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Tandem amplification of SCCmec can drive high level methicillin resistance in MRSA

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Key words: *Staphylococcus*; SCCmec; beta-lactam; antibiotic; resistance; mechanism

Running title: Amplification of SCCmec is a new mechanism of high level β -lactam resistance
in MRSA

24 **Abstract.** Hospital-associated methicillin-resistant *Staphylococcus aureus* strains typically
25 express high level, homogenous (HoR) β -lactam resistance, whereas community-associated
26 MRSA (CA-MRSA) more commonly express low level heterogeneous (HeR) resistance.
27 Expression of the HoR phenotype typically requires both increased expression of
28 the *mecA* gene, carried on the *Staphylococcus* cassette chromosome *SCCmec* element, and
29 additional mutational event(s) elsewhere on the chromosome. Here the oxacillin
30 concentration in a chemostat culture of the CA-MRSA strain USA300 was increased from 8
31 $\mu\text{g/ml}$ to 130 $\mu\text{g/ml}$ over 13 days to isolate highly oxacillin resistant derivatives. A stable,
32 small colony variant, designated HoR34, which had become established in the chemostat
33 culture was found to have acquired mutations in *gdpP*, *clpX*, *guaA* and *camS*. Closer
34 inspection of the genome sequence data further revealed that reads covering *SCCmec* were
35 ~ 10 times over-represented compared to other parts of the chromosome. qPCR confirmed
36 >10 -fold higher levels of *mecA* DNA on the HoR34 chromosome, and MinION genome
37 sequencing verified the presence of 10 tandem repeats of the *SCCmec* element. qPCR
38 further demonstrated that sub-culture of HoR34 in varying concentrations of oxacillin (0–
39 100 $\mu\text{g/ml}$) was accompanied by accordion-like contraction and amplification of the *SCCmec*
40 element. Although slower growing than USA300, HoR34 out-competed the parent strain in
41 the presence of sub-inhibitory oxacillin. These data identify tandem amplification of the
42 *SCCmec* element as a new mechanism of high-level methicillin resistance in MRSA, which
43 may provide a competitive advantage for MRSA under antibiotic selection.
44

45 **Introduction**

46 In recent decades, the overall incidence of methicillin resistant *Staphylococcus aureus*
47 infections has greatly increased due to the emergence of community-associated MRSA (CA-
48 MRSA), which are increasingly displacing hospital associated-MRSA (HA-MRSA) strains in
49 healthcare settings (1). Methicillin resistance is mediated by the *mecA*-encoded low affinity
50 penicillin binding protein 2a carried on the mobile *Staphylococcus* cassette chromosome
51 *mec* element (SCC*mec*). Heterogeneity is a feature of *S. aureus* methicillin resistance (2). In
52 general clinical CA-MRSA isolates exhibit low level, heterogeneous methicillin resistance
53 (HeR) under laboratory growth conditions, whereas HA-MRSA isolates can exhibit high-level,
54 homogeneous methicillin resistance (HoR). HeR strains can express a HoR phenotype after
55 selection on elevated concentrations of β -lactam antibiotics, via mechanism(s) involving the
56 stringent response and altered c-di-AMP signalling (2).

57 In general, the capacity of pathogens like MRSA to become resistant to new drugs only
58 becomes apparent months or years after their introduction into clinical practice, during
59 which time exposure of the pathogen to new drugs gradually increases, as does the
60 likelihood that endogenous resistance will emerge. This clinical scenario can be mimicked in
61 the laboratory using standard, batch culture techniques to isolate bacterial mutants
62 exhibiting resistance to an antimicrobial drug. However, such artificial culture conditions can
63 mask the impact of acquired antimicrobial resistance (AMR) on bacterial fitness (3, 4), a
64 phenomenon that plays a significant role in determining maintenance and spread of the
65 AMR genotype in natural bacterial populations, and affects the disease-causing capacity of
66 the pathogen. Here we used a continuous-growth chemostat to address this limitation by
67 creating a more dynamic and competitive environment from which to isolate
68 physiologically-relevant β -lactam resistant mutants. A USA300 culture was exposed to

69 increasing concentrations of oxacillin (8-130 $\mu\text{g/ml}$) over a thirteen-day period. Among the
70 hyper-resistant mutants isolated was a stable small colony variant in which the tandem
71 amplification of the *SCCmec* element was identified as a new mechanism of high-level β -
72 lactam resistance in MRSA.

73 **Results and discussion**

74 **Isolation of USA300 oxacillin hyper-resistant mutants.** A USA300 nutrient broth culture was
75 grown in a chemostat for 13 days. A sub-MIC concentration of oxacillin was used at the start
76 of the chemostat culture and increased on an incremental, daily basis up to 130 µg/ml
77 (equivalent to 800 µg/ml on Mueller Hinton, BHI or nutrient agar), as described in the
78 methods. Isolated hyper resistant mutants were readily differentiated into i) white coloured
79 small colony variants and ii) regular-sized, pigmented colonies (Fig. 1A). Using population
80 analysis profiling as described previously (5), all the mutants were shown to be
81 homogeneously resistant (HoR) (data not shown) and exhibited oxacillin MICs = 800 µg/ml.
82 Further analysis revealed that the small colony mutants appeared to be phenotypically
83 similar, exhibiting the same biofilm forming capacity and repressed β-haemolysis (data not
84 shown). In contrast the faster growing HoR mutants appeared to be heterogeneous,
85 exhibiting different levels of biofilm forming capacity and β-haemolytic activity on sheep
86 blood agar (data not shown). Whole genome sequencing further revealed a variety of
87 different mutations in nine HoR mutants recovered from the chemostat (Table 1). These
88 included four mutants with Ser₆₇Lys amino acid substitutions in DacA, the diadenylate
89 cyclase responsible for synthesis of c-di-AMP, which has previously been implicated in
90 the HoR phenotype (2, 6, 7), four mutants with five different mutations in genes
91 encoding predicted lipoproteins and one mutant with a Glu₂₂₇Gln substitution in a
92 predicted ABC transporter designated *abcA* (8). Mutation of the *abcA* gene has
93 previously been shown to increase β-lactam resistance and is associated with
94 upregulation of the adjacent *pbpD* gene, which encodes penicillin binding protein 4 (8).

95 In addition to a small colony size (Fig. 1A), impaired growth (Fig. 1B) and expression of
96 hyper-resistance to oxacillin, a representative SCV HoR, designated HoR34, also exhibited
97 altered cell morphology including defective septa formation (Fig. 1C) and an approximately
98 2-fold increase in cell wall thickness (18.6 ± 1.8 nm in USA300 versus 36.1 ± 4.2 nm in
99 HoR34)(Fig. 1D). Whole genome sequence analysis of HoR34 and the parent USA300 strain
100 compared to the publically-available USA300 FPR3757 genome, revealed that plasmid
101 pUSA02 (which carries tetracycline resistance) had been lost and identified non-
102 synonymous mutations in the *gdpP* (c-di-AMP phosphodiesterase (2, 7)), *guaA* (GMP
103 synthetase (9)), *clpX* (chaperone protein (10)) and *camS* (membrane lipoprotein (11)) genes
104 (Table 1). GdpP is an c-di-AMP phosphodiesterase responsible for turnover of c-di-AMP
105 synthesised by DacA, and has previously been implicated in the HoR phenotype (2, 7) but
106 not a small colony phenotype, which is clinically important in persistent infections (12).
107 Therefore to determine if the *guaA*, *clpX* or *camS* mutations (alone or in combination) were
108 involved in the small colony size of HoR34, the mutant was subjected to daily subculture in
109 the absence of antibiotic selection for 2 weeks in an effort to isolate fast-growing
110 revertants. The SCV phenotype of HoR34 was stable and no fast growing revertants were
111 isolated even after repeated attempts. However the oxacillin MIC of the passaged HoR34
112 strain, designated HoR34p, was reduced from 800 $\mu\text{g/ml}$ to 300 $\mu\text{g/ml}$, indicating that
113 although the strain continued to be hyper-resistant, oxacillin resistance levels in this strain
114 can be regulated.

115 To further tease out the contributions of the *guaA*, *clpX*, *camS* and *gdpP* mutations to the
116 HoR34 phenotypes, wild type alleles of the four genes, including their upstream promoter
117 sequences, were cloned on the medium copy number *E. coli-Staphylococcus* shuttle plasmid
118 pLI50 and introduced into HoR34. The multicopy *clpX* plasmid was unstable in HoR34 and

119 rapidly lost in the absence of antibiotic selection. Furthermore imposition of continuous
120 antibiotic selection for the *pcpX* plasmid in HoR34 appeared to be accompanied by the
121 selection of compensatory mutations, as evidenced by the rapid emergence of fast growing
122 colonies among the HoR34 small colony variants. Although we were unable to progress this
123 complementation experiment further, two previous studies have shown that mutation of
124 *clpX* is associated with increased resistance to β -lactam antibiotics (albeit not to the levels
125 measured in HoR34) (10, 13), suggesting that the *clpX* mutation may contribute in part to
126 increased oxacillin resistance in HoR34. The remaining complementation experiments
127 revealed that neither *gdpP*, nor the *camS* and *guaA* genes had any significant effect on the
128 colony morphology (data not shown) or oxacillin MIC of HoR34, as measured by Etest (Fig.
129 2A) and agar dilutions (data not shown). Furthermore, the doubling times for HoR34 (31.9
130 min), HoR34 *pguaA* (33.0 min), HoR34 *pgdpP* (31.7 min) and HoR34 *pcamS* (28.9 min) were
131 all substantially slower than USA300 (22.6 min) and HoR34 grown in Ox 0.5 μ g/ml (32.70
132 min), but not significantly different from each other indicating that the *guaA*, *camS* and
133 *gdpP* mutations alone did not affect growth rate. Because GdpP and c-di-AMP signalling also
134 contributes to the regulation of autolytic activity (2, 14), we further investigated this
135 phenotype. Consistent with previous studies, the *gdpP* mutation in HoR34 was associated
136 with increased autolytic activity that was successfully complemented only by *gdpP* and not
137 *camS* or *guaA* (Fig. 2B). The potential roles of the identified mutations in *guaA*, *clpX* and
138 *camS* in the HoR phenotype remain unclear but they may have emerged initially to support
139 growth or maintain fitness at relatively lower oxacillin concentrations during the early
140 stages of growth in the chemostat. It seems unlikely that the mutations in *camS*, *clpX* or
141 *guaA* are accompanied by any gain of function; the *clpX* and *camS* genes contain mutations
142 introducing stop codons (Table 1), while predicted loss of function mutations in *guaA* have

143 previously been implicated in the HoR phenotype (7). Taken together, these data suggest
144 that the mutations in *guaA*, *clpX*, *camS* and *gdpP*, at least on their own, are not responsible
145 for the HoR34 oxacillin hyper-resistance phenotype, and raised the possibility that other
146 genomic rearrangements were responsible for this phenotype.

147 **Chromosomal amplification of the SCCmec element in HoR34.** A number of recent studies
148 have indicated that large regions of the *S. aureus* chromosome can undergo duplication and
149 amplification events (15, 16). To investigate if such genomic rearrangements had taken
150 place in HoR34, read coverage across the genome was analysed. Illumina sequence reads
151 covering the SCCmec element were >10 times over-represented compared to other parts of
152 the chromosome (Fig. 3). LightCycler qPCR confirmed 10-fold higher levels of *mecA* in HoR34
153 gDNA samples compared to USA300 (data not shown). To determine whether the SCCmec
154 element had amplified on the chromosome or excised and re-integrated at multiple sites
155 around the chromosome, we attempted to assemble the Illumina sequence reads
156 corresponding to the SCCmec element into contigs. However these efforts were hampered
157 by the short reads. To address this we re-sequenced the HoR34 genome using MinION
158 technology, which generates sequence reads of 10Kb onto which the Illumina sequence
159 reads were mapped. The combined MinION/Illumina sequence data revealed the presence
160 of 10 tandem SCCmec element repeats on the HoR34 chromosome (Fig. 4). All 10 copies of
161 SCCmec were completely intact and no additional DNA sequences were identified at the join
162 sites. Oligonucleotide primers designed to span the join sites of tandem SCCmec elements
163 amplified PCR produced of the predicted size from HoR34 but not USA300, whereas control
164 primers targeting *mecA* amplified PCR products of the predicted size from both HoR34 and
165 USA300 (Fig. 5A).

166 **Stability of the SCCmec amplification event.** The reduction in oxacillin MIC in the HoR34
167 strain passaged in BHI media (from 800 to 300 $\mu\text{g}/\text{ml}$) indicated that the amplified SCCmec
168 elements may be unstable in the absence of antibiotic selection. To measure SCCmec copy
169 number, LightCycler qPCR was used to compare the relative abundance of *mecA* in HoR34
170 grown in the presence and absence of oxacillin. These experiments revealed that the *mecA*
171 copy number in the HoR34p strain that had been passaged daily in antibiotic-free BHI media
172 for 2 weeks (oxacillin MIC = 300 $\mu\text{g}/\text{ml}$), was only 3-fold higher than USA300 (Fig. 5B),
173 indicating that up to seven of the amplified SCCmec elements were excised/lost from the
174 original chemostat isolate during this time. Interestingly the doubling time of HoR34p (32.70
175 min) was not significantly different to that of HoR34 (31.9 min), indicating that a reduction
176 in the number of amplified SCCmec elements was not sufficient to alleviate the growth
177 defect. However further passage of HoR34p in 0.5, 64 and 100 $\mu\text{g}/\text{ml}$ oxacillin was
178 accompanied by a significant, concentration-dependent increase in *mecA* copy numbers (up
179 to 17-fold compared to USA300)(Fig. 5B), and an increase in MIC to ≥ 800 $\mu\text{g}/\text{ml}$. PCR
180 amplification and sequencing was used to confirm that the identified mutations in the *guaA*,
181 *gdpP*, *clpX* and *camS* genes of HoR34 had not reverted to wild type following passage in
182 antibiotic free media (data not shown). These data suggest that recombination between the
183 tandem SCCmec elements in HoR34 facilitates accordion-like contraction and expansion in
184 response to oxacillin exposure. Consistent with these qPCR data, Western blot analysis of
185 HoR34 grown in 0, 0.5, 64 and 100 $\mu\text{g}/\text{ml}$ oxacillin also revealed concentration-dependent
186 increases in PBP2a expression (Fig. 5C).

187 To investigate why a small colony variant may have been selected and maintained in the
188 chemostat, we performed competition experiments between USA300 and HoR34.
189 Predictably USA300 out-competed the slower-growing HoR34 in the absence of antibiotic

190 selection (Fig. 5D). However in the presence of sub-inhibitory oxacillin (0.5 $\mu\text{g/ml}$), HoR34
191 strongly outcompeted the wild type (Fig. 5D). Collectively these data identify tandem
192 amplification of the *SCCmec* element as a new mechanism of high-level methicillin
193 resistance in MRSA, which may provide a competitive advantage for MRSA under antibiotic
194 selection.

195 **Concluding remarks.** Several genetic mechanisms may have contributed alone or in
196 combination to the *SCCmec* amplification event in HoR34. Expression of the *ccr* recombinase
197 genes which excise *SCCmec* (17) can be increased by β -lactams and vancomycin (18),
198 potentially generating multiple, extrachromosomal copies of *SCCmec* capable of subsequent
199 reintegration. This possibility is supported by a recent study which identified a replication
200 initiator gene upstream of the *ccr* recombinase genes suggesting that the element may be
201 replicative (19). Other mechanisms that may have contributed to the *SCCmec* amplification,
202 alone or in combination with Ccr-mediated excision, include RecA-dependent non-equal
203 homologous recombination or RecA-independent mechanisms such as recombination
204 between single-stranded repetitive sequence on sister chromatids at the replication fork
205 (20). The absence of repeat sequences flanking the *SCCmec* amplification may also suggest
206 that an initial double-strand break (DSB), followed by RecA-dependent DSB repair during
207 rolling circle replication may drive the production of long tandem arrays in a single
208 generation, which have previously been implicated in fast adaption to drug treatment (21).
209 Following the initial *SCCmec* duplication/amplification, the long stretches of homology are
210 likely to facilitate RecA-mediated expansion and contraction of the element in different
211 concentrations of oxacillin, as recently observed in a *S. lugdenensis* strain carrying an
212 amplified *isd* locus (16). Recombination events leading to partial deletion of the *SCCmec*
213 locus have been described previously. For instance, increased vancomycin resistance has

214 been linked to site-specific insertion sequence-mediated excision of *SCCmec* (22), suggesting
215 that distinct RecA-independent mechanisms may favour high or low copy numbers of *mecA*
216 in high β -lactam or vancomycin environments, respectively.

217 Even though multiple copies of *SCCmec* were maintained by HoR34 following repeated
218 subculture in the absence of oxacillin selection, no evidence for *SCCmec* amplification was
219 found in a search of 404 MRSA genomes using read coverage of the *mecA* gene normalised
220 with read coverage of three single copy genes (data not shown). The clinical relevance of
221 this data merits further investigation, particularly given that β -lactams are not typically part
222 of the treatment regimen for MRSA infections. However, this may change in view of ongoing
223 clinical trials showing the therapeutic value of combining flucloxacillin and vancomycin for
224 the treatment of MRSA sepsis (23, 24). Our growth competition experiments revealed the
225 increased competitiveness of HoR34 in the presence of oxacillin was balanced by a
226 significant loss of competitiveness in the absence of antibiotic selection, suggesting that
227 MRSA strains carrying multiple *SCCmec* elements are unlikely to be maintained under
228 physiological conditions or in clinical environments where exposure to antibiotics is
229 sporadic. Taken together our data identify chromosomal amplification of the *SCCmec*
230 element as a new mechanism that may be used by MRSA to adapt to, and be more
231 competitive in, high oxacillin environments.

232 **Materials and Methods**

233 **Strains and culture conditions.** Strains used in this study are listed in Table 2 and were
234 grown at 37°C in LB (Sigma), BHI (Oxoid), Mueller Hinton (Oxoid) or nutrient (Oxoid) broth
235 supplemented with ampicillin (50 µg/ml), oxacillin (0.5, 64, 100 or 130 µg/ml),
236 chloramphenicol (10 µg/ml) or erythromycin (10 µg/ml) as indicated. *S. aureus* strains were
237 also grown on BHI agar media plates supplemented with oxacillin concentrations up to 1200
238 µg/ml.

239

240 **Measurement of oxacillin minimum inhibitory concentration (MIC).** The oxacillin MIC for
241 the *S. aureus* strains used in this study was determined in accordance with the Clinical
242 Laboratory Standards Institute (CLSI) guidelines and using E-tests strips from Biomerieux on
243 Mueller Hinton agar (Oxoid) containing 2% NaCl.

244

245 **Isolation of USA300 oxacillin hyper-resistant mutants using chemostat system.** The
246 community-associated CA-MRSA strain, USA300 FPR3757, which expresses a HeR
247 phenotype with an oxacillin minimum inhibitory concentration (MIC) on brain heart infusion
248 or nutrient agar of 32 µg/ml was used in this study. A 580 ml capacity laboratory reactor
249 containing 500 ml of nutrient broth (Oxoid) was used as described previously (25). USA300
250 was inoculated into the chemostat and allowed to grow to stationary phase for 2 days at
251 37°C in the absence of any antibiotic selection or media replacement. A growth media
252 reservoir containing 20 l of nutrient broth was then connected to the chemostat and fed to
253 the chemostat using a peristaltic pump at a flow rate of 100 ml/h, replacing the entire
254 nutrient broth volume of the chemostat every 5h. After 24 hours continuous culture growth
255 in the absence of antibiotic selection, the nutrient broth in the feeding tank was
256 supplemented with oxacillin at a concentration of 8 mg/l. Thereafter the oxacillin
257 concentration in the growth medium reservoir was increased in a step-wise manner every
258 day reaching a final concentration of 130 mg/l on Day 12. Culture samples were collected
259 aseptically from the chemostat after 24 hours culture at each oxacillin concentration before
260 being serially diluted and inoculated onto BHI agar supplemented with oxacillin 100 µg/ml.
261 The MICs of colonies recovered from these plates were determined on BHI agar
262 supplemented with oxacillin ranging from 100-1000 µg/ml. All isolates examined were

263 hyper-resistant and capable of robust growth on BHI agar supplemented with 800 µg/ml
264 oxacillin. Phenotypic and whole genome sequence analysis of the hyper-resistant mutants is
265 described in the supplementary methods.

266

267 **Haemolysis, biofilm and autolysis assays.** Beta haemolysis was assessed on BHI agar
268 supplemented with 5% sheep blood following overnight growth at 37°C and a further 24
269 hours at 4°C. Semi-quantitative measurements of biofilm formation were determined under
270 static conditions using Nunclon Hydrophilic tissue culture treated 96 well polystyrene plates
271 (Nunc, Denmark) as described previously (26). Triton X-100 induced autolysis was measured
272 essentially as described previously (27). Each experiment was repeated at least three times
273 and average data presented.

274

275 **Transmission Electron Microscopy (TEM).** Overnight BHI cultures were diluted 1:200 in
276 fresh BHI and grown at 37°C to an $A_{600} = 1.0$. 10 ml culture aliquots were subjected to
277 centrifugation at $8,000 \times g$, and the cell pellets were re-suspended in fixation solution (2.5%
278 glutaraldehyde in 0.1 M cacodylate buffer [pH 7.4]) and incubated overnight at 4°C. The
279 fixed cells were further treated with 2% osmium tetroxide, followed by 0.25% uranyl acetate
280 for contrast enhancement. The pellets were then dehydrated in increasing concentrations
281 of ethanol as described above for the SEM cell preparation, followed by pure propylene
282 oxide, and transferred to a series of resin and propylene oxide mixtures (50:50, 75:25, pure
283 resin) before being embedded in Epon resin. Thin sections were cut on an ultramicrotome.
284 Images were analysed using AMT v.542 software using a Hitachi H7000 instrument. At least
285 3 to 5 measurements of cell wall thickness were performed on each cell and 88 cells were
286 measured for each sample.

287

288 **PCR and Quantitative PCR.** Amplification of the *mecA* gene and the *SCCmec* junctions in
289 HoR34 was achieved using the following primers (Table 3): *mecA*_Fwd and *mecA*_Rev (for
290 *mecA*) and *SCCmec*JNFwd and *SCCmec*JnRev (for the *SCCmec* junctions). Quantitative PCR
291 (qPCR) for *mecA* was performed on the Roche LightCycler 480 instrument using the
292 LightCycler 480 Sybr Green Kit (Roche) and the following primers: *mecA*1_Fwd and
293 *mecA*2_Rev. Cycling conditions were 95 °C for 5 minutes and followed by 45 cycles of 95 °C
294 for 10 seconds, 58 °C for 20 seconds and 72°C for 20 seconds. Melt curve analysis was

295 performed at 95 °C for 5 seconds followed by 65 °C for one minute up to 97 °C at a ramp rate
296 of 0.11c/sec with five readings taken for every degree of temperature increase. The *gyrB*
297 gene was used as an internal standard for all reactions using previous described primers (2).
298 For each reaction, the ratio of *mecA* and *gyrB* transcript number was calculated as follows:
299 $2^{(Ct_{gyrB} - Ct_{mecA})}$. Each qPCR experiment was performed at least three times and average data
300 and standard errors are presented.

301
302 **Analysis of PBP2a expression:** Total cell protein preparations were prepared from overnight
303 cultures grown in 0, 0.5, 64 or 100 µg/ml oxacillin. Cell pellets were re-suspended in distilled
304 water containing 5 µg/ml lysostaphin, 10 units of DNase I, and 50 µl of 10% SDS before
305 being incubated at 37°C for 30 minutes. Insoluble material was pelleted by centrifugation
306 and the supernatant used for Western blotting. Protein concentration was assessed using
307 the Pierce BCA protein assay kit (Thermo Scientific). Protein samples were separated on a
308 10% SDS gel (Thermo Scientific) and transferred to nitrocellulose membranes (Thermo
309 Scientific) using a TE 70 semidry transfer unit (Amersham). Anti-PBP2a antibodies (Abnova)
310 were used at a 1:2000 dilution. A 1:200 dilution of protein G-horseradish peroxidase (HRP)
311 conjugate (Sigma) was used to detect bound antibody and visualisation was achieved using
312 a colorimetric detection system (Bio-Rad).

313
314 **Complementation of HoR34 with *gdpP*, *guaA*, *camS* and *clpX*.** The *gdpP*, *guaA*, *camS* and
315 *clpX* genes were amplified from USA300 genomic DNA by PCR using primers listed in Table
316 2, before being cloned into the cloning vector pDrive (Quigen) in *Escherichia coli* TOP10. The
317 sequence of inserts in recombinant plasmids was verified by Sanger sequencing (Source
318 Biosciences) before being subcloned on *EcoRI* or *BamHI/HindIII* restriction fragments into
319 the *E. coli* - *Staphylococcus* shuttle plasmid pLI50. The plasmids were transformed by
320 electroporation into the restriction-deficient strain RN4220, and subsequently into HoR34.
321 All plasmid-harboring strains were cultured in medium supplemented with 100 µg/ml
322 ampicillin (*E. coli*) or 10 µg/ml chloramphenicol (*S. aureus*) for plasmid selection.

323
324 **Growth competition experiments.** Overnight cultures of USA300 and HoR34 cultures were
325 diluted to $A_{600} = 0.05$ in fresh BHI media and grown for 6h. The cell density of both
326 exponential phase cultures was adjusted to $A_{600} = 0.1$ in 500 ml flasks containing 50ml BHI or
327 BHI supplemented with 0.5 µg/ml oxacillin and incubated at 37°C with shaking. The number

328 of colony forming units in samples collected at 0, 2, 4, 8, 24 and 48h was determined by
329 plating serial dilutions on BHI agar. Colonies formed by each strain were readily
330 differentiated based on their tetracycline resistance and appearance i.e. the HoR34 colonies
331 were tetracycline sensitive and had a white-coloured, small colony phenotype whereas
332 USA300 colonies were regular sized, tetracycline resistant and pigmented.

333

334 **Statistical analysis.** Two-tailed, two-sample equal variance Student's t-Tests were used to
335 determine statistically significant differences in assays performed during this study. A *P*
336 value <0.05 was deemed significant.

337

338 **Quality control of genome sequence data.** Read quality was assessed by screening the read
339 length, nucleotide and quality score distributions using FastQC
340 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and the FASTX-Toolkit
341 (http://hannonlab.cshl.edu/fastx_toolkit/index.html). The DNA reads were trimmed based
342 on quality scores. Potential adaptor sequence was removed using Trimmomatic v0.32 (28),
343 which scanned reads using a four-base sliding window and trimmed reads where the
344 average Phred base quality of the window was below 30. All ambiguous 'N' bases and reads
345 shorter than 35 bp were removed. The first 20 bases of the DNA reads were removed
346 because they had a nucleotide content that deviated from the expected 25% rate for each
347 base. The DNA reads were corrected using BayesHammer (29) to reduce sequencing errors
348 that can reduce the alignment quality, increase false positive SNP rates and reduce the
349 number of valid SNPs (30). These steps retained 84% of the initial DNA reads among HoR
350 isolates from the chemostat yielding median quality values > 30 across the reads. Insert
351 sizes were an average of 185. Read lengths after trimming and filtering averaged 185 bp and
352 the average coverage per sample on the chromosome, calculated using the Bedtools
353 `genomecov` function (31) on mapped reads, ranged from 47 to 197.

354 **Genome assembly.** The error-corrected paired and unpaired reads for each DNA sample
355 were assembled using SPAdes v3.1.1 [5] with k-mers 21, 33, 55, 77, 99 and 127 and the
356 'careful' parameter, which minimized the number of mismatches in the contigs (32). The
357 resulting assemblies were compared to the reference USA300_FPR3757 (PMID:16517273)
358 chromosome using QUAST v2.3 (33). The GC content of each assembly was 32.6%, and there

359 were between 31 and 51 scaffolds per assembly, with N50 values > 200 Kb. One or two
360 short gaps (<500 bp) were found in each assembly that could not be fully closed using
361 Gapfiller (34).

362 **Single nucleotide polymorphism (SNP) calling using assembly and read-mapping.** The
363 chromosome and three plasmids (GenBank accessions NC_007790-NC_007793) were
364 indexed with *k*-mer of thirteen and step size of two using SMALT v5.7
365 (<http://www.sanger.ac.uk/science/tools/smalt-0>). The error-corrected DNA reads were
366 mapped to the genome with SMALT, which applied a Smith-Waterman sequence alignment
367 algorithm. The SAM (sequence alignment/map) files were converted to BAM (binary
368 alignment/map) files using Samtools v0.1.18 (35). The BAM files were then coordinate-
369 sorted, the paired and unpaired files were merged, and PCR duplicate reads were removed.
370 Candidate SNPs were detected where the base quality (BQ) was >25, the mapping quality
371 (MQ) was >30, and the read depth was <100 using Samtools Mpileup v0.1.18, Bcftools
372 v0.1.17-dev, and the Samtools v0.1.11 vcfutils.pl function. The read depth allele frequency
373 of the non-reference allele (RDAF) and local coverage were estimated using Samtools Pileup
374 v0.1.11.

375 To call SNPs using an assembly-based approach, the scaffolds produced by SPAdes were
376 aligned to the USA300 reference genome using nucmer in the MUMmer v3.23 package. This
377 was followed by eliminating conflicting repeat copies using the 'delta-filter' command and
378 the 'show-snps' command to call SNPs and indels. The union of SNPs called by nucmer and
379 SNPs called by Bcftools was used as a candidate SNP set. These sites were queried across all
380 samples using the Samtools Pileup files to find false negative SNPs uncalled by nucmer or
381 Bcftools. The RDAF of the non-reference alleles was reported for each SNP using Samtools
382 Pileup output. Each candidate SNP was assessed using the following additional criteria:

383

- 384 1) SNP Quality (SQ) >30
- 385 2) read coverage >5
- 386 3) forward-reverse read coverage ratio between 0.1 and 0.9
- 387 4) non-reference read allele frequency >0.1
- 388 5) 2+ forward reads

389 6) 2+ reverse reads

390 Results were converted to variant call format (VCF) and annotated. SNPs were homozygous
391 if the RDAF was ≥ 0.85 and heterozygous if $0.1 < \text{RDAF} < 0.85$. Insufficient read depth
392 coverage was present to predict SNPs with $\text{RDAF} < 0.1$.

393 **Indel calling using split-read mapping.** Deletions and short insertions (indels) were called
394 using the samtopindel script to convert the BAM files, and then with Pindel (36) to only keep
395 indels with at least ten supporting reads. The RDAF of the indels smaller than the read
396 length were calculated using the BAM files in IGV (number of reads with indel at locus / all
397 reads at the locus). For indels greater than one bp in length, the sum of the number of reads
398 with the indel was divided by the sum of the number of reads at each site in the indel. This
399 approach may be limited by uneven coverage at a locus. If the indel was longer than the
400 read length, then a lack of read coverage at the sites predicted to have the mutation was
401 considered evidence of the deletion and the RDAF was set to one.

402 **Variant annotation.** The functional effect of SNPs and indels was estimated by annotation
403 with SnpEff v4.0e (37) using the 'Staphylococcus_aureus_USA300_FPR3757_uid58555'
404 database file from the SnpEff database. Results were manually checked using the reference
405 genome annotation.

406 **Copy number variation detection using read coverage.** Copy number variants (CNVs) were
407 screened using the BAM files containing reads with $\text{MQ} > 30$ to reduce false positive rates
408 [12–14]. Coverage was calculated for every base using genomecov in Bedtools with the '-d'
409 flag (31) so that the median chromosomal coverage could be calculated for each sample.
410 Genome-wide coverage levels were analysed in 10 Kb and 25 Kb windows and plotted as 5
411 Kb sliding windows with a 2.5 Kb step using the Bedtools makewindows function (31).
412 Coverage for each window was normalised by dividing it by the median coverage of the
413 chromosome to produce a copy number estimate. Windows with copy number ≥ 2 were
414 reported. The copy number of plasmids was determined by dividing the median read
415 coverage of the plasmid by the median read coverage of the chromosome.

416 **MinION long-read genome sequencing.** To evaluate the number of SCCmec copies and their
417 location contiguous with or excised from the chromosome, genomic DNA from HoR34 was

418 amplified to generate long reads using a Oxford Nanopore Technologies (ONT) MinION.
419 MinION sequencing library construction was carried out according to manufacturer's
420 instructions and as previously described (38). The library was sequenced on an R7.3 MinION
421 flowcell using the 2D sequencing protocol. The run produced 26859 FAST5 files, which were
422 processed using poRe (39), yielding 17254 2D reads. These reads were used with the MiSeq
423 data in a hybrid assembly using SPAdes (32) and SSPACE-LongRead (40) to produce a single
424 contig.

425

426

427

428 **Figure Legends**

429

430 **Figure 1. Growth and cell morphology phenotypes of USA300 and HoR34.** **A.** Small colony
431 variants and other isolates recovered from the chemostat culture after 13 days at a final
432 oxacillin concentration of 130 mg/l grown on BHI agar for 24 h. **B.** Growth curve of USA300
433 and HoR34 grown for 20 hours in BHI media at 37°C with vigorous aeration. The number of
434 colony forming units per ml in culture samples removed at regular intervals was determined
435 by plating on BHI agar. **C.** Cell morphology of USA300 and HoR34 imaged using transmission
436 electron microscopy (TEM) at 8,000× magnification. **D.** Cell wall thickness of USA300 and
437 HoR34 determined using TEM at 100,000× magnification and AMT v.542 imaging software.

438

439 **Figure 2. Oxacillin susceptibility and autolysis phenotypes of USA300 and HoR34.** **A.**
440 Oxacillin MIC of USA300, HoR34 and HoR34 carrying plasmids pLI50 (control), *pgdpP*, *pguaA*
441 and *pcamS* determined using Etests. **B.** Autolytic activity in USA300 and HoR34. USA300,
442 HoR34, HoR34 carrying plasmids pLI50 (control), *pgdpP*, *pguaA* and *pcamS*, and a USA300
443 JE2 *atl* mutant (negative control) were grown to early exponential phase in BHI at 37°C and
444 washed in PBS and adjusted to $A_{600} = 1.0$ in 0.01% Triton X-100. The A_{600} was measured
445 initially and at 15 min intervals thereafter with shaking incubation at 37°C. Autolytic activity
446 is expressed as a percentage of the initial A_{600} . Average results from three independent
447 experiments shown.

448

449 **Figure 3.** Copy number as determined by Illumina sequence read coverage across *SCCmec*
450 for USA300 (Sample 1A_S1), HoR34 (Sample 8A_S8, highlighted with blue box) and eight
451 other isolates from the chemostat culture. The position on the chromosome is indicated,
452 with *SCCmec* coordinates between 0.034 and 0.057 Mb. The blue lines depict locally
453 weighted scatterplot smoothing (lowess) applied to the data points (black). Note that the y-
454 axis for HoR34 differs from the other samples.

455

456 **Figure 4.** Chromosomal organisation of HoR34 depicting expansion of the *SCCmec* element
457 and locations of *gdpP*, *clpX*, *camS* and *guaA* mutations. On the circular map, the inner track
458 shows copy number of 10 kb non-overlapping loci across the genome with loci that had
459 copy number greater than two shown in red and those with copy number less than two

460 shown in blue. The next track shows black blocks illustrating different regions on the
461 genome e.g. *SCCmec* and *ACME*. Single nucleotide polymorphisms are shown on the third
462 track. Missense mutations are labelled in green whereas stop gain mutations are labelled in
463 blue. Genes are shown in the outermost tracks. Genes transcribed in the forward (5' -> 3')
464 direction are labelled in green and are in the outside track whereas those transcribed in the
465 reverse direction are labelled in red.

466

467 **Figure 5. Chromosomal amplification of *SCCmec* can drive high level oxacillin resistance. A.**

468 PCR amplification across the *SCCmec* junctions in HoR34. Amplification of the *mecA* gene in
469 both USA300 and HoR34 was used as a control. **B.** Comparison of relative *mecA* abundance
470 by LightCycler qPCR in USA300 and HoR34 grown for 24 h in BHI supplemented with 0, 0.5,
471 64 or 100 mg/ml oxacillin. **C.** Comparison of relative PBP2a expression by Western blot
472 analysis in USA300 and HoR34 grown in BHI and BHI supplemented with 0, 0.5, 64 or 100
473 mg/ml oxacillin. **D.** Competitive growth of USA300 and HoR34 over 48 hours in BHI and BHI
474 supplemented with oxacillin (0.5 mg/ml). The CFU of each strain was enumerated on BHI
475 agar to count all bacteria and BHI oxacillin (30 mg/ml) to count HoR34. The ratio of the two
476 strains in each culture is shown. The data presented are mean and SD of three experiments.
477 Statistical evaluation was performed using a paired two tailed t-test.

478

479

480

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483 Fingleton for assistance with the chemostat experiment and analysis of HoR34.

484 **Table 1. Genetic alterations in USA300 oxacillin hyper-resistant mutants from the**
 485 **chemostat culture**

Isolate, growth characteristic	Genome position	Nucleotide change	Amino acid change	Locus tag/gene
HoR20, fast-growing	703854	G-C	Glu ₂₂₇ Gln	RS03375/ <i>abcA</i>
HoR18, 21, 27, 36; fast-growing	110748, 110752,111618, 111630, 111648	Multiple	Multiple	RS00520-RS00525 /uncharacterized lipoprotein genes
HoR33, 41, 43, 46; fast-growing	2288896	G-A	Ser ₆₇ Lys	RS11640/ <i>dacA</i>
HoR34, slow-growing	19122	A-C	Thr ₂₆₀ Pro	<i>gdpP</i> (c-di-AMP phosphodiesterase (14))
	44078	C-T	Ala ₂₁₄ Val	<i>guaA</i> (GMP synthetase (9))
	441379	G-T	Glu ₅₁₁ Asp	<i>guaA</i>
	1775825	C-A	Glu ₃₇ STOP	<i>clpX</i> (Chaperone with ClpP-dependent role in protein degradation and ClpP-independent role in protein folding (10))
	2046530	G-A	Gln ₃₀₅ STOP	<i>camS</i> (membrane lipoprotein involve in sex pheromone biosynthesis (11))

486

487

488 **Table 2. Bacterial strains and plasmids used in this study**

Strains/plasmids	Relevant Details
<i>S. aureus</i>	
RN4220	Restriction-deficient laboratory <i>S. aureus</i> .
USA300	CA-MRSA expressing heterogeneous resistance to oxacillin
HoR34	USA300 derivative expressing high level resistance to oxacillin
ATCC 29213	MSSA strain for susceptibility testing
JE2 <i>atl::erm</i>	Transposon mutation in the major autolysin gene <i>atl</i> of strain JE2, a USA300 derivative used in the construction of the Nebraska Transposon mutant library (41). Exhibits impaired autolytic activity.
<i>E. coli</i>	<i>E. coli</i> Top10 cloning strain.
Plasmids	
pLI50	<i>E. coli-Staphylococcus</i> shuttle vector. Ap ^r (<i>E. coli</i>), Cm ^r (<i>Staphylococcus</i>).
pDrive	<i>E. coli</i> cloning vector

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491

492

493 **Table 3. Oligonucleotide primers used in this study**

Target Gene	Primer Name	Primer Sequence (5'-3')
<i>gdpP</i>	<i>gdpP_Fwd</i>	GCCGAATGCAGTAACGATTT
	<i>gdpP_Rev</i>	TTGTTGGCGTTCTTGTTTTG
<i>guaA</i>	<i>guaA_Fwd</i>	AGAGGACAAAGCGCCTAAGA
	<i>guaA_Rev</i>	CCTTACCCTTTTCCGCCT
<i>clpX</i>	<i>clpX_Fwd</i>	AACGCAAAGTTCGTTGAAGG
	<i>clpX_Rev</i>	TGAGCGTCAACTTGATTGG
<i>camS</i>	<i>camS_Fwd</i>	GCTGGTGAAGATGCAGTTT
	<i>camS_Rev</i>	CCTGGTGCATTTGTTGAACTG
<i>mecA</i>	<i>mecA_Fwd</i>	CATATCGTGAGCAATGAACTGA
	<i>mecA_Rev</i>	CATCGTTACGGATTGCTTCA
SCC <i>mec</i> Junction	SCC <i>mec</i> In_Fwd	CTTGCTGGGTGCTATTTGA
	SCC <i>mec</i> In_Rev	CGCTGTCTTCTGTATTTCTG
<i>mecA</i>	<i>mecA1_Fwd</i>	TGCTCAATATAAAATTAACAAACTACGGTAAC
	<i>mecA1_Rev</i>	GAATAATGACGCTATGATCCCAA
<i>gyrB</i>	<i>gyrB_Fwd</i>	CCAGGTAATTAGCCGATTGC
	<i>gyrB_Rev</i>	AAATCGCCTGCGTTCTAGAG

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630

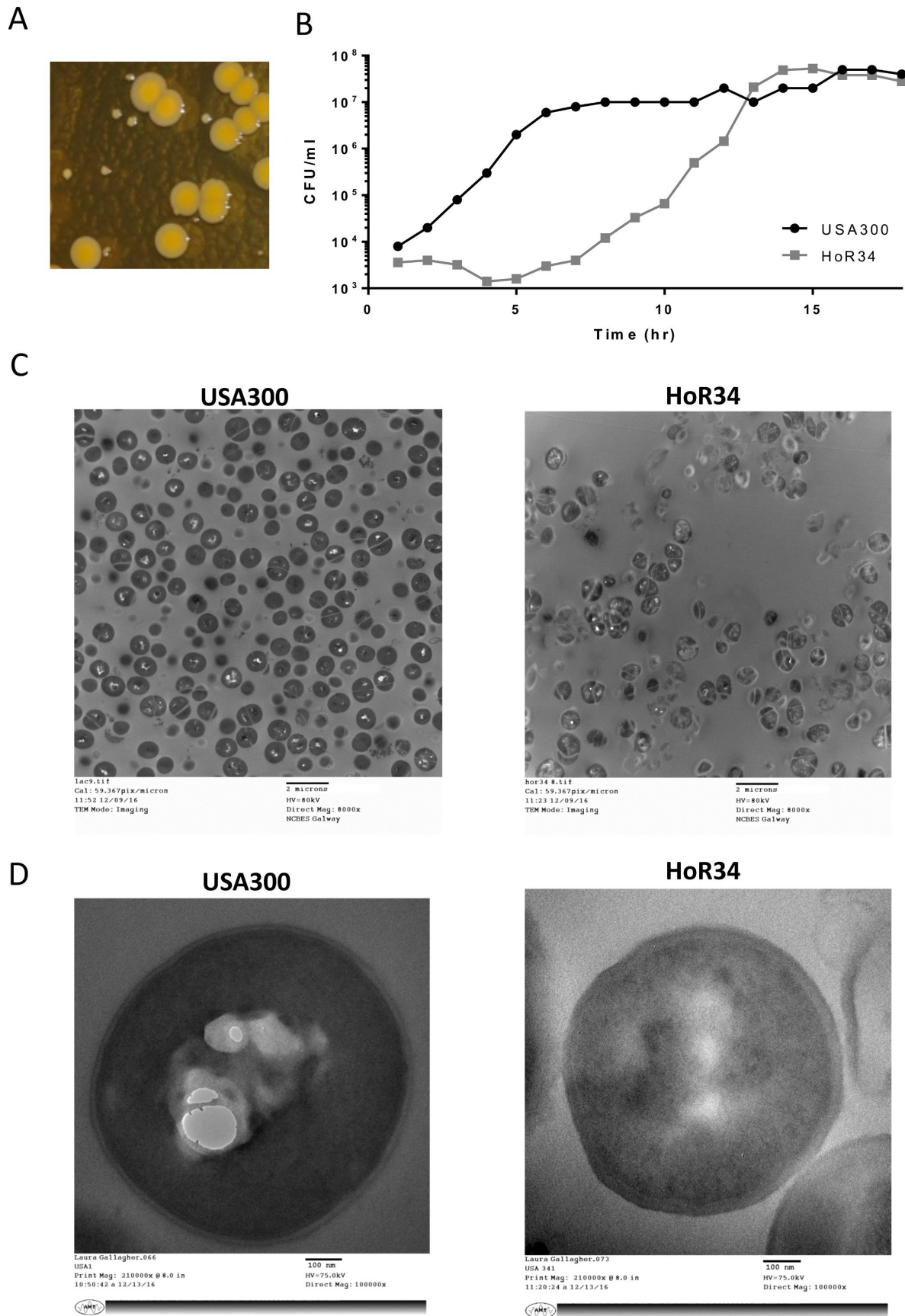


Fig. 1

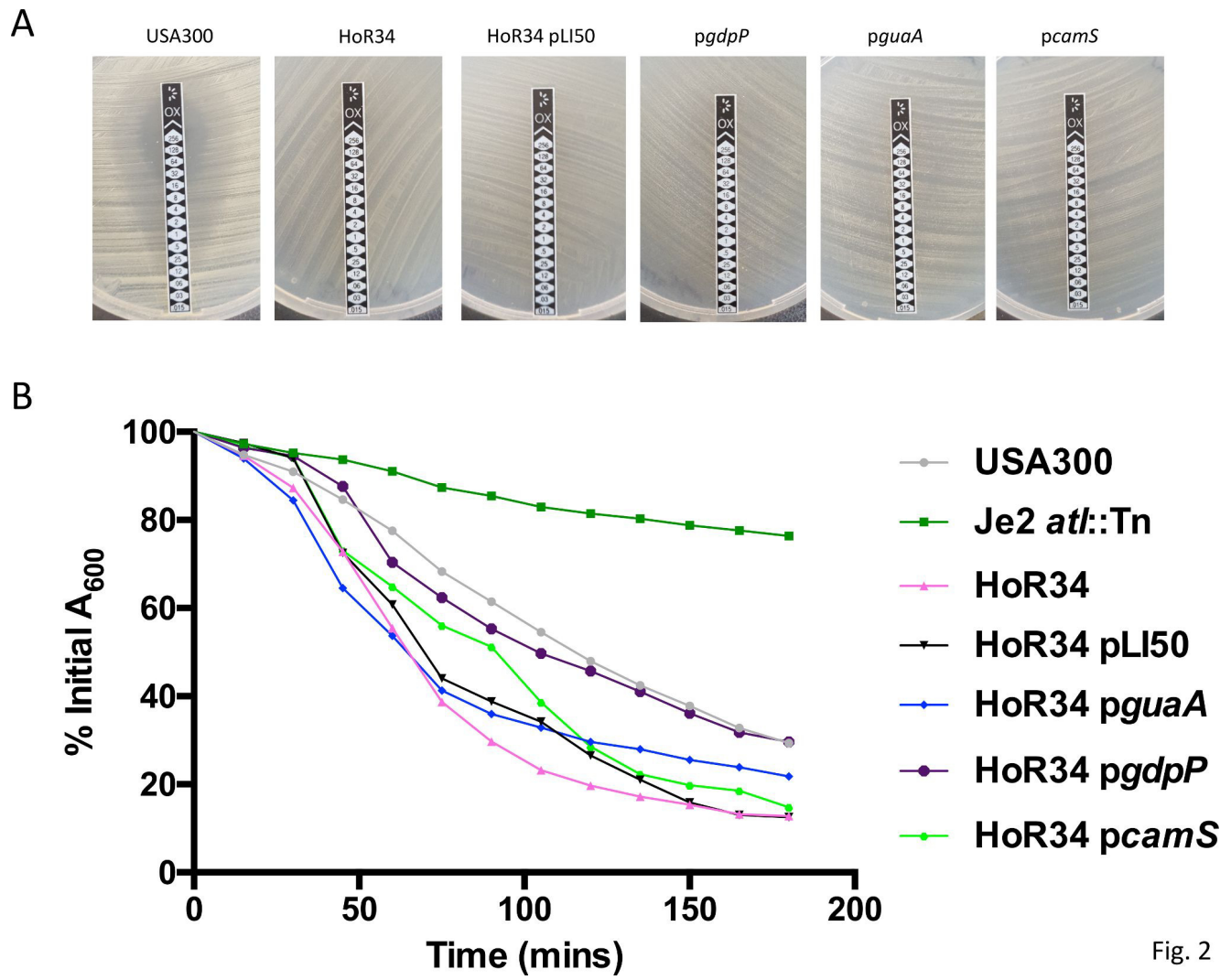


Fig. 2

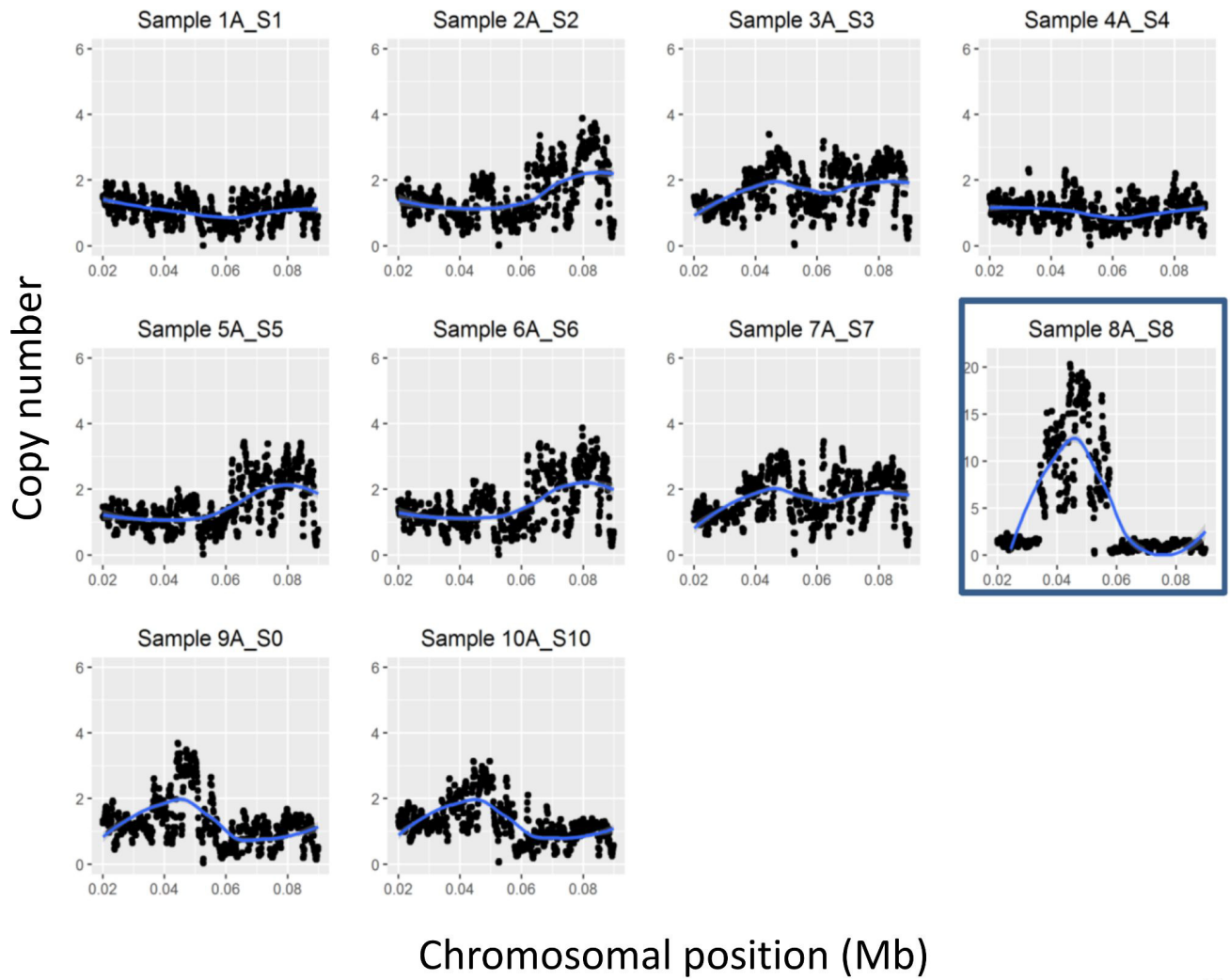


Fig. 3

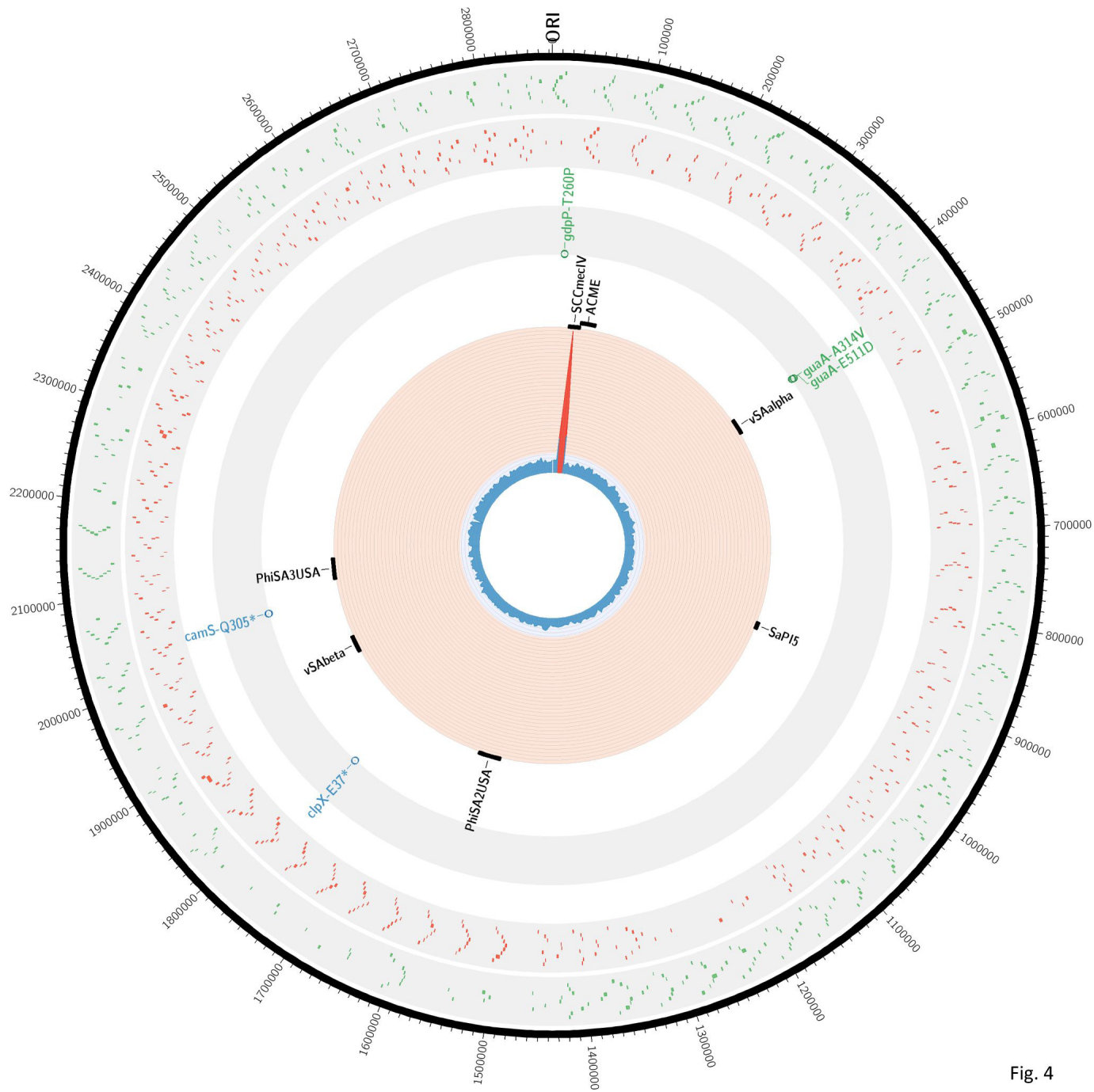


Fig. 4

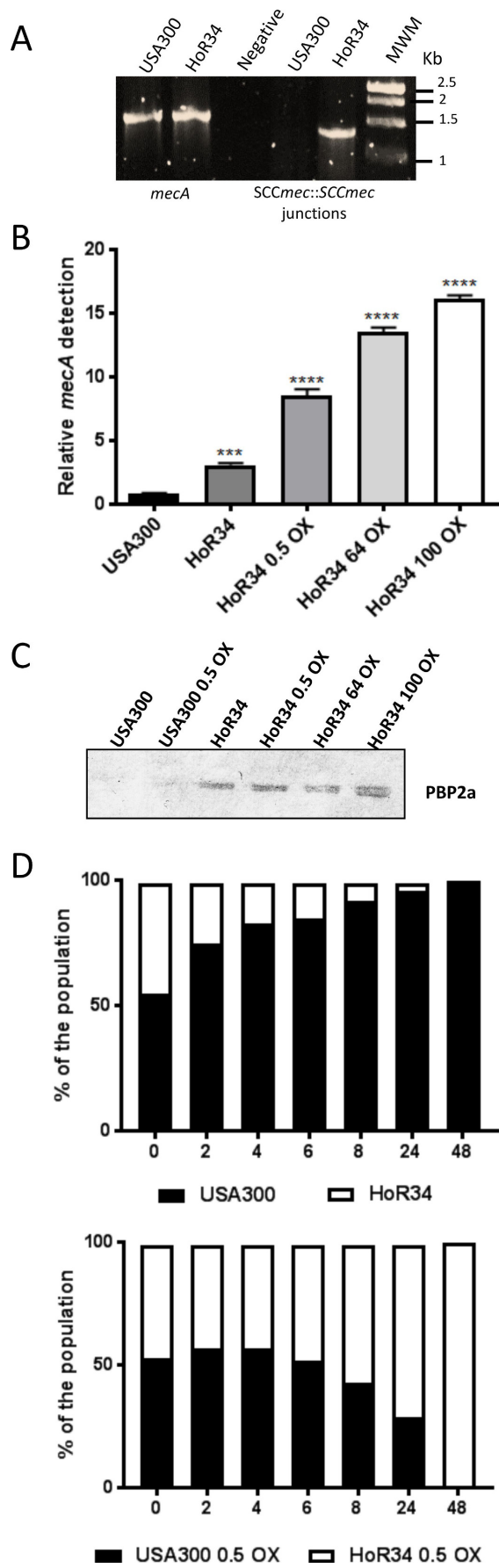


Fig. 5