Impaired alanine transport or exposure to D-cycloserine increases the susceptibility of MRSA to β-lactam antibiotics

Laura A. Gallagher[1], Rebecca K. 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]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: Re-sensitization of MRSA 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-sensitized MRSA to β-lactam antibiotics. The *cycA* mutation also resulted in hyper-susceptibility to DCS, an alanine analogue antibiotic that inhibits alanine racemase and D-alanine ligase required for D-alanine incorporation into cell wall peptidoglycan (PG). CycA functions as an alanine permease, and impaired alanine transport correlated with increased susceptibility to oxacillin and DCS. The *cycA* mutation or exposure to DCS were both associated with the accumulation of muropeptides with tripeptide stems lacking the terminal D-ala-D-ala and reduced PG crosslinking, prompting us to investigate synergism between β-lactams and DCS. DCS re-sensitised MRSA to β-lactams *in vitro* and significantly enhanced MRSA eradication by oxacillin in a mouse bacteraemia model. These findings reveal alanine transport as a new therapeutic target to enhance the susceptibility of MRSA to β-lactam antibiotics.

Abstract word count: 161

40 Manuscript word count: 3,494

41

42 **Introduction**

43 Whilst many bacteria can exhibit resistance to select antimicrobials, isolates of the human
44 pathogen *Staphylococcus aureus* can express resistance to all licensed anti-staphylococcal
45 drugs. This results in significant morbidity and mortality, with up to 20% of patients with
46 systemic methicillin resistant *S. aureus* (MRSA) infections dying, despite receiving treatment
47 with anti-staphylococcal drugs [1]. As part of our efforts to identify improved therapeutic
48 approaches for MRSA infections, we recently described the novel use of β-lactam antibiotics
49 to attenuate the virulence of MRSA-induced invasive pneumonia and sepsis [2]. We
50 demonstrated that oxacillin-induced repression of the Agr quorum-sensing system and
51 altered cell wall architecture resulted in downregulated toxin production and increased MRSA
52 killing by phagocytic cells, respectively [2]. Supporting this *in vitro* data, a randomised
53 controlled trial involving 60 patients showed that the β-lactam antibiotic flucloxacillin in
54 combination with vancomycin shortened the duration of MRSA bacteraemia from 3 days to
55 1.9 days [3, 4].

56 Because expression of methicillin resistance in *S. aureus* impacts fitness and virulence and is
57 a regulated phenotype, further therapeutic interventions may also be possible. The
58 complexity of the methicillin resistance phenotype is evident among clinical isolates of MRSA,
59 which express either low-level, heterogeneous (HeR) or homogeneous, high-level methicillin
60 resistance (HoR) [5-7]. Exposure of HeR isolates to β-lactam antibiotics induces expression of
61 *mecA,* which encodes the alternative penicillin binding protein 2a (PBP2a) and can select for
62 mutations in accessory genes resulting in a HoR phenotype, including mutations that affect
63 the stringent response and c-di-AMP signalling [8-12]. Because accessory genes can influence
64 the expression of methicillin resistance in MRSA, targeting the pathways associated with such
65 genes may identify new ways to increase the susceptibility of MRSA to β-lactams. To pursue
66 this, we performed a forward genetic screen to identify loci that impact the expression of
67 resistance to β-lactam antibiotics in MRSA. Using the Nebraska Transposon Mutant Library,
68 which comprises 1,952 sequence-defined transposon insertion mutants [13], inactivation of
69 a putative amino acid permease gene, *cycA*, was found to reduce resistance to cefoxitin, the
70 β-lactam drug recommended by the Clinical and Laboratory Standards Institute for measuring
71 *mecA*-mediated methicillin resistance in MRSA isolates. Amino acid transport and

susceptibility to oxacillin and D-cycloserine (DCS) were compared in the wild-type and *cycA* mutant grown in chemically defined media (CDM), CDM supplemented with glucose (CDMG) and other complex media. The activity of DCS and β-lactams, alone and in combination, against MRSA was measured *in vitro* and in a mouse model of bacteraemia. Peptidoglycan analysis was performed to compare the impact of the *cycA* mutation or exposure to DCS on cell wall structure and crosslinking. Our experiments suggest that therapeutic strategies targeting alanine transport, which was required for resistance to β-lactams and DCS, and a re-evaluation of DCS may be important as part of efforts to restore the efficacy of β-lactam antibiotics against MRSA.

Results

Mutation of *cycA* increases the susceptibility of MRSA to β-lactam antibiotics and D-cycloserine. To identify new ways of controlling expression of methicillin resistance, we sought to identify novel mutations involved in this phenotype. An unbiased screen of the NTML to identify mutants with increased susceptibility to cefoxitin identified NE810 (SAUSA300_1642) (Fig. S1A), which also exhibited a >500-fold increase in susceptibility to oxacillin (Fig. S1B). NE810 was previously identified among several NTML mutants reported to be more susceptible to amoxicillin [14], but was not investigated further. Expression of *mecA* was not affected in NE810 and genome sequence analysis revealed an intact SCC*mec* element and the absence of any other mutations (data not shown). NE810 was successfully complemented (Fig. S1B), and transduction of the SAUSA300_1642 allele into several MRSA strains from a number of clonal complexes and with different SCC*mec* types was also accompanied by increased cefoxitin and oxacillin susceptibility (Table 1).

SAUSA300_1642 is annotated as a serine/alanine/glycine transporter with homology to CycA in *Mycobacterium tuberculosis* [15, 16] required for D-cycloserine (DCS) resistance in *Mycobacteria* [15, 16]. In contrast to the observations in *Mycobacteria*, our data showed that NE810 and several unrelated MRSA strains carrying the *cycA* mutation were significantly more susceptible to DCS than the wild type JE2 (Fig. S1C, Table 1). The *cycA* mutation also reduced the DCS MIC of the MSSA strains 8325-4 and ATCC29213 from 32 to 4 μg/ml. DCS inhibits alanine racemase (Alr) that converts L-alanine to D-alanine and the Ddl D-alanyl:D-alanine ligase [17]. A mutant in the putative *ddl* SAUSA300_2039 gene is not available in the NTML library, suggesting that it may be essential. However, the *alr* mutant NE1713 was significantly more susceptible to cefoxitin and DCS (data not shown), consistent with an important role for D-alanine in resistance to both antibiotics.

CycA is an alanine permease required for D-ala-D-ala incorporation into the peptidoglycan stem peptide. To investigate the role of CycA as a potential permease, JE2 and NE810 were grown for 8 h in chemically defined media containing 14mM glucose (CDMG) and amino acid consumption in spent media was measured. Although no growth rate or yield difference was noted between JE2 and NE810 in CDMG (Fig. 1A), alanine uptake by NE810 was significantly impaired compared to JE2 (Fig. 2B). Utilisation of other amino acids by NE810 and JE2, including serine and glycine, were similar (Fig. S2). Impaired alanine transport in the *cycA*

113 mutant grown in CDMG correlated with increased susceptibility to oxacillin (1 μg/ml) (Fig. 1C)
114 and DCS (1 μg/ml) (Fig. 1D). These data demonstrate for the first time that CycA in *S. aureus*
115 functions as an alanine permease.
116 Quantitative peptidoglycan compositional analysis was performed using UPLC analysis of
117 muramidase-digested muropeptide fragments extracted from exponential phase cultures of
118 JE2 and NE810 grown for 220 mins in TSB media (Fig. S3). The PG profile of the *cycA* mutant
119 revealed a significant accumulation of tripeptides compared to wild-type JE2 (Fig. 2A,B),
120 which was associated with a significant reduction in crosslinking (Fig. 2C). In NE810, the dimer,
121 trimer and tetramer fractions were decreased, which was accompanied by a concomitant
122 increase in the monomer fraction (Fig. 2D). Consistent with this data, exposure of JE2 to DCS
123 8μg/ml was also associated with a similar accumulation in muropeptides with tripeptide
124 stems (Fig. 2B), reduced cross-linking (Fig. 2C), increased muropeptide monomers and
125 reduced dimers, trimers and tetramers (Fig. 2D). DCS had a strong dose-dependent effect on
126 the accumulation of muropeptides with tripeptide stems, reduced cross-linking and
127 accumulation of monomers (Fig. 2A-D). Sub-inhibitory (0.25× MIC) and 4× MIC DCS
128 concentrations, were previously shown to be associated with incorporation of an incomplete
129 stem peptide (tripeptide) [17] and reduced D-ala-D-ala levels [18], respectively. These data
130 show that impaired D-ala incorporation in the *cycA* mutant or following exposure to DCS is
131 accompanied by reduced PG cross-linking and increased β-lactam susceptibility.
132
133 **Mutation of *cycA* or exposure to D-cycloserine increases the susceptibility of MRSA to β-**
134 **lactam antibiotics.** Previously reported synergy between DCS and β-lactam antibiotics [17,
135 19] suggests that impaired alanine uptake in the *cycA* mutant may have the same impact on
136 cell wall biosynthesis as DCS-mediated inhibition of Alr and Ddl. To further investigate this,
137 we compared the activity of DCS and β-lactam antibiotics, alone and in combination, against
138 JE2 and NE810. Checkerboard microdilution assay fractional inhibitory concentration indices
139 (ΣFICs ≤0.5) revealed synergy between DCS and several licensed β-lactam antibiotics with
140 different PBP selectivity against JE2 and USA300 FPR3757 (Table 1). Oxacillin and nafcillin
141 were not included in checkerboard assays because measurement of their MICs involves
142 supplementing the media with 2% NaCl, which distorts the MIC of DCS (data not shown).
143 Using the MRSA strains JE2, USA300, DAR173, DAR22, DAR169 and their corresponding *cycA*
144 mutants, the kinetics of killing by DCS, oxacillin and cefoxitin, alone and in combination was

145 measured over 24h using antibiotic concentrations corresponding to 0.125×, 0.25× and 0.5×
146 MICs. Recovery of growth in media supplemented with oxacillin or cefoxitin alone was evident
147 after 8 h (Fig. 3), reflecting the selection and expansion of HoR mutants as described
148 previously [2, 18, 20]. Recovery of growth in cultures exposed to DCS alone was also evident
149 (Fig. 3), which may correlate with our observation that mutants resistant to DCS (on BHI agar
150 supplemented with 128 μg/ml DCS) arise at a rate of approximately 5.5×10^{-8} per cell per
151 generation (data not shown). Using combinations of DCS and oxacillin or cefoxitin at 0.125×
152 MIC did not achieve a $\geq 2 \log^{10}$ reduction in the number of CFU/ml (data not shown). However,
153 at 0.5× MIC for strains JE2, USA300, DAR173 and DAR22, DCS (16 μg/ml)/oxacillin (32 μg/ml)
154 and DCS (16 μg/ml)/cefoxitin (32 μg/ml) combinations achieved a $\geq 5 \log^{10}$ reduction in the
155 number of CFU/ml compared to oxacillin, cefoxitin or DCS alone (Fig. 3). For strain DAR169,
156 DCS/β-lactam combinations at 0.25× MIC was sufficient to achieve a $\geq 5 \log^{10}$ reduction in
157 CFUs recovered compared to the individual antibiotics (Fig. 3). DCS/β-lactam combinations at
158 0.5× MIC were also able to achieve $\geq 5 \log^{10}$ reduction in the number of CFU/ml against the
159 methicillin resistant *S. epidermidis* (MRSE) strain RP62A [21] compared to either antibiotic
160 alone (Fig. S4). Checkerboard experiments with fourteen MRSA strains and MRSE strain
161 RP62A further revealed synergy (ΣFICs ≤0.5) between DCS and a range of β-lactam antibiotics
162 with different penicillin binding protein (PBP) specificity, namely cefoxitin (PBP4), cefaclor
163 (PBP3), cefotaxime (PBP2), piperacillin-tazobactin (PBP3/β-lactamase inhibitor) and
164 imipenem (PBP1) (Table 1).
165 This synergy appears to be specific to β-lactams and no synergy (ΣFICs > 0.5) was measured
166 between DCS and several antibiotics that are used topically or systemically for the
167 decolonization or treatment of patients colonized/infected with *S. aureus* or MRSA
168 (clindamycin, trimethoprim, mupirocin, ciprofloxacin), or several antibiotics to which *S.*
169 *aureus* isolates commonly exhibit resistance (tobramycin, kanamycin and spectinomycin)
170 (Table S1). Furthermore the *cycA* mutation had no impact on susceptibility to any of these
171 non-β-lactam antibiotics (apart from *ermB*-encoded clindamycin resistance on the
172 transposon).
173
174 **Combination therapy with DCS and oxacillin significantly reduces the bacterial burden in**
175 **the kidneys and spleen of mice infected with MRSA**. The virulence of the NE810 mutant and
176 the therapeutic potential of oxacillin in combination with DCS in the treatment of MRSA

177 infections were assessed in mice. Treatment with oxacillin or DCS alone significantly reduced
178 the number of CFUs recovered from the kidneys of mice infected with JE2 (Fig. 4).
179 Furthermore the oxacillin/DCS combination was significantly more effective than either
180 antibiotic alone and the combination was equally effective in reducing the bacterial burden
181 in the kidneys of animals infected with JE2 or NE810 when compared to no treatment
182 ($p \leq 0.0001$) (Fig. 4) demonstrating the capacity of DCS to significantly potentiate the activity
183 of β-lactam antibiotics against MRSA under *in vivo* conditions. Unexpectedly, oxacillin- or DCS-
184 mediated eradication of NE810 infections in the kidneys was similar to JE2 (Fig. 4). In the
185 spleen, only oxacillin/DCS combination treatment was associated with a significant reduction
186 in the number of CFUs recovered from mice infected with JE2 or NE810 (Fig. S5).
187
188 **Alanine transport and resistance to oxacillin and DCS in chemically defined medium are not**
189 **dependent on *cycA*.** The failure of oxacillin or DCS treatment to enhance the eradication of
190 NE810 infections in the mouse bacteraemia model prompted us to further characterise the
191 growth conditions used for the *in vitro* antibiotic susceptibility assays. Specifically we
192 investigated the role of glucose, which we previously reported to increase the growth
193 requirement for amino acids [22], and which we reasoned may be important for CycA alanine
194 permease activity. Growth of JE2 and NE810 was similar in CDM lacking glucose (Fig. 5A) and
195 uptake of alanine (Fig. 5B) and other amino acids (Fig. S6) was unchanged in NE810 compared
196 to JE2. Furthermore NE810 and JE2 grew equally well in CDM supplemented with oxacillin and
197 DSC (Fig. 5C and D). These data explain in part why the *cycA* mutant does not exhibit increased
198 β-lactam and DCS susceptibility in the mouse bacteraemia model and further reveal the
199 strong correlation between alanine transport and susceptibility to oxacillin and DCS.

200 **Discussion**

201 The exploitation of antibiotic re-purposing as part of concerted efforts to address the
202 antimicrobial resistance crisis has been hampered by a lack of mechanistic data to explain
203 demonstrated therapeutic potential and the perception that studies attempting to identify
204 new uses for existing drugs are not hypothesis-driven. In this study, we identified an alanine
205 permease (CycA) required for full expression of resistance to β-lactam antibiotics and DCS.
206 Loss of function of this alanine transporter significantly increased the susceptibility of MRSA
207 to β-lactam antibiotics, an outcome that could be reproduced through exposure to DCS, which
208 targets the Alr and Ddl enzymes in the early steps of cell wall biosynthesis.

209 The potential of β-lactam/DCS combinations for treatment of MRSA infections follows a
210 recent report that DCS can also potentiate the activity of vancomycin against a laboratory-
211 generated vancomycin highly-resistant *S. aureus* (VRSA) strain *in vitro* and in a silkworm
212 infection model [23]. The excellent safety profile of β-lactam antibiotics makes these drugs
213 particularly attractive as components of combination antimicrobial therapies. When used in
214 the treatment of tuberculosis DCS (trade name Seromycin, The Chao Centre) is typically
215 administered orally in 250 mg tablets twice daily for up to two years. At this dosage, the DCS
216 concentration in blood serum is generally 25-30 μg/ml, which is similar to the concentrations
217 used in our *in vitro* and *in vivo* experiments. The known neurological side effects associated
218 with DCS therapy [24, 25] mean that this antibiotic is unlikely to be considered for the
219 treatment of MRSA infections unless alternative therapeutic approaches have been
220 exhausted. Oxacillin/DCS combination therapy was significantly more effective than DCS or
221 oxacillin alone over a 5-day therapeutic window suggesting that further studies on using DCS
222 to augment β-lactams as a treatment option for recalcitrant staphylococcal infections is
223 merited.

224 Mutation of *cycA* increases the susceptibility of MRSA to β-lactam antibiotics and results in
225 hyper-susceptibility to D-cycloserine, whereas a *cycA* point mutation in *M. bovis* contributes,
226 in part, to increased DCS resistance presumably by interfering with transport into the cell [16].
227 In *E. coli*, *cycA* mutations can also result in increased resistance or have no effect on DCS
228 susceptibility depending on the growth media [26-30], suggesting that CycA is primarily
229 important for DCS resistance under conditions when its contribution to amino acid transport

230 is also important. Our data showing that mutation of *cycA* was not associated with increased
231 DCS resistance strongly suggests that CycA has no role in uptake of this antibiotic in *S. aureus*.
232 Under growth conditions where CycA is required for alanine transport (in nutrient/glucose-
233 replete media), mutation of *cycA* or DCS-exposure have similar effects on the structure of *S.*
234 *aureus* peptidoglycan (Fig. 8). Consistent with previous studies in *S. aureus* [17] and in *M.*
235 *tuberculosis* [31], our studies showed a dose-dependent accumulation of muropeptides with
236 a tripeptide stem in MRSA exposed to DCS. The *cycA* mutation was also associated with the
237 increased accumulation of muropeptides with a tripeptide stem. These data indicate that a
238 reduced intracellular alanine pool or inhibition of Alr and Ddl is associated with reduced D-
239 ala-D-ala incorporation into the PG stem peptide. The increased accumulation of tripeptides
240 in turn interferes with normal PBP transpeptidase activity and offers a plausible explanation
241 for increased susceptibility to β-lactam antibiotics. The importance of the terminal stem
242 peptide D-ala-D-ala for β-lactam resistance has previously been reported. Mutation of the
243 *murF*-encoded ligase, which catalyses of the D-ala-D-ala into the stem peptide also increased
244 β-lactam (but not DCS) susceptibility [32, 33]. Similarly growth of a HoR MRSA strain in media
245 supplemented with high concentrations of glycine was accompanied by replacement of the
246 D-ala-D-ala with D-ala-gly and decreased methicillin resistance [34].
247 Impaired uptake of alanine in CDMG correlated with increased susceptibility to oxacillin and
248 DCS, suggesting that alanine utilisation via CycA is important to make D-alanine available for
249 cell wall biosynthesis and consequently resistance to β-lactams. Consistent with this, NE810
250 also exhibited increased oxacillin susceptibility in BHI, TSB and MH media. However no change
251 in alanine transport or susceptibility to oxacillin and DCS was measured in CDM lacking
252 glucose, which may explain the failure of oxacillin and DCS to more efficiently eradicate NE810
253 infections in the mouse bacteraemia model. The availability of nutrients such as glucose and
254 amino acids varies in different niches colonised by *S. aureus* during infection ranging from
255 glucose-rich in organs such as the liver [35], to glucose-depleted in established abscesses [36].
256 In turn this impacts the role of amino acids as carbon sources [22, 37], and potentially the
257 activity of CycA in alanine transport and β-lactam susceptibility. Furthermore, normal alanine
258 transport in the *cycA* mutant grown in CDM indicates that an alternative alanine transport
259 mechanism(s) may be active under these growth conditions (Fig. 6). Identification of this
260 alternative alanine permease may be important in the development of therapeutic strategies

261 targeting alanine transport to increase β-lactam susceptibility in MRSA, while elucidation of
262 the role of glucose in the control of alanine transport should provide new insights into β-
263 lactam resistance.

264 **Materials and Methods**

265 **Bacterial strains, growth conditions and antimicrobial susceptibility testing.** Bacterial strains
266 used in this study (Table S2) were grown in Luria Bertoni (LB) (Sigma), brain heart infusion
267 (BHI) (Oxoid), Mueller Hinton (MH) (Oxoid), nutrient (Oxoid), sheep blood BHI, chemically
268 defined media (CDM) [38] or CDM supplemented with 14mM glucose (CDMG) [38].
269 Minimum inhibitory concentrations (MICs) were determined in accordance with the Clinical
270 Laboratory Standards Institute (CLSI) guidelines using plate and broth dilution assays in MH
271 or MH containing 2% NaCl for oxacillin and nafcillin. Oxacillin MICs were also measured using
272 E-tests strips from Oxoid on MH agar containing 2% NaCl. Quality control strains ATCC29213
273 and ATCC25923 were used for broth dilution and disk diffusion oxacillin and cefoxitin MIC
274 assays, respectively.

275 **Identification of cefoxitin susceptible MRSA mutant NE810.** Cefoxitin (30μg) disks (Oxoid)
276 were used to measure susceptibility of NTML mutants. The zone diameters for JE2 was 18mm
277 NE1868 (*mecA*::Em^r) was >35mm and NE810 was 22mm. The *cycA* transposon insertion in
278 NE810 was verified by PCR using the primers NE810_Fwd and NE810_Rev (Table S3). Phage
279 80α was used to transduce the NE810 *cycA* allele into JE2 and other strains. Genome
280 sequencing was performed by MicrobesNG using the USA300_FPR3757 genome as a
281 reference. To complement NE810, *cycA* was amplified from JE2 on a 1608 bp fragment using
282 primers NE810F1_Fwd and NE810F1_Rev (Table S3) and cloned into pLI50 using the Clontech
283 In-fusion kit.

284 **Amino acid transport studies.** Amino acid analysis in spent media from cultures grown in
285 CDM or CDMG was performed as described previously [22].

286 **Analysis of peptidoglycan composition in NE810 and JE2 treated with D-cycloserine**.
287 Independent quadruplicate 50ml cultures were grown to A_{600}=0.5, dosed with DCS at 0, 8, 20
288 or 32 μg/ml for 100 mins, then harvested and resuspended in 5ml PBS (Fig. S3) before
289 peptidoglycan was extracted as described previously [39]. Mass spectrometry was performed
290 on a Waters Xevo G2-XS QTof mass spectrometer. Structural characterization of
291 muropeptides was determined based on their MS data and MS/MS fragmentation pattern,
292 matched with PG composition and structure reported previously [34, 40-42].

293 **Antibiotic synergy analysis using the microdilution checkerboard assay.** Antibiotic synergism
294 was measured using the checkerboard microdilution method in 96-well plates inoculated with
295 5×10^5 CFU/ml, and growth or no growth was assessed after 24 h at 37°C. The fractional
296 inhibitory concentration index (ΣFIC) was calculated for each drug combination in triplicate
297 experiments with an FIC index of ≤0.5 considered synergistic.

298 **Kill curve assays.** Overnight cultures adjusted to 10^7 CFU/ml were exposed to 0.125×, 0.25×,
299 and 0.5× MIC of oxacillin, cefoxitin and DCS alone or in combination, and the number of
300 colony forming units (CFU)/ml enumerated at 0, 2, 4, 8 and 24 h. Data is presented at the
301 antibiotic concentrations where synergy was measured i.e. 0.5× MIC for JE2, USA300,
302 DAR173, DAR22, DAR113, BH1CC, and RP62A, and 0.25× MIC for DAR169. Synergism was
303 defined as a ≥2 $\log^{10}$ decrease in the number of CFU/ml in cell suspensions exposed to DCS/β-
304 lactam combinations compared to the most effective individual drug after 8 h.

305 **Mouse infection experiments.** 6- to 8-week-old, age matched, outbred CD1 female mice
306 (Charles River, UK) were used in a non-lethal model of bacteremia. JE2 and NE810 cultures
307 were grown to A_{600}=0.5 in BHI, washed in PBS, adjusted to 1×10^8 CFU/ml. Mice were infected
308 intravenously (via the tail vein) with 5×10^6 CFU ($n = 10$ mice per group). The infections were
309 left untreated (PBS control) or treated with either 75 mg oxacillin/Kg/12 hours, 30 mg
310 DCS/Kg/12 hours or a combination of both (first antibiotic dose administered 16 hours post
311 infection), before being sacrificed after 5 days. Bacteria present in homogenised spleens and
312 kidneys recovered from the mice were enumerated on blood agar.

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

318 **Statistical analysis**. Two-tailed Student's t-Tests and one-way ANOVA with Kruskal-Wallis test
319 followed by Dunn's multiple comparisons test in the GraphPad Prism application (for the
320 mouse infection experiments) were used to determine statistically significant differences in
321 assays performed during this study. A p value <0.05 was deemed significant.

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

Footnotes

Financial support. This study was funded by grants from the Irish Health Research Board (HRA_POR/2012/51, HRA-POR-2015-1158) and Science Foundation Ireland (16/TIDA/4137) to (J.P.O'G), the UK Medical Research Council (MR/M020045/1) to (A.K), the National Institutes of Health (AI083211) to (P.D.F) and Svenska Forskningsrådet Formas to (F.C).

Disclaimer. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

***Potential conflicts of interest*.** The authors declare no conflicts of interest relating to this study.

Correspondence: Prof James P. O'Gara, Discipline of Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland. Tel: +353 91 492250; Email: jamesp.ogara@nuigalway.ie

338 **Figure Legends**

339 **Figure 1. Mutation of *cycA* impairs alanine uptake. A.** Growth of JE2 and NE810 in chemically
340 defined media supplemented with glucose (CDMG). Cell density was measured at A_{600}. **B.**
341 Alanine consumption by JE2 and NE810 grown aerobically in CDMG. Residual amino acid was
342 measured in spent media after 0, 4, 6 and 8 h growth. **C and D.** Growth of JE2 and NE810
343 cultures for 12 h in CDMG supplemented with 1 μg/ml oxacillin (F) or 1 μg/ml D-cycloserine
344 (G). Cell density was measured at A_{600}.

345 **Figure 2. Mutation of *cycA* or D-cycloserine (DCS) treatment impacts peptidoglycan peptide**
346 **stem length and reduces cell wall crosslinking. A.** Representative UV chromatograms of
347 peptidoglycan from wild-type JE2, NE810 and JE2 treated with increasing concentrations of
348 DCS (8, 20 and 32 μg/ml). Muropeptides with tripeptide stems are numbered 1-3. The
349 Proposed structures of the three muropeptides with tripeptide stems identified in NE810 and
350 DCS-treated JE2 cells. NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid; L-Ala, L-
351 alanine; D-Gln, D-glutamine; D-Glu, D-glutamic; L-Lys, L-lysine. The theoretical and observed
352 neutral masses determined by MS are indicated. **B.** Relative abundance of muropeptides with
353 tripeptides in the stem. **C.** Relative crosslinking efficiency. **D.** Relative proportions of cell wall
354 muropeptide fractions based on oligomerization. All errors bars represent 95% confidence
355 interval (n = 4). Significant differences determined using Students t-test are denoted using
356 asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

357 **Figure 3.** *In vitro* kill curves for D-cycloserine (DCS), oxacillin and cefoxitin with JE2, USA300
358 FPR3757, DAR173, DAR22, DAR169 and their isogenic *cycA* mutants. Antibiotics at the
359 concentrations indicated (μg/ml) were added to suspensions of overnight bacterial cultures
360 adjusted to 10^7 CFU/ml in BHI (A_{600}=0.05), incubated at 37°C and the number of CFU/ml
361 enumerated at 0, 2, 4, 8 h. The data presented are the mean of three independent
362 experiments, and standard error of the mean is shown. Antibiotic synergism was defined as a
363 ≥2 $\log^{10}$ decrease in the number of CFU/ml in cell suspensions exposed to DCS/β-lactam
364 combinations compared to the most effective individual antibiotic alone.

365 **Figure 4. Combination therapy with D-cycloserine and oxacillin significantly reduces the**
366 **bacterial burden in the kidneys of mice infected with MRSA.** The number of colony-forming
367 units (CFU) recovered from the kidneys of mice infected by tail vein injection with 5×10^6 JE2
368 or NE810 (CycA) and left untreated or treated with 75mg of oxacillin (Ox)/kg, 30mg of DCS/kg
369 or a combination of both Ox and DCS delivered subcutaneously every 12 hours for 5 days. The
370 first antibiotic dose was given 16 hours after infection. Significant differences determined
371 using one-way ANOVA with Kruskal-Wallis test followed by Dunn's multiple comparisons
372 test are denoted using asterisks (*$p\leq0.05$, **$p\leq0.01$, ****$p\leq0.0001$). The limit of detection
373 (50 colonies) is indicated with a hashed red line.

374 **Figure 5. Alanine transport and resistance to oxacillin and D-cycloserine in chemically**
375 **defined medium are *cycA*-independent**. **A.** Growth of JE2 and NE810 in chemically defined
376 medium lacking glucose (CDM). Cell density was measured at A_{600}. **B.** Alanine consumption by

377 JE2 and NE810 grown aerobically in CDM. Residual amino acid was measured in spent media
378 after 0, 4, 6 and 8 h growth. **C and D.** Growth (cell density at A_{600}) of JE2 and NE810 cultures
379 for 12 h in CDM supplemented with 1 μg/ml oxacillin (C) or 1 μg/ml DCS (D).

380 **Figure 6.** Proposed model depicting how impaired alanine transport associated with mutation
381 of CycA or exposure to DCS can inhibit the D-alanine pathway for peptidoglycan biosynthesis
382 leading to increased susceptibility to β-lactam antibiotics.

383 **References**

384

385 1. Clatworthy AE, Pierson E, Hung DT. Targeting virulence: a new paradigm for antimicrobial therapy.
386 Nat Chem Biol **2007**; 3:541-8.
387 2. Waters EM, Rudkin JK, Coughlan S, et al. Redeploying beta-lactam antibiotics as a novel
388 antivirulence strategy for the treatment of methicillin-resistant Staphylococcus aureus infections. J
389 Infect Dis **2017**; 215:80-7.
390 3. Davis JS, Sud A, O'Sullivan MV, et al. Combination of Vancomycin and beta-Lactam Therapy for
391 Methicillin-Resistant Staphylococcus aureus Bacteremia: A Pilot Multicenter Randomized Controlled
392 Trial. Clin Infect Dis **2016**; 62:173-80.
393 4. Tong SY, Nelson J, Paterson DL, et al. CAMERA2 - combination antibiotic therapy for methicillin-
394 resistant Staphylococcus aureus infection: study protocol for a randomised controlled trial. Trials
395 **2016**; 17:170.
396 5. Chambers HF. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical
397 implications. Clin Microbiol Rev **1997**; 10:781-91.
398 6. Chambers HF, Hackbarth CJ. Effect of NaCl and nafcillin on penicillin-binding protein 2a and
399 heterogeneous expression of methicillin resistance in Staphylococcus aureus. Antimicrob Agents
400 Chemother **1987**; 31:1982-8.
401 7. Sabath LD, Wallace SJ. The problems of drug-resistant pathogenic bacteria. Factors influencing
402 methicillin resistance in staphylococci. Annals of the New York Academy of Sciences **1971**; 182:258-
403 66.
404 8. Griffiths JM, O'Neill AJ. Loss of function of the gdpP protein leads to joint beta-lactam/glycopeptide
405 tolerance in Staphylococcus aureus. Antimicrobial agents and chemotherapy **2012**; 56:579-81.
406 9. Dengler V, McCallum N, Kiefer P, et al. Mutation in the C-di-AMP cyclase dacA affects fitness and
407 resistance of methicillin resistant Staphylococcus aureus. PloS one **2013**; 8:e73512.
408 10. Geiger T, Goerke C, Fritz M, et al. Role of the (p)ppGpp synthase RSH, a RelA/SpoT homolog, in
409 stringent response and virulence of Staphylococcus aureus. Infection and immunity **2010**; 78:1873-83.
410 11. Geiger T, Kastle B, Gratani FL, Goerke C, Wolz C. Two small (p)ppGpp synthases in Staphylococcus
411 aureus mediate tolerance against cell envelope stress conditions. J Bacteriol **2014**; 196:894-902.
412 12. Mwangi MM, Kim C, Chung M, et al. Whole-genome sequencing reveals a link between beta-
413 lactam resistance and synthetases of the alarmone (p)ppGpp in Staphylococcus aureus. Microb Drug
414 Resist **2013**; 19:153-9.
415 13. Fey PD, Endres JL, Yajjala VK, et al. A genetic resource for rapid and comprehensive phenotype
416 screening of nonessential Staphylococcus aureus genes. MBio **2013**; 4:e00537-12.
417 14. Mlynek KD, Callahan MT, Shimkevitch AV, et al. Effects of Low-Dose Amoxicillin on Staphylococcus
418 aureus USA300 Biofilms. Antimicrobial agents and chemotherapy **2016**; 60:2639-51.
419 15. Desjardins CA, Cohen KA, Munsamy V, et al. Genomic and functional analyses of Mycobacterium
420 tuberculosis strains implicate ald in D-cycloserine resistance. Nat Genet **2016**; 48:544-51.
421 16. Chen JM, Uplekar S, Gordon SV, Cole ST. A point mutation in cycA partially contributes to the D-
422 cycloserine resistance trait of Mycobacterium bovis BCG vaccine strains. PLoS One **2012**; 7:e43467.
423 17. Sieradzki K, Tomasz A. Suppression of beta-lactam antibiotic resistance in a methicillin-resistant
424 Staphylococcus aureus through synergic action of early cell wall inhibitors and some other antibiotics.
425 J Antimicrob Chemother **1997**; 39 Suppl A:47-51.
426 18. Pozzi C, Waters EM, Rudkin JK, et al. Methicillin resistance alters the biofilm phenotype and
427 attenuates virulence in Staphylococcus aureus device-associated infections. PLoS Pathog **2012**;
428 8:e1002626.
429 19. Soper TS, Manning JM. Synergy in the antimicrobial action of penicillin and beta-chloro-D-alanine
430 in vitro. Antimicrobial agents and chemotherapy **1976**; 9:347-9.
431 20. Plata KB, Riosa S, Singh CR, Rosato RR, Rosato AE. Targeting of PBP1 by beta-lactams determines
432 recA/SOS response activation in heterogeneous MRSA clinical strains. PLoS One **2013**; 8:e61083.

21. Conlon KM, Humphreys H, O'Gara JP. icaR encodes a transcriptional repressor involved in environmental regulation of ica operon expression and biofilm formation in Staphylococcus epidermidis. J Bacteriol **2002**; 184:4400-8.
22. Halsey CR, Lei S, Wax JK, et al. Amino Acid Catabolism in Staphylococcus aureus and the Function of Carbon Catabolite Repression. MBio **2017**; 8.
23. Tabuchi F, Matsumoto Y, Ishii M, et al. D-cycloserine increases the effectiveness of vancomycin against vancomycin-highly resistant Staphylococcus aureus. J Antibiot (Tokyo) **2017**; 70:907-10.
24. Fujihira T, Kanematsu S, Umino A, Yamamoto N, Nishikawa T. Selective increase in the extracellular D-serine contents by D-cycloserine in the rat medial frontal cortex. Neurochem Int **2007**; 51:233-6.
25. Batson S, de Chiara C, Majce V, et al. Inhibition of D-Ala:D-Ala ligase through a phosphorylated form of the antibiotic D-cycloserine. Nat Commun **2017**; 8:1939.
26. Baisa G, Stabo NJ, Welch RA. Characterization of Escherichia coli D-cycloserine transport and resistant mutants. J Bacteriol **2013**; 195:1389-99.
27. Curtiss R, 3rd, Charamella LJ, Berg CM, Harris PE. Kinetic and genetic analyses of D-cycloserine inhibition and resistance in Escherichia coli. Journal of bacteriology **1965**; 90:1238-50.
28. Russell RR. Mapping of a D-cycloserine resistance locus in escherichia coli K-12. Journal of bacteriology **1972**; 111:622-4.
29. Wargel RJ, Hadur CA, Neuhaus FC. Mechanism of D-cycloserine action: transport mutants for D-alanine, D-cycloserine, and glycine. Journal of bacteriology **1971**; 105:1028-35.
30. Feher T, Cseh B, Umenhoffer K, Karcagi I, Posfai G. Characterization of cycA mutants of Escherichia coli. An assay for measuring in vivo mutation rates. Mutat Res **2006**; 595:184-90.
31. Prosser GA, Rodenburg A, Khoury H, et al. Glutamate Racemase Is the Primary Target of beta-Chloro-d-Alanine in Mycobacterium tuberculosis. Antimicrob Agents Chemother **2016**; 60:6091-9.
32. Sobral RG, Ludovice AM, Gardete S, Tabei K, De Lencastre H, Tomasz A. Normally functioning murF is essential for the optimal expression of methicillin resistance in Staphylococcus aureus. Microb Drug Resist **2003**; 9:231-41.
33. Sobral RG, Ludovice AM, de Lencastre H, Tomasz A. Role of murF in cell wall biosynthesis: isolation and characterization of a murF conditional mutant of Staphylococcus aureus. J Bacteriol **2006**; 188:2543-53.
34. de Jonge BL, Chang YS, Gage D, Tomasz A. Peptidoglycan composition of a highly methicillin-resistant Staphylococcus aureus strain. The role of penicillin binding protein 2A. J Biol Chem **1992**; 267:11248-54.
35. Li C, Sun F, Cho H, et al. CcpA mediates proline auxotrophy and is required for Staphylococcus aureus pathogenesis. J Bacteriol **2010**; 192:3883-92.
36. Thurlow LR, Joshi GS, Richardson AR. Peroxisome Proliferator-Activated Receptor gamma Is Essential for the Resolution of Staphylococcus aureus Skin Infections. Cell Host Microbe **2018**; 24:261-70 e4.
37. Lehman MK, Nuxoll AS, Yamada KJ, Kielian T, Carson SD, Fey PD. Protease-Mediated Growth of Staphylococcus aureus on Host Proteins Is opp3 Dependent. MBio **2019**; 10.
38. Hussain M, Hastings JG, White PJ. A chemically defined medium for slime production by coagulase-negative staphylococci. Journal of medical microbiology **1991**; 34:143-7.
39. Alvarez L, Hernandez SB, de Pedro MA, Cava F. Ultra-Sensitive, High-Resolution Liquid Chromatography Methods for the High-Throughput Quantitative Analysis of Bacterial Cell Wall Chemistry and Structure. Methods Mol Biol **2016**; 1440:11-27.
40. De Jonge BL, Gage D, Xu N. The carboxyl terminus of peptidoglycan stem peptides is a determinant for methicillin resistance in Staphylococcus aureus. Antimicrob Agents Chemother **2002**; 46:3151-5.
41. Kuhner D, Stahl M, Demircioglu DD, Bertsche U. From cells to muropeptide structures in 24 h: peptidoglycan mapping by UPLC-MS. Sci Rep **2014**; 4:7494.
42. Boneca IG, Xu N, Gage DA, de Jonge BL, Tomasz A. Structural characterization of an abnormally cross-linked muropeptide dimer that is accumulated in the peptidoglycan of methicillin- and cefotaxime-resistant mutants of Staphylococcus aureus. J Biol Chem **1997**; 272:29053-9.

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.

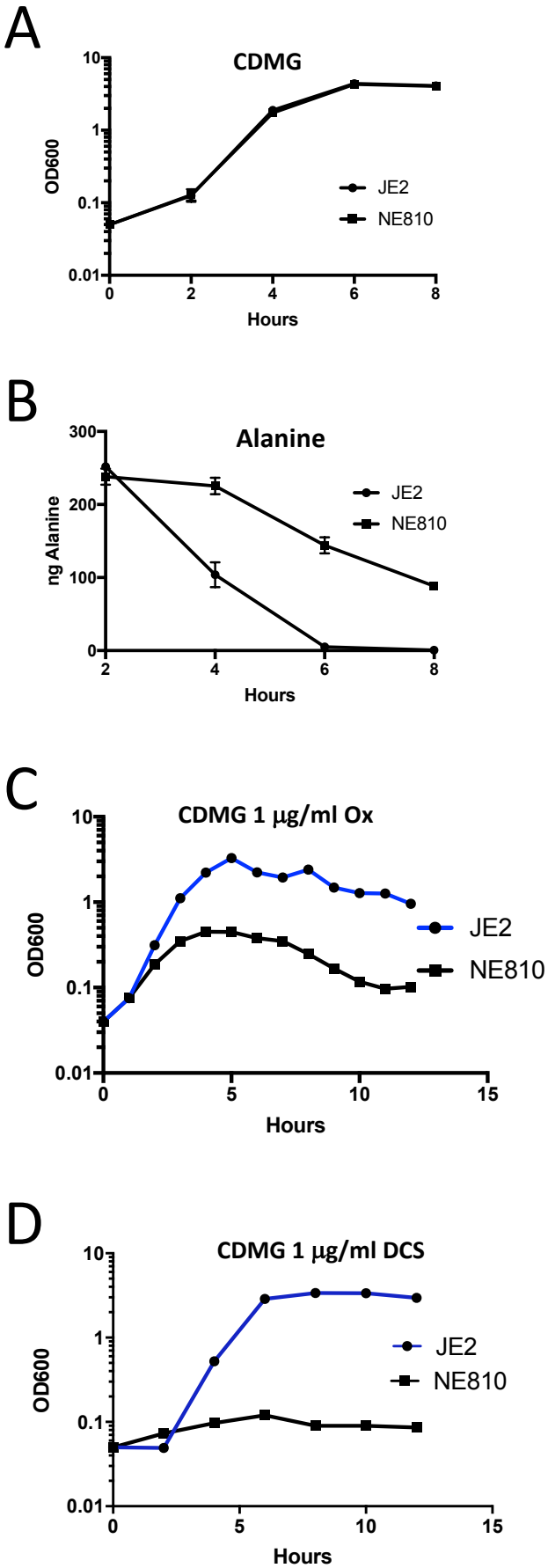
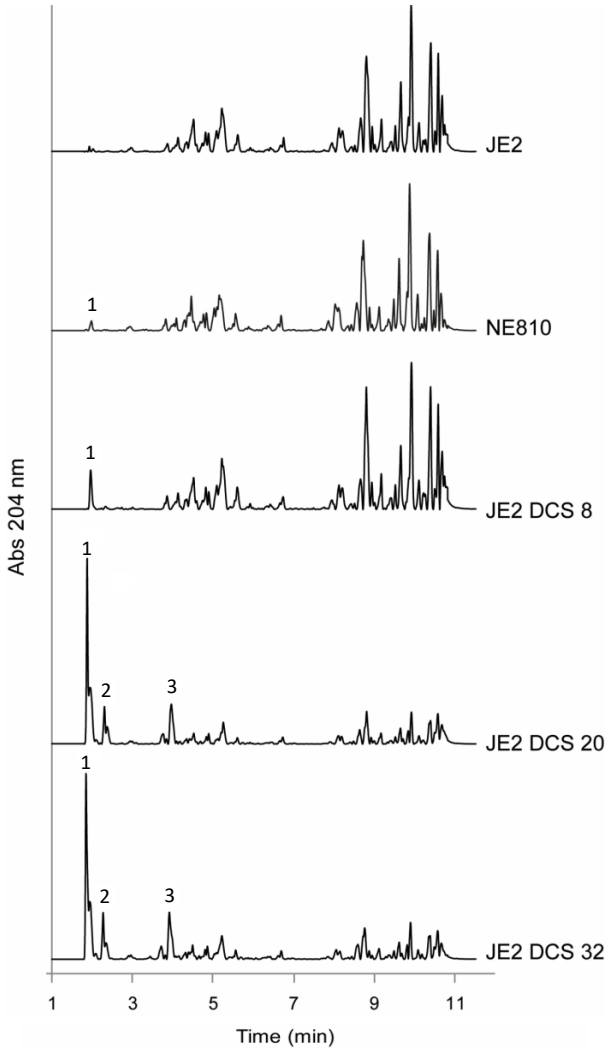
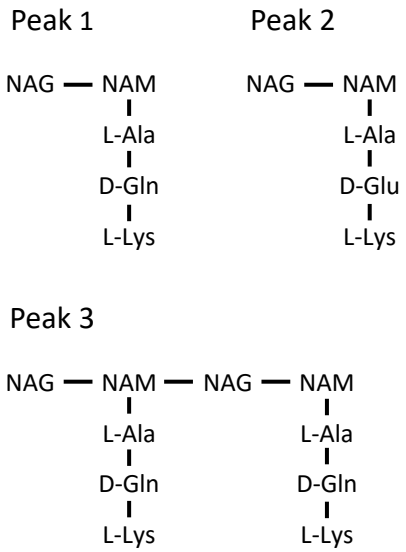

A
CDMG
OD600
10
1
0.1
0.01
0
2
4
6
8
Hours
JE2
NE810
B
Alanine
ng Alanine
300
200
100
0
2
4
6
8
Hours
JE2
NE810
C
CDMG 1 µg/ml Ox
OD600
10
1
0.1
0.01
0
5
10
15
Hours
JE2
NE810
D
CDMG 1 µg/ml DCS
OD600
10
1
0.1
0.01
0
5
10
15
Hours
JE2
NE810

Fig. 1

Fig. 2

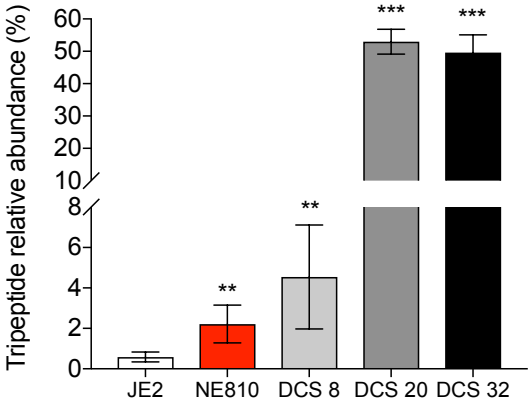
A



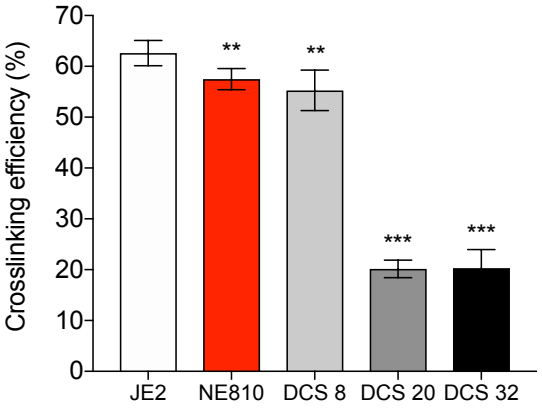


Peak	Muropeptide	Neutral mass (Da)	
		Theoretical	Observed
1	Monomer tripeptide (Gln)	825.3967	825.3976
2	Monomer tripeptide (Glu)	826.3815	826.3818
3	Chain of two monomers tripeptide (Gln)	1630.7673	1630.7627

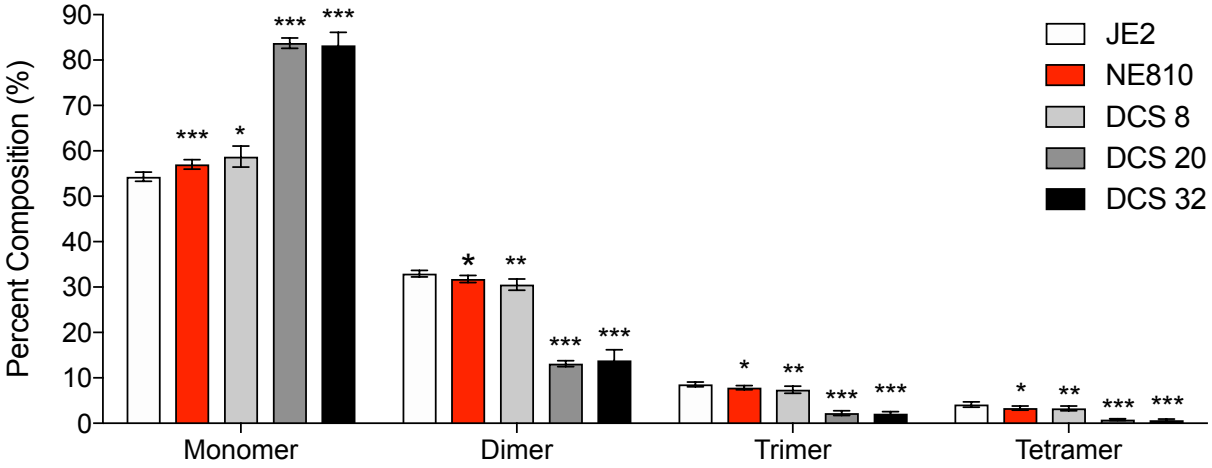
B



C



D



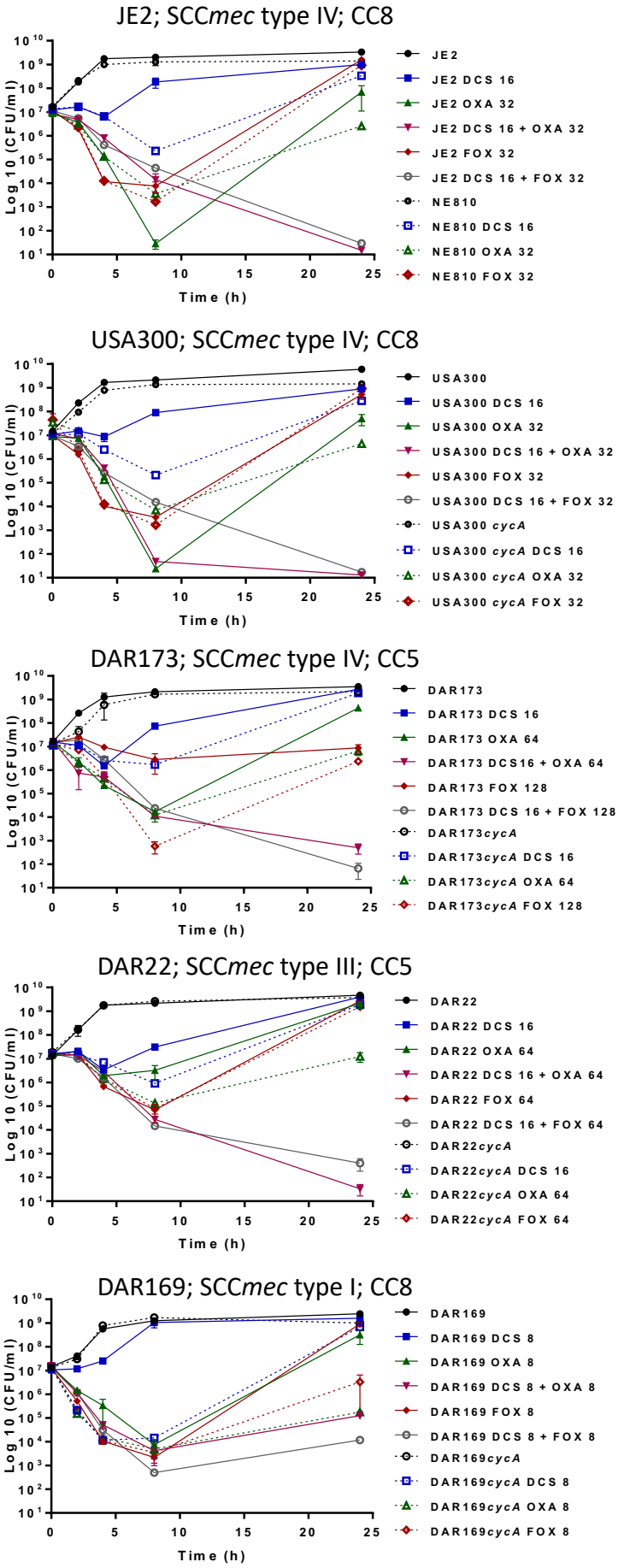

JE2; SCCmec type IV; CC8
Log 10 (CFU/ml)
Time (h)
JE2
JE2 DCS 16
JE2 OXA 32
JE2 DCS 16 + OXA 32
JE2 FOX 32
JE2 DCS 16 + FOX 32
NE810
NE810 DCS 16
NE810 OXA 32
NE810 FOX 32
USA300; SCCmec type IV; CC8
Log 10 (CFU/ml)
Time (h)
USA300
USA300 DCS 16
USA300 OXA 32
USA300 DCS 16 + OXA 32
USA300 FOX 32
USA300 DCS 16 + FOX 32
USA300 cycA
USA300 cycA DCS 16
USA300 cycA OXA 32
USA300 cycA FOX 32
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
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
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. 3

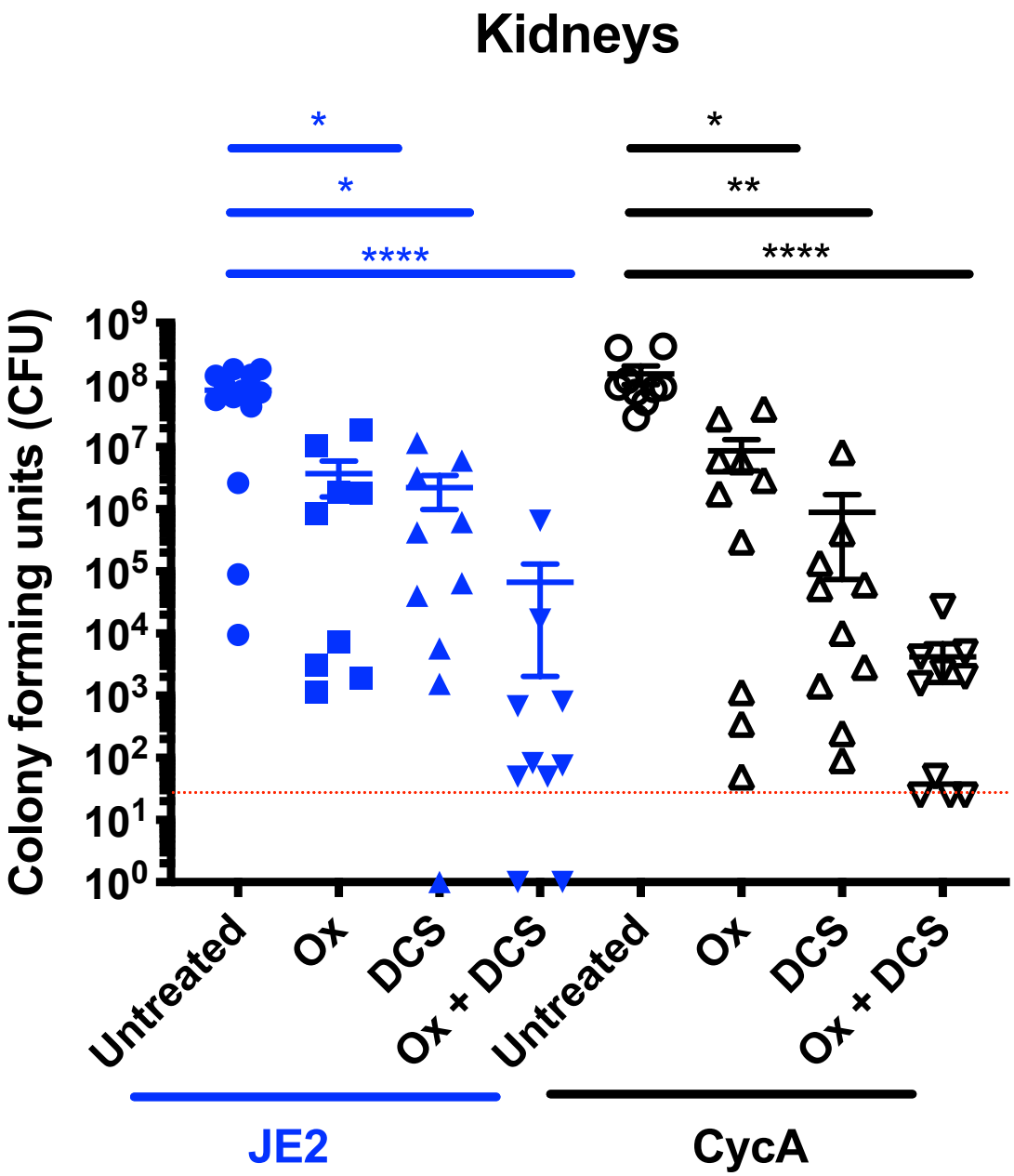

Kidneys
*
*
****
*
**
****
Colony forming units (CFU)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Untreated
Ox
DCS
Ox + DCS
Untreated
Ox
DCS
Ox + DCS
JE2
CycA

Fig. 4

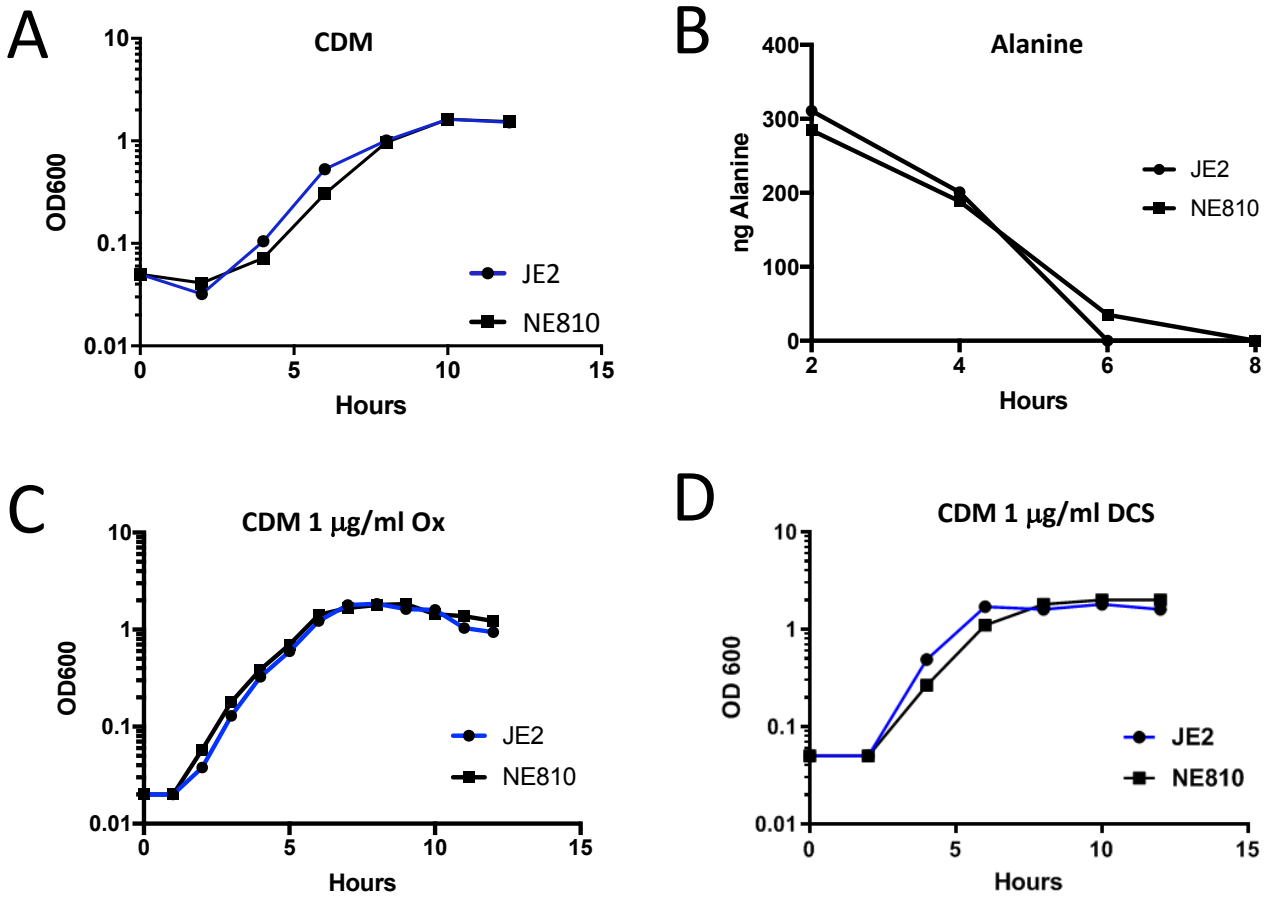

A
CDM
OD600
10
1
0.1
0.01
0
5
10
15
Hours
JE2
NE810
B
Alanine
ng Alanine
400
300
200
100
0
2
4
6
8
Hours
JE2
NE810
C
CDM 1 µg/ml Ox
OD600
10
1
0.1
0.01
0
5
10
15
Hours
JE2
NE810
D
CDM 1 µg/ml DCS
OD 600
10
1
0.1
0.01
0
5
10
15
Hours
JE2
NE810

Fig. 5

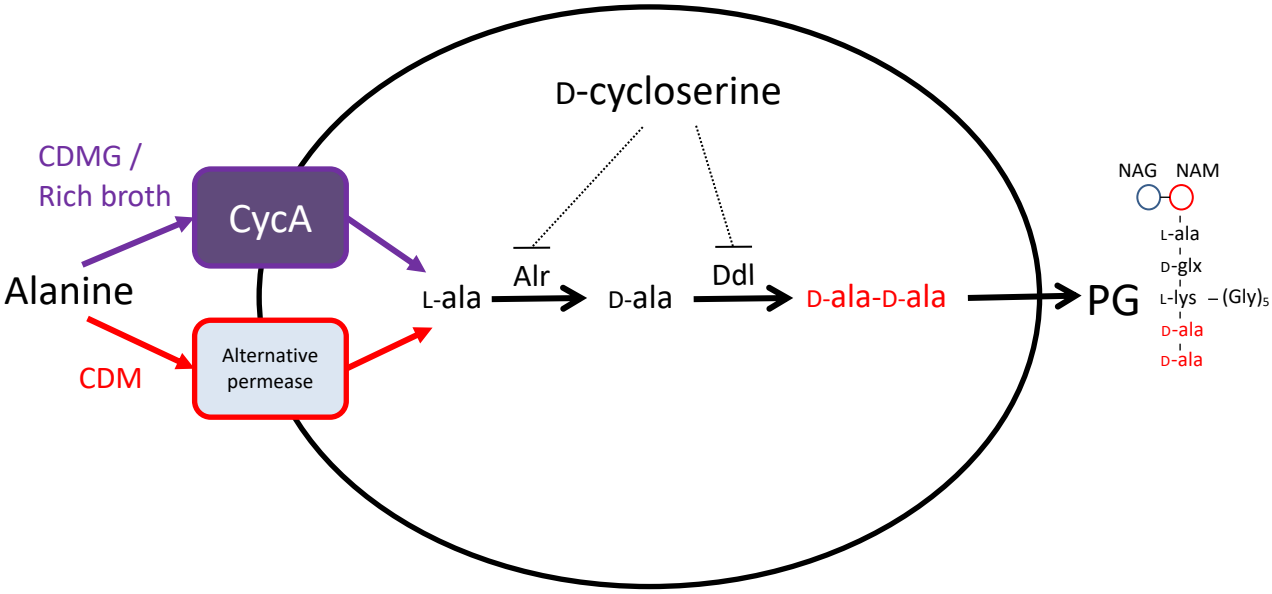

D-cycloserine
CDMG /
Rich broth
CycA
Alanine
CDM
Alternative
permease
L-ala
Alr
D-ala
Ddl
D-ala-D-ala
PG
NAG
NAM
L-ala
D-glx
L-lys
– (Gly)5
D-ala
D-ala

Fig. 6

SUPPLEMENTARY DATA

Impaired alanine transport or exposure to D-cycloserine increases the susceptibility of MRSA to β-lactam antibiotics

Laura A. Gallagher[1], Rebecca K. 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]*

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. 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 [43]. 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 [44]
COL	MRSA reference strain; SCC*mec* type I; CC8 [45]
BH14(04)	MRSA clinical isolate; SCC*mec* type IV; CC22 [44]
BH8(03)	MRSA clinical isolate; SCC*mec* type IV; CC22 [44]
BH6(03)	MRSA clinical isolate; SCC*mec* type II; CC8 [44]
DAR113	MRSA reference isolate; SCC*mec* type IV; CC22 [44, 46]
DAR13	MRSA reference isolate; SCC*mec* type IV; CC8 [44, 46]
DAR45	MRSA reference isolate; SCC*mec* type II; CC30 [44, 46]
DAR202	MRSA reference isolate; SCC*mec* type III; CC239 [44, 46]
DAR173	MRSA reference isolate; SCC*mec* type IV; CC5 [44, 46]
DAR173 *cycA*	DAR173 *cycA* mutant (*cycA*::Tn allele from NE810)
DAR22	MRSA reference isolate; SCC*mec* type III; CC5 [44, 46]
DAR22 *cycA*	DAR22 *cycA* mutant (*cycA*::Tn allele from NE810)
DAR169	MRSA Reference strain; SCCmec type I; CC8 [44, 46]
DAR169 *cycA*	DAR169 *cycA* mutant (*cycA*::Tn allele from NE810)
8325-4	NCTC 8325 derivative cured of prophages [47], 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. [21]
E. coli	*E. coli* HST08
Plasmids	
pLI50	*E. coli-Staphylococcus* shuttle vector. Ap^r (*E. coli*), Cm^r. (*Staphylococcus*)
p*cycA*	pLI50 carrying *cycA* from JE2

Table S3. 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

Supplementary Figures

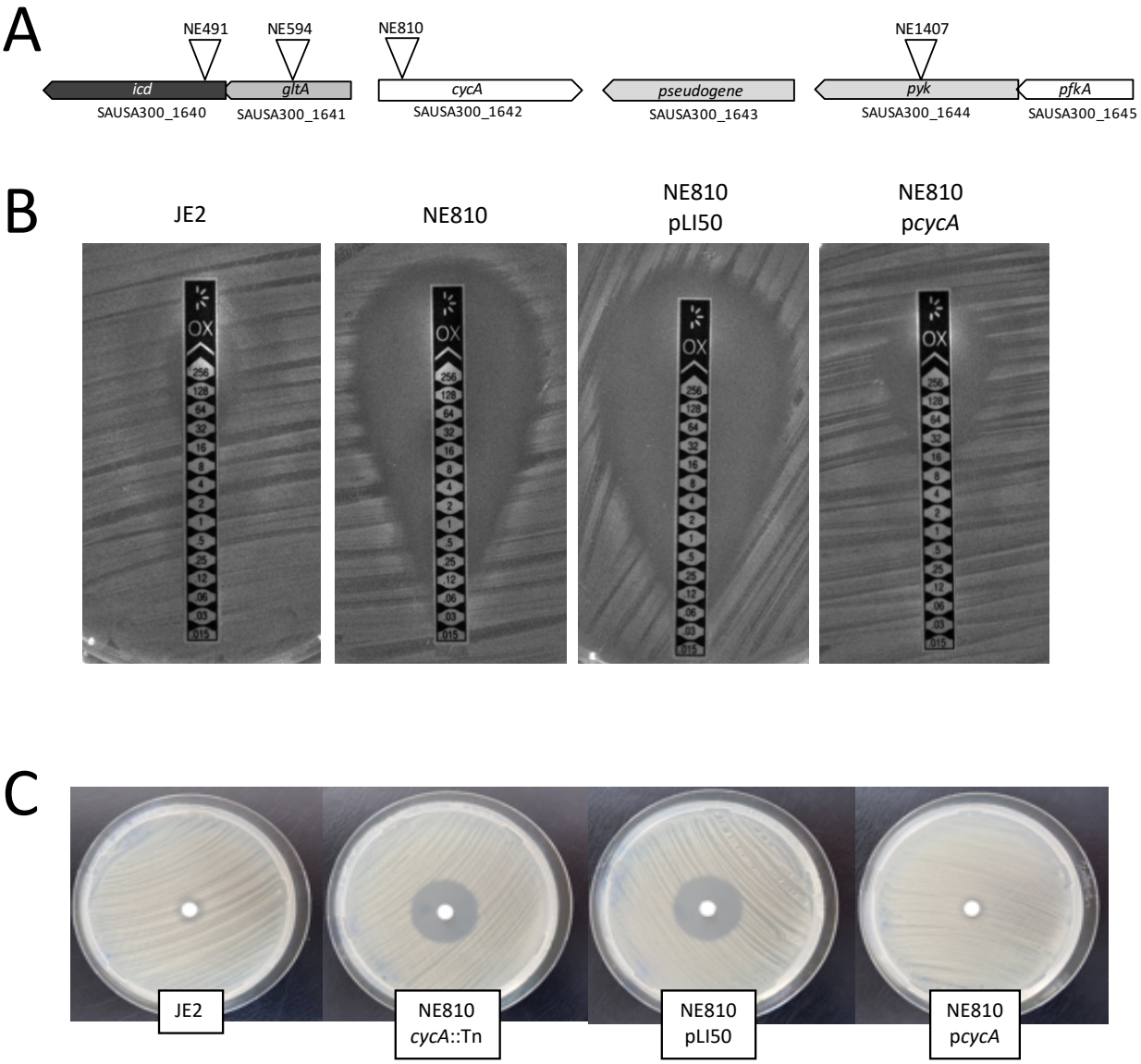


Figure S1. Mutation of *cycA* increases the susceptibility of MRSA to β-lactam antibiotics and D-cycloserine. A. Chromosomal location of *cycA* and neighbouring genes *icd* (isocitrate dehydrogenase), *gltA* (citrate synthase), *pyk* (pyruvate kinase) and *pfkA* (6-phosphofructokinase). The locations of transposon insertions in NE810, NE491, NE594 and NE1497 mutants from the Nebraska library are indicated. **B.** E-test measurement of oxacillin minimum inhibitory concentrations (MICs) in JE2 (wild type), NE810 (*cycA*::Tn), NE810 carrying pLI50 (control) and p*cycA*. **C.** Comparison of zones of inhibition around D-cycloserine 30µg disks on lawns of JE2, NE810 (*cycA*::Tn), NE810 pLI50 (control) and NE810 p*cycA* grown on Mueller Hinton (MH) agar.

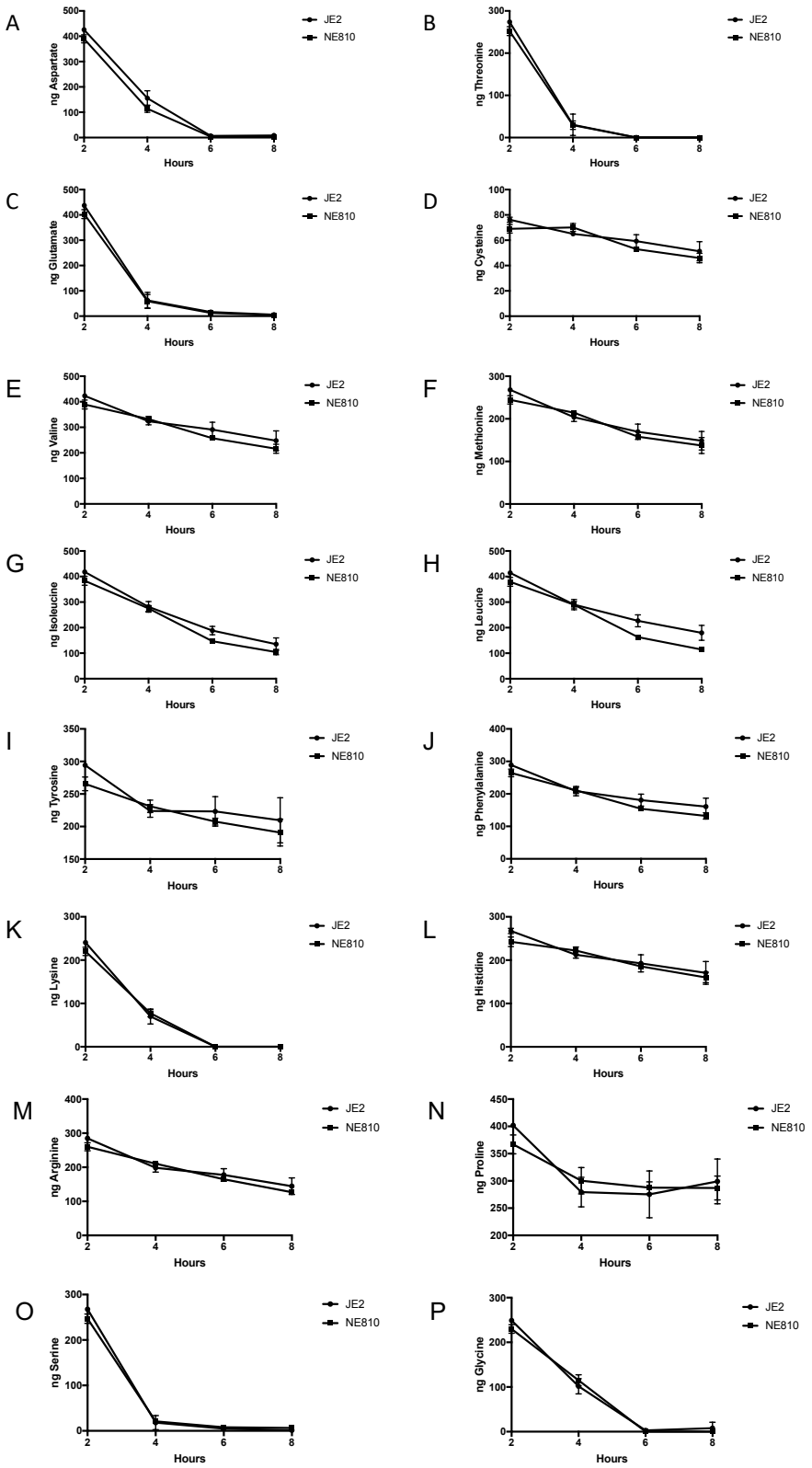


Figure S2. Amino acid consumption by JE2 and NE810 grown aerobically in chemically defined media containing 14mM of glucose (CDMG). Residual amino acids were measured in spent media after 0, 4, 6 and 8 h growth.

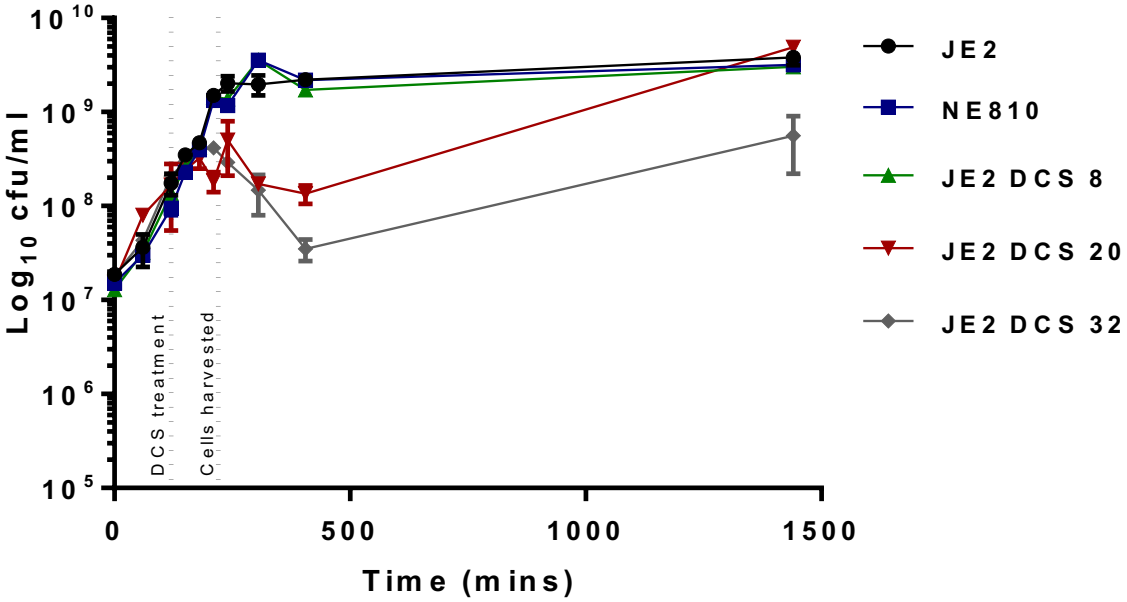


Figure S3. Preparation of cell suspensions for peptidoglycan extraction and structural analysis by UPLC-MS. 50 ml flask cultures were inoculated into fresh BHI media from overnight cultures at a starting cell density of A_{600}=0.05 and incubated at 37°C. The number of CFU/ml was enumerated every 1-2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

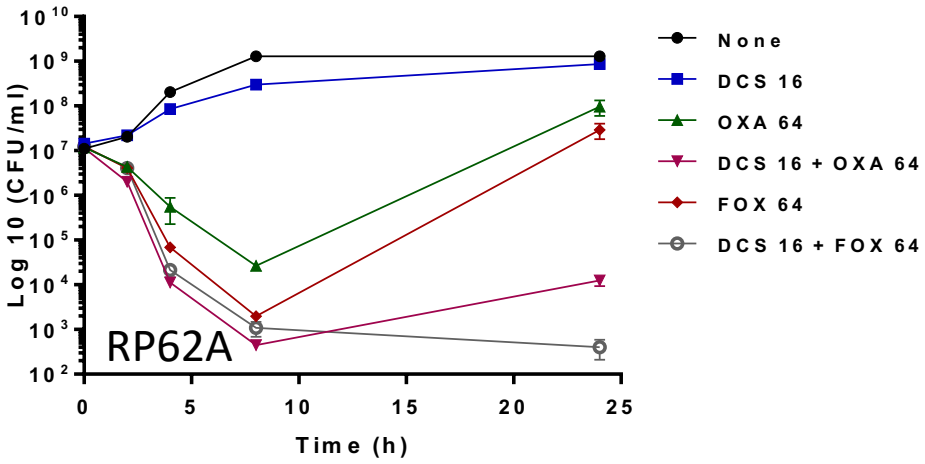


Figure S4. *In vitro* kill curves for D-cycloserine (DCS), oxacillin and cefoxitin with methicillin resistant *S. epidermidis strain* RP62A. Antibiotics at the concentrations indicated (equivalent to 0.5×MIC) were added to suspensions of overnight bacterial cultures adjusted to 10^7CFU/ml in BHI, incubated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

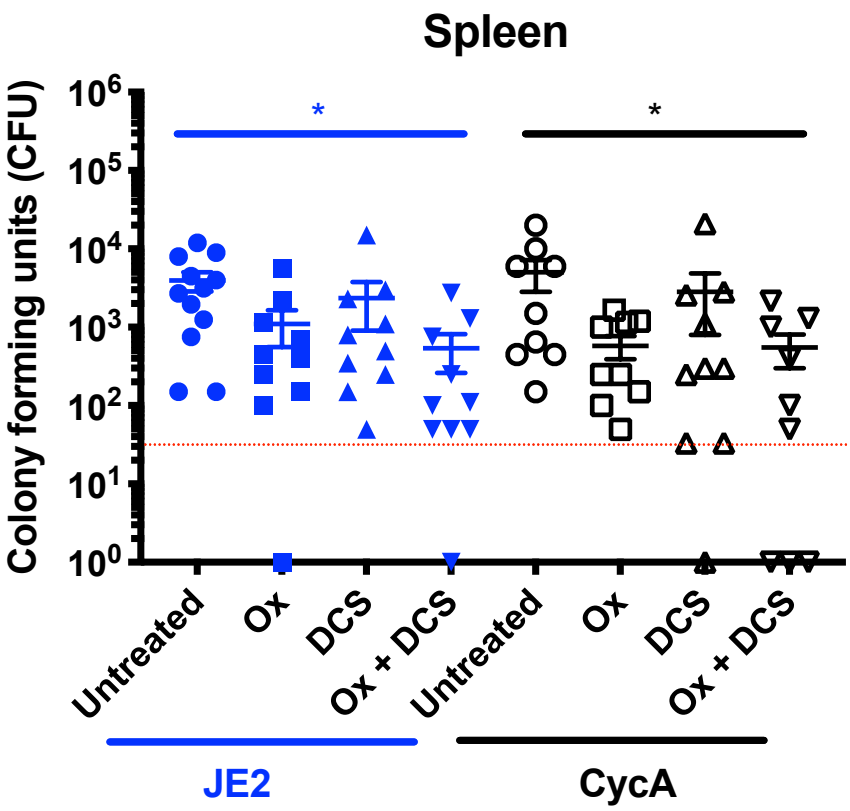


Figure S5. Combination therapy with D-cycloserine and oxacillin significantly reduces the bacterial burden in the spleen of mice infected with MRSA. The number of colony-forming units (CFU) recovered from the spleens of mice infected by tail vein injection with 5×10^6 JE2 or NE810 (CycA) and left untreated 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.

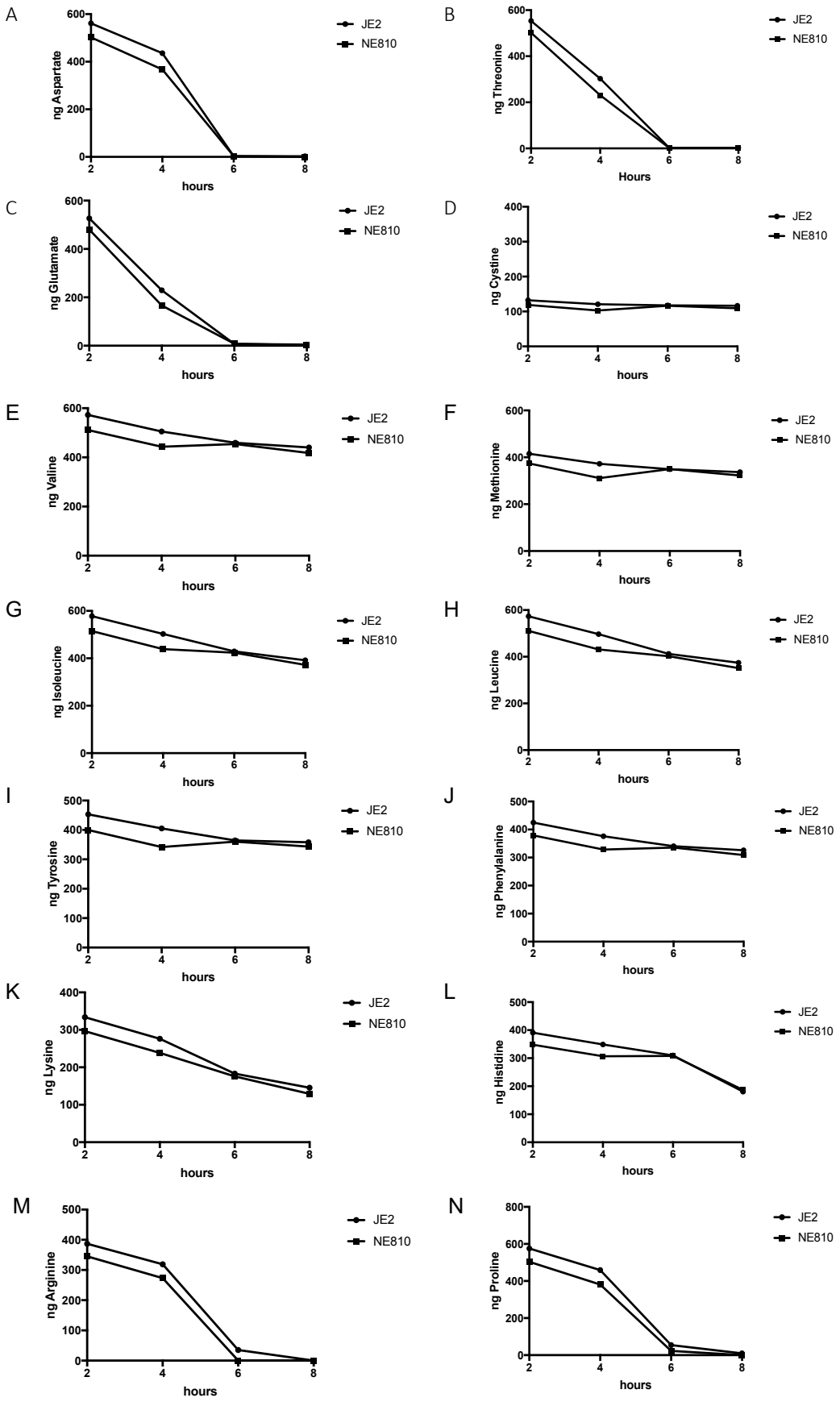


Figure S6. Amino acid consumption by JE2 and NE810 grown aerobically in chemically defined media lacking glucose(CDM). Residual amino acids were measured in spent media after 0, 4, 6 and 8 h growth.