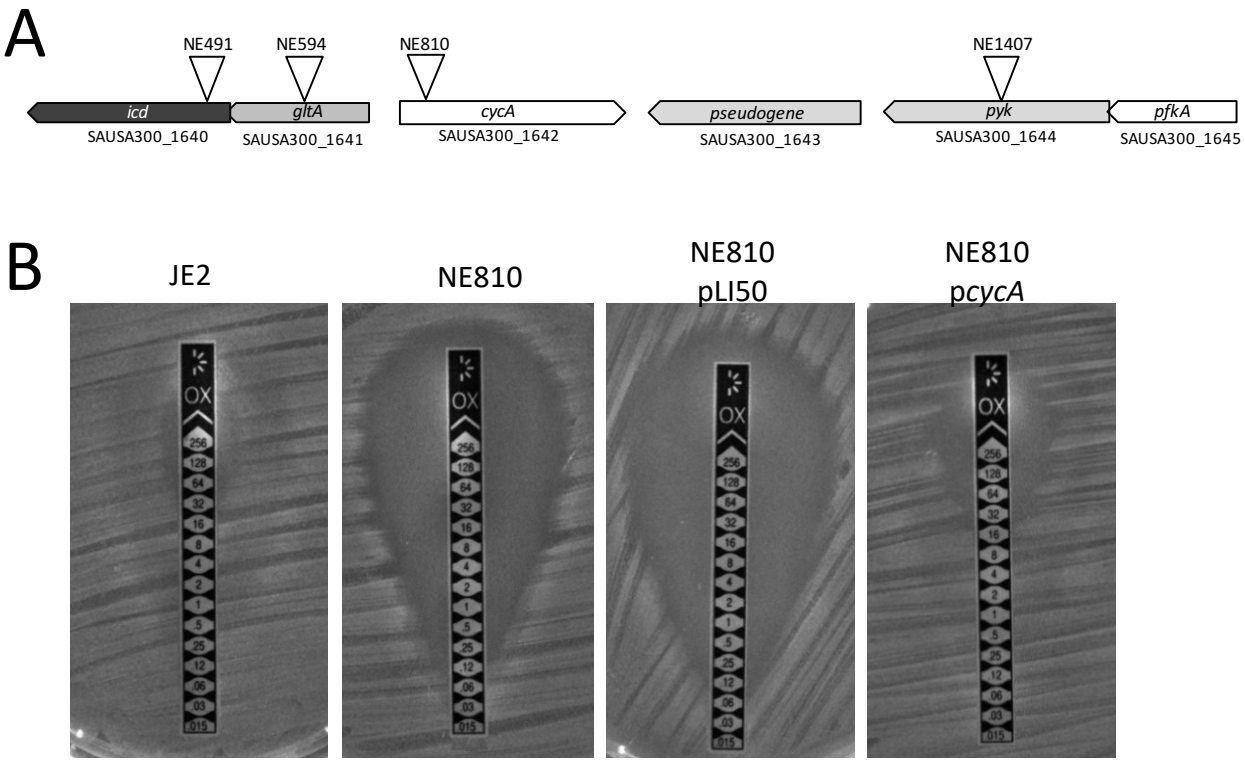
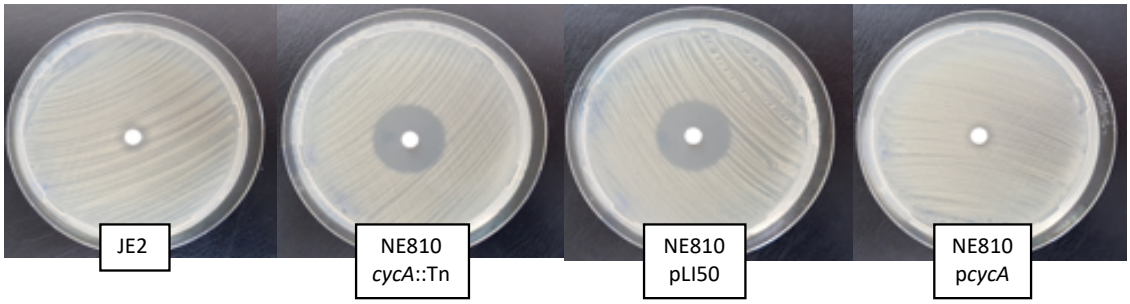
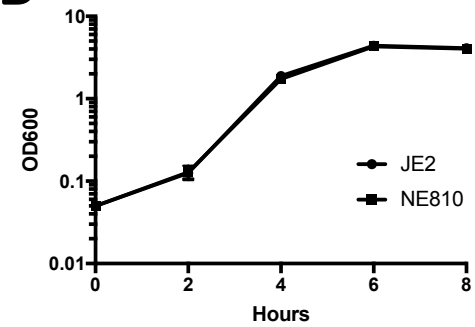

A
NE491
NE594
NE810
NE1407
icd
gltA
cycA
pseudogene
pyk
pfkA
SAUSA300_1640
SAUSA300_1641
SAUSA300_1642
SAUSA300_1643
SAUSA300_1644
SAUSA300_1645
B
JE2
NE810
NE810 pLI50
NE810 pcycA
OX
256
128
64
32
16
8
4
2
1
.5
.25
.12
.06
.03
.015

Fig. 1

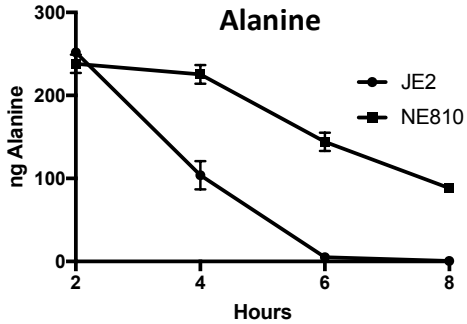
A


JE2
NE810
cycA::Tn
NE810
pLI50
NE810
pcycA

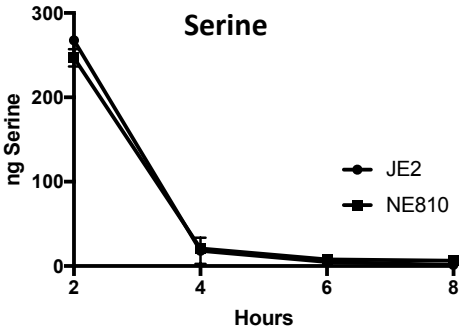
B


OD600
10
1
0.1
0.01
0
2
4
6
8
Hours
JE2
NE810

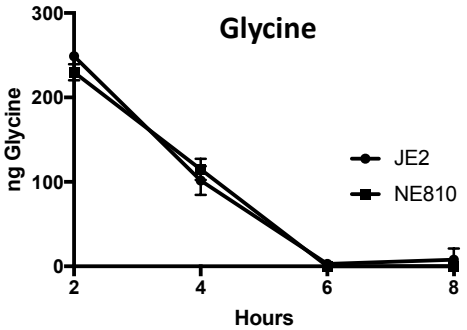
C


Alanine
ng Alanine
300
200
100
0
2
4
6
8
Hours
JE2
NE810

D


Serine
ng Serine
300
200
100
0
2
4
6
8
Hours
JE2
NE810

E


Glycine
ng Glycine
300
200
100
0
2
4
6
8
Hours
JE2
NE810

F

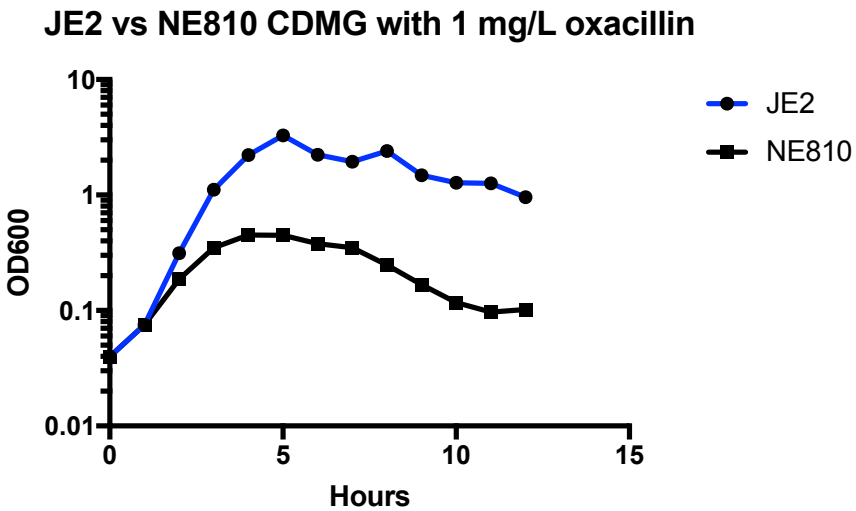

JE2 vs NE810 CDMG with 1 mg/L oxacillin
OD600
10
1
0.1
0.01
0
5
10
15
Hours
JE2
NE810

Fig. 2

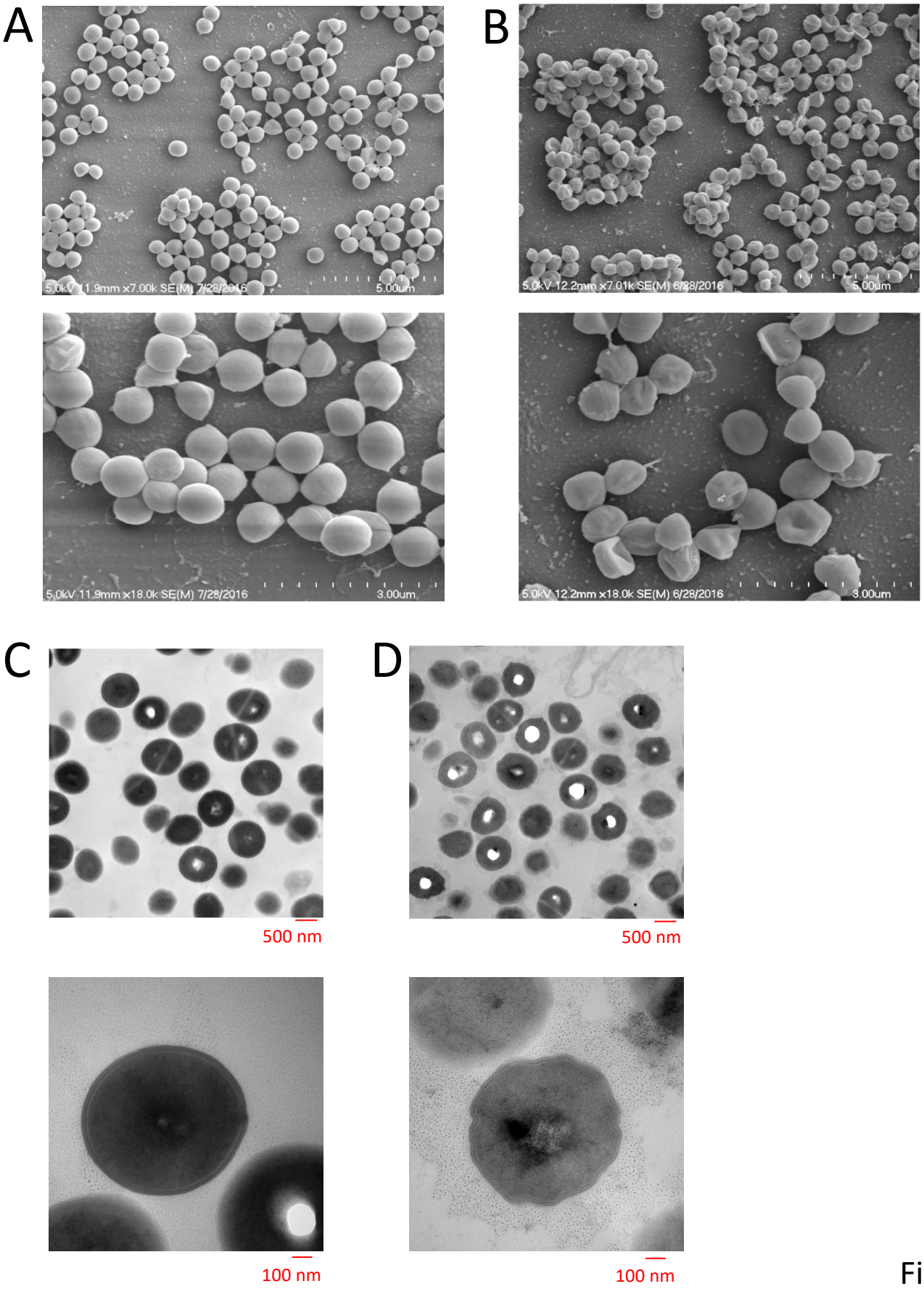
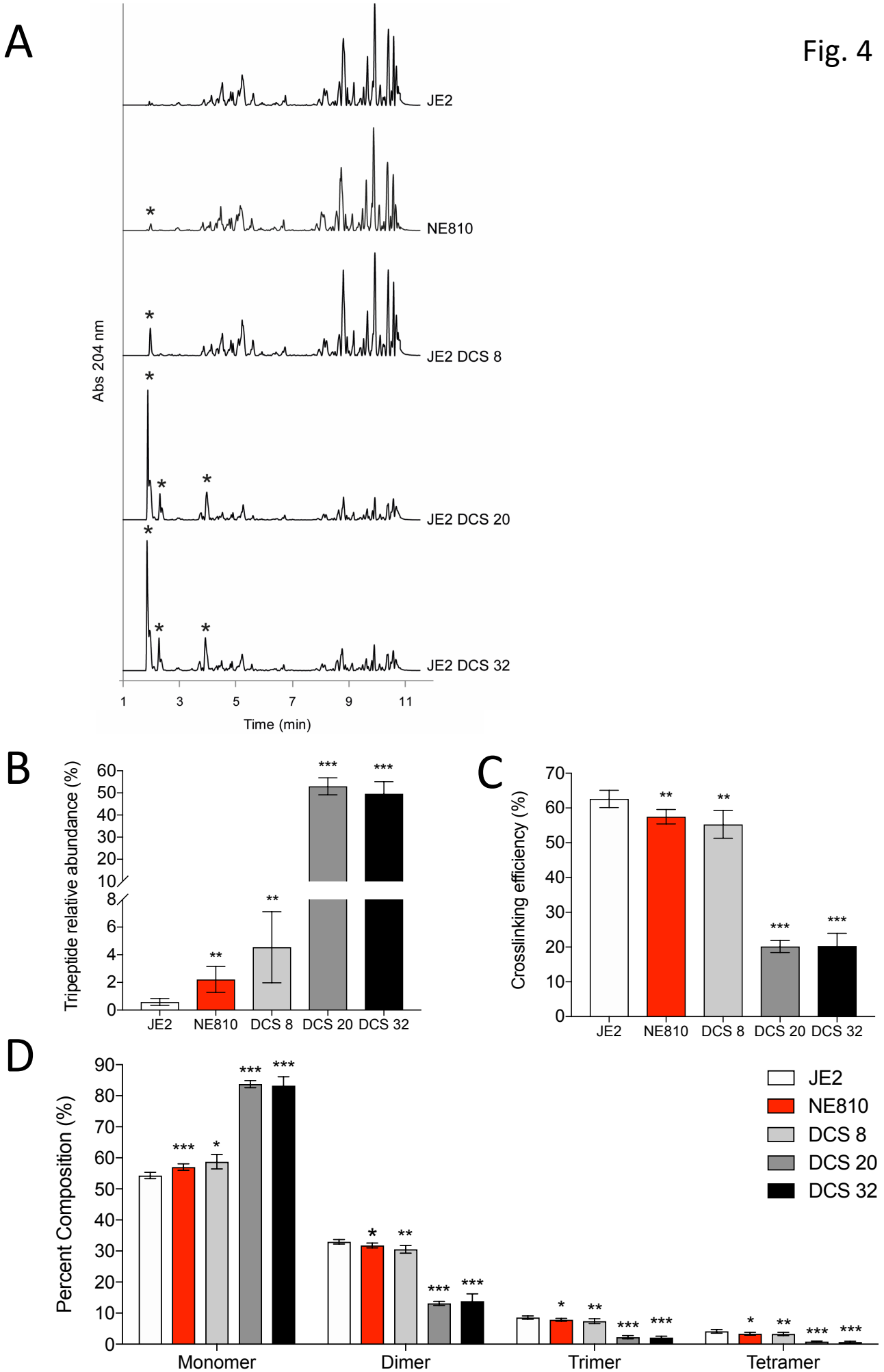
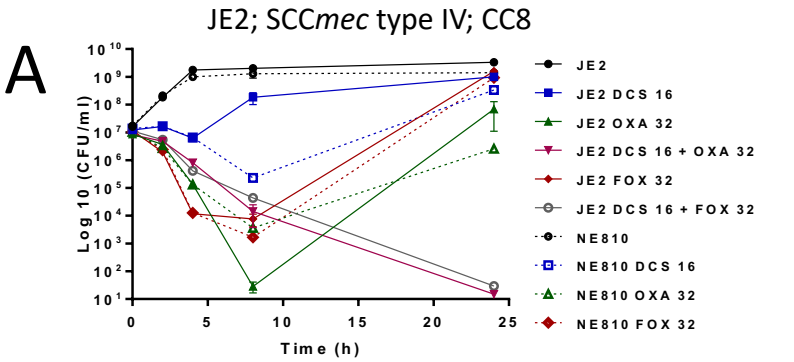
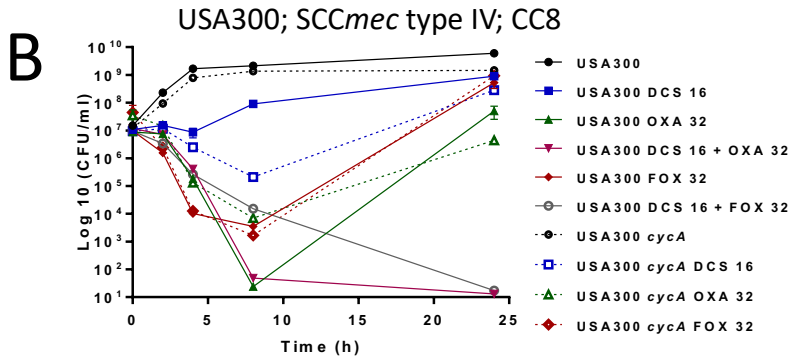

A
5.0kV 11.9mm x7.00k SE(M) 7/28/2016
5.00um
B
5.0kV 12.2mm x7.01k SE(M) 6/28/2016
5.00um
5.0kV 11.9mm x18.0k SE(M) 7/28/2016
3.00um
5.0kV 12.2mm x18.0k SE(M) 6/28/2016
3.00um
C
500 nm
100 nm
D
500 nm
100 nm

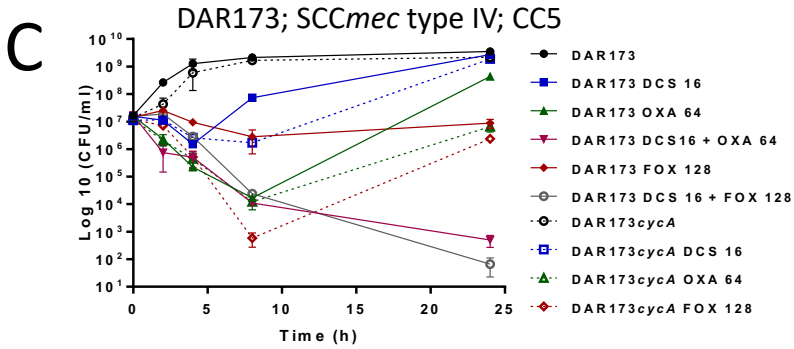
Fig. 3

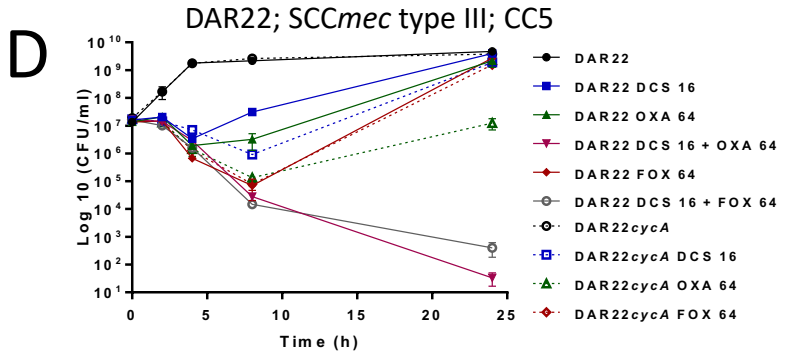
Fig. 4


A
JE2
NE810
JE2 DCS 8
JE2 DCS 20
JE2 DCS 32
Abs 204 nm
Time (min)
B
Tripeptide relative abundance (%)
JE2
NE810
DCS 8
DCS 20
DCS 32
C
Crosslinking efficiency (%)
D
Percent Composition (%)
Monomer
Dimer
Trimer
Tetramer


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

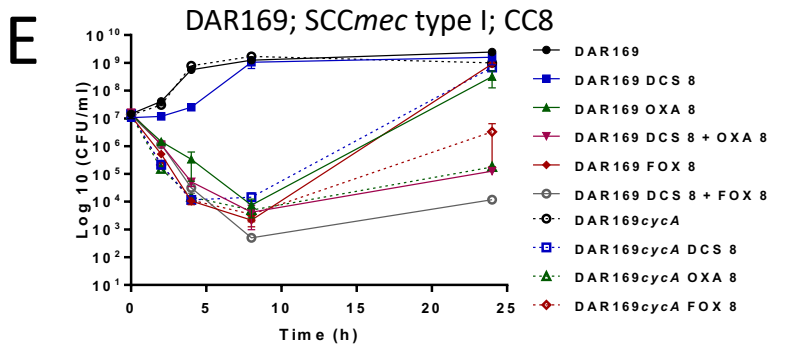
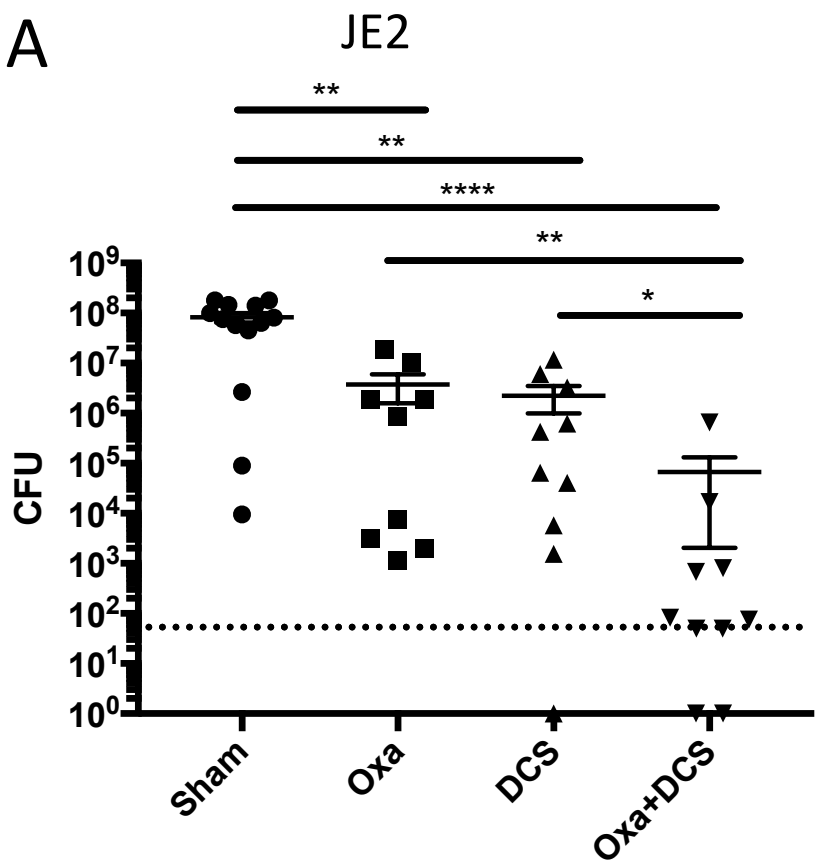

E
DAR169; SCCmec type I; CC8
Log 10 (CFU/ml)
Time (h)
DAR169
DAR169 DCS 8
DAR169 OXA 8
DAR169 DCS 8 + OXA 8
DAR169 FOX 8
DAR169 DCS 8 + FOX 8
DAR169cycA
DAR169cycA DCS 8
DAR169cycA OXA 8
DAR169cycA FOX 8

Fig. 5


A
JE2
**
**
****
**
*
CFU
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Sham
Oxa
DCS
Oxa+DCS

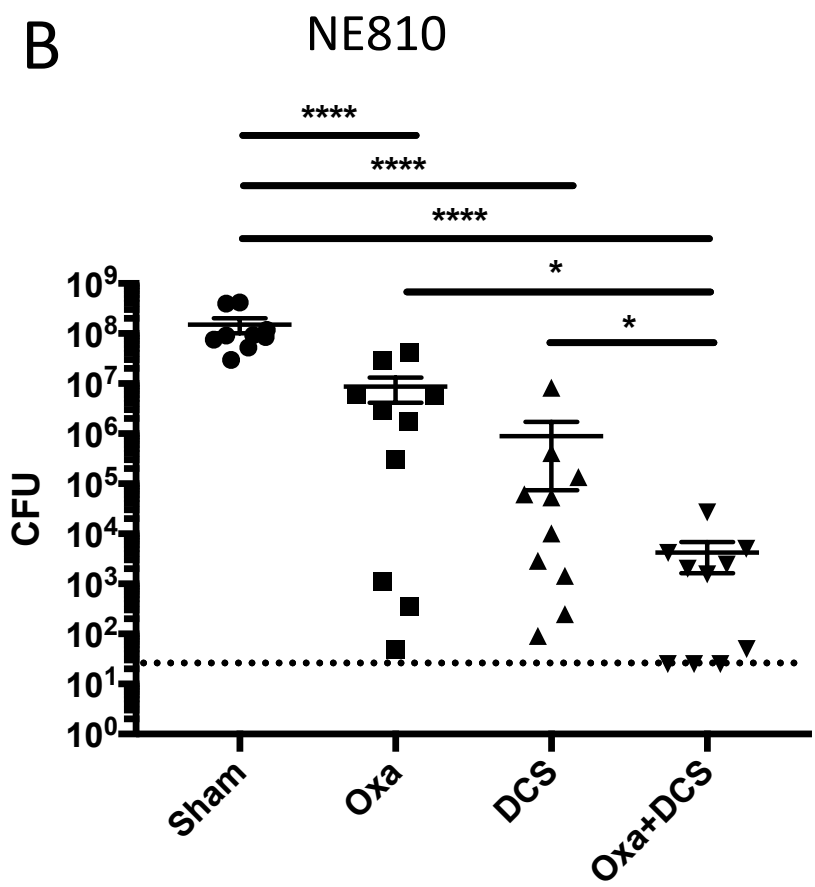

B
NE810
****
****
****
*
*
CFU
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Sham
Oxa
DCS
Oxa+DCS

Fig. 6

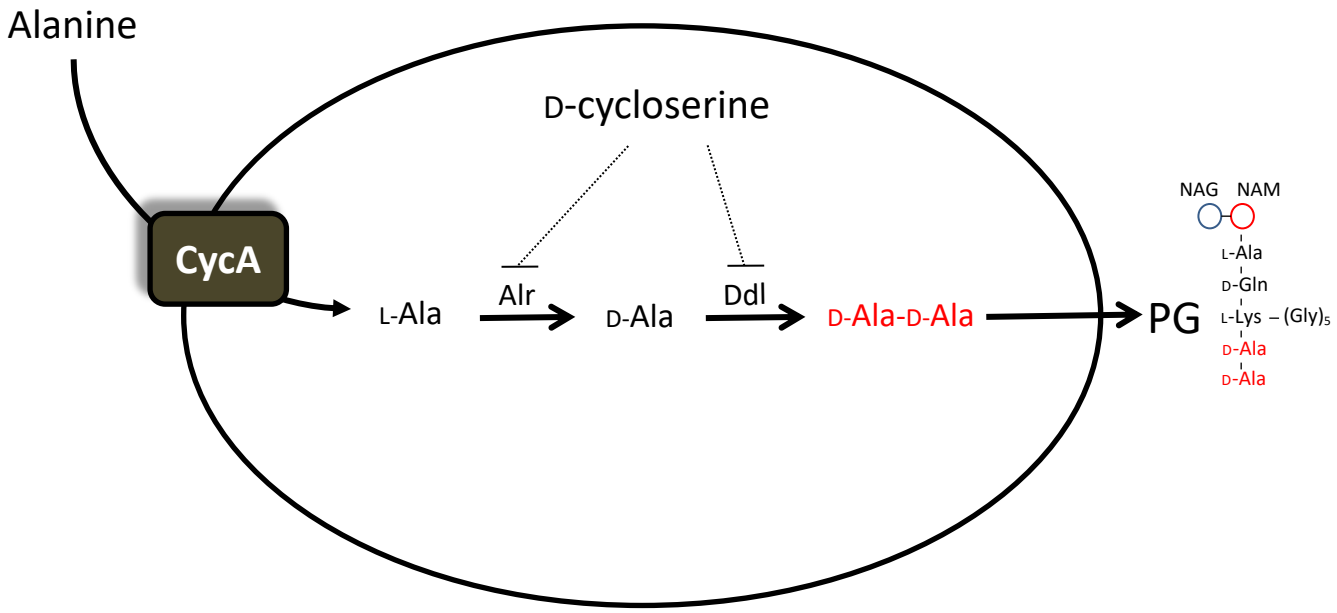

Alanine
D-cycloserine
CycA
L-Ala
Alr
D-Ala
Ddl
D-Ala-D-Ala
PG
NAG
NAM
L-Ala
D-Gln
L-Lys – (Gly)5
D-Ala
D-Ala

Fig. 7

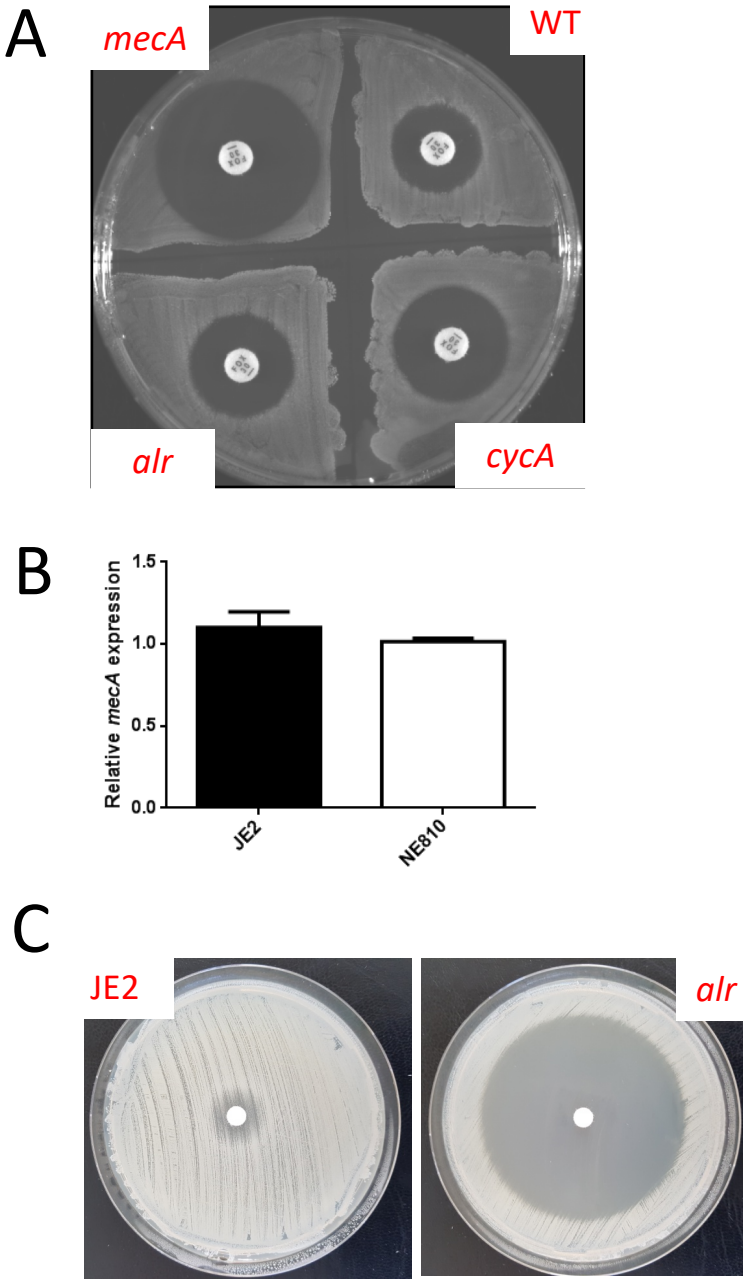


Figure S1. A. Susceptibility of JE2 (wild type), NE1868 (*mecA*::Tn), NE810 (*cycA*::Tn), and NE1713 (*alr*::Tn) grown on MH agar to cefoxitin (FOX, 30μg disks). **B.** Comparison of relative *mecA* gene expression by LightCycler RT-PCR in JE2 and NE810 grown to A_{600}=3 in BHI media. The data are the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

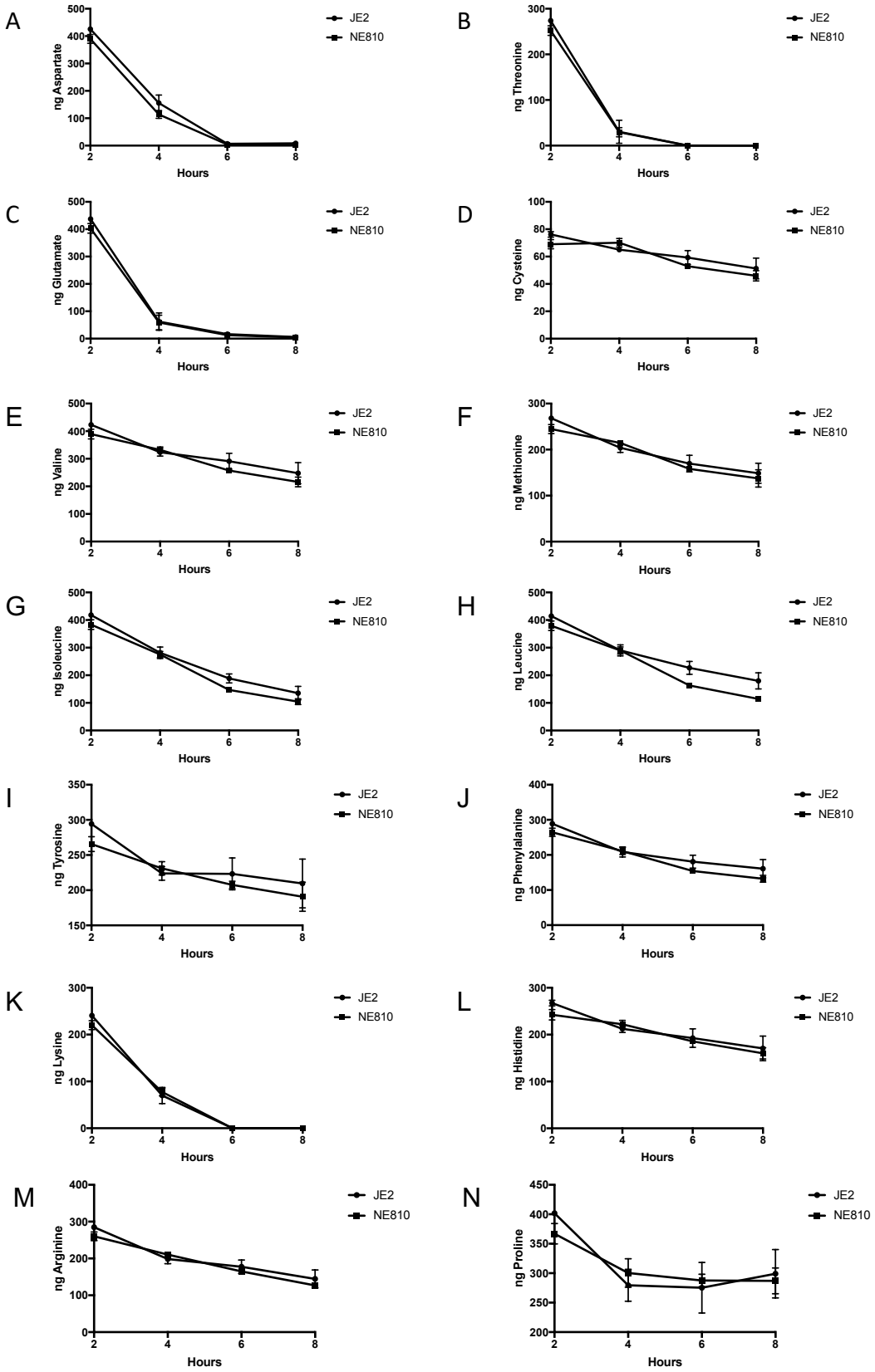


Figure S2. Amino acid consumption by JE2 and NE810 grown aerobically in chemically defined media containing 14mM of glucose (CDMG). Residual amino acids were measured in spent media after 0, 4, 6 and 8 h growth.

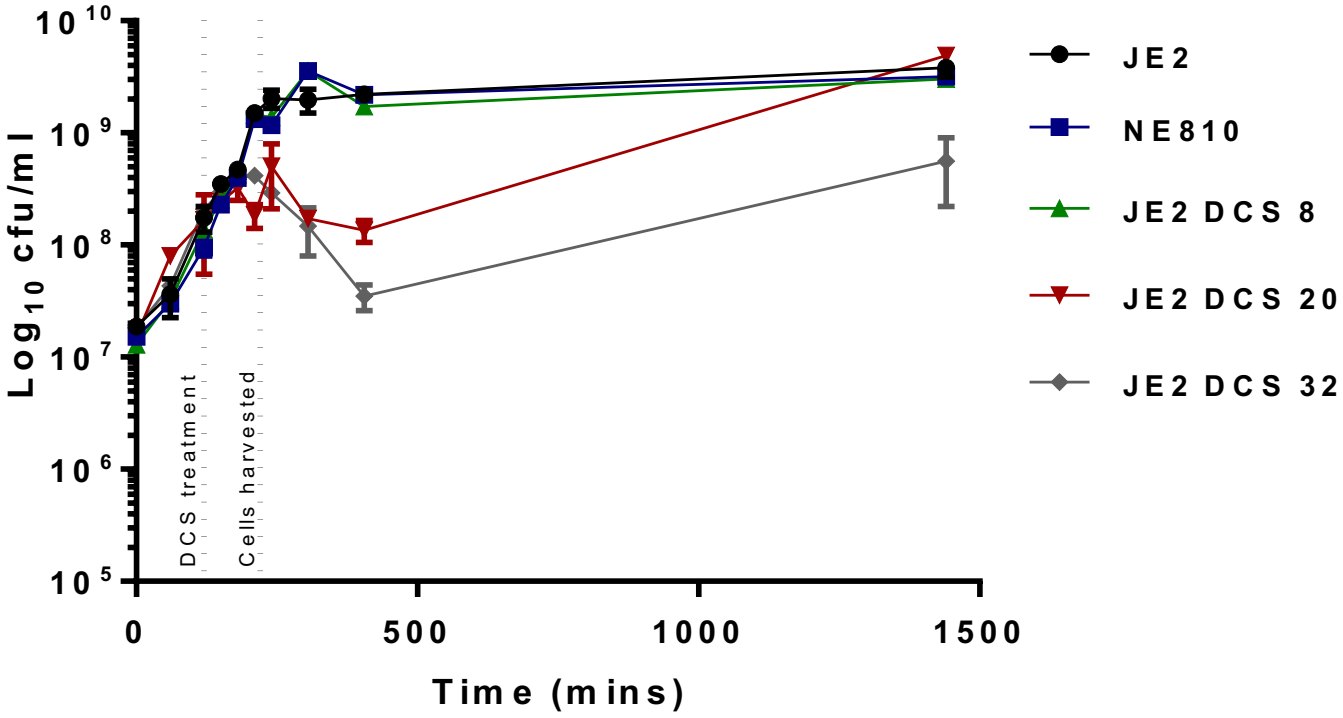


Figure S3. Preparation of cell suspensions for peptidoglycan extraction and structural analysis by UPLC-MS. 50 ml flask cultures were inoculated from overnight broths at a starting cell density of A_{600}=0.05 and incubated at 37°C. The number of CFU/ml was enumerated every 1-2 h for 6 h and again after 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.

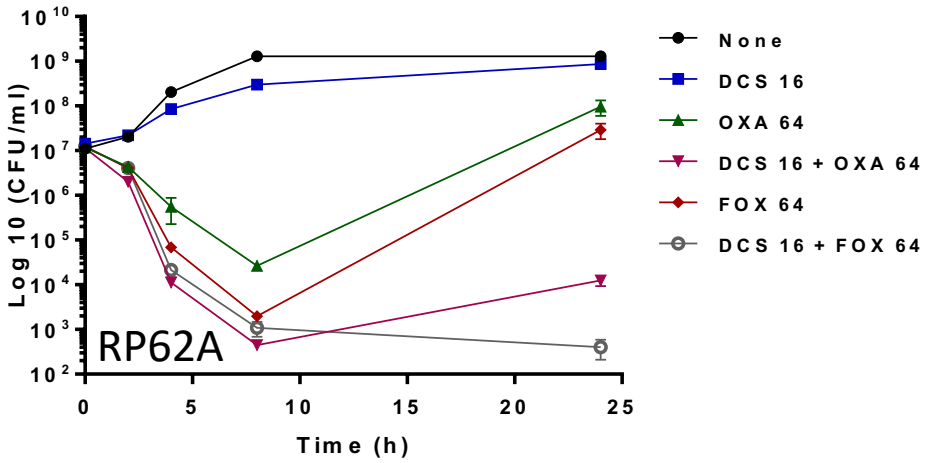
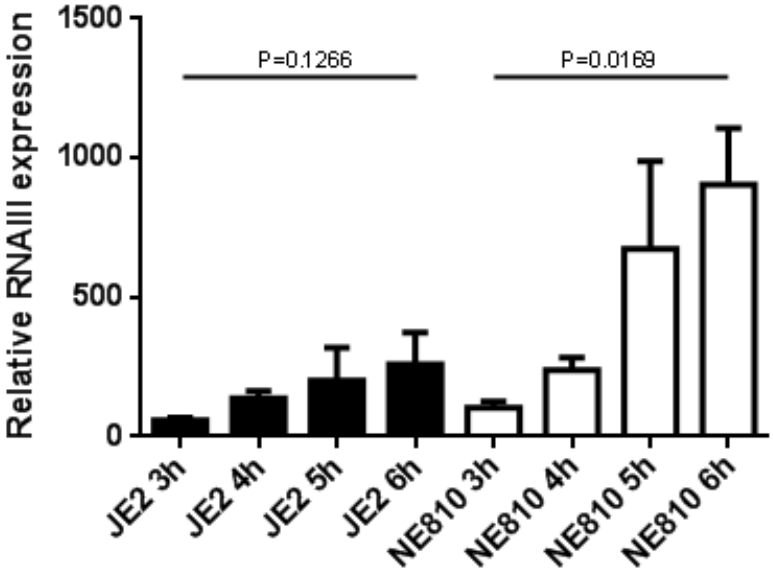


Figure S4. *In vitro* kill curves for D-cycloserine (DCS), oxacillin and cefoxitin with methicillin resistant *S. epidermidis strain* RP62A. Antibiotics at the concentrations indicated (equivalent to 0.5× MIC) were added to suspensions of overnight bacterial cultures adjusted to 10^7 CFU/ml in BHI, incubated 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

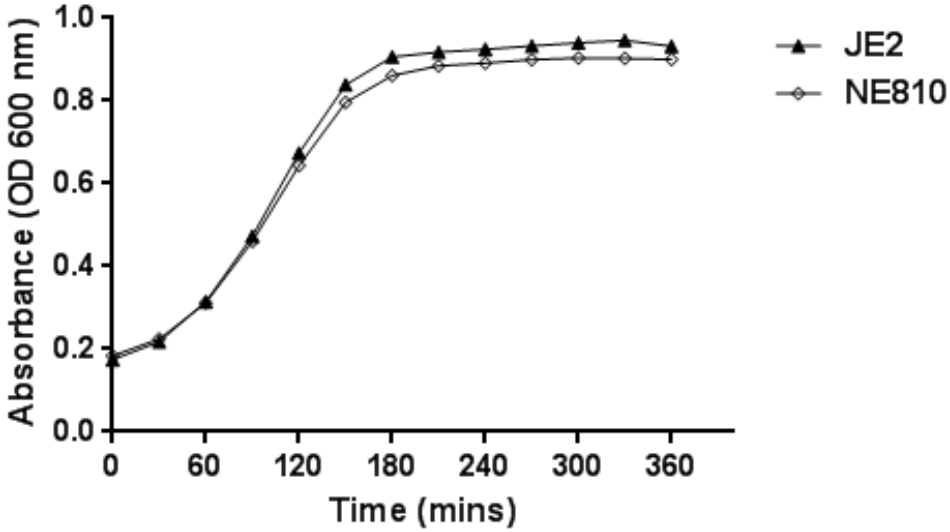


Figure S5. A. Zones of haemolysis of JE2 and NE810 grown on sheep blood agar for 24 hours. **B**. Comparison of relative *agr (RNAIII)* gene expression by LightCycler RT-PCR in JE2 and NE810 grown for 3, 4, 5 and 6 h in BHI media. The data are the average of at least three independent experiments and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