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Demographic fluctuation of community-acquired antibiotic-resistant *Staphylococcus aureus* lineages: potential role of flimsy antibiotic exposure

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Running Title: Antibiotic exposure increases CA-MRSA fitness

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29

30 **ABSTRACT**

31 Community-acquired (CA) –as opposed to hospital acquired- methicillin-resistant
32 *Staphylococcus aureus* (MRSA) lineages arose worldwide during the 1990s. To determine which
33 factors, including selective antibiotic pressure, govern the expansion of two major lineages of
34 CA-MRSA, namely “USA300” in Northern America and the “European ST80” in North Africa,
35 Europe and the Middle East, we explored virulence factor expression, and fitness levels with or
36 without antibiotics. The sampled strains were collected in a temporal window representing
37 various steps of the epidemics, reflecting predicted effective population size as inferred from
38 whole genome analysis. In addition to slight variations in virulence factor expression and biofilm
39 production that might influence the ecological niches of these lineages, competitive fitness
40 experiments revealed that the biological cost of resistance to methicillin, fusidic-acid and
41 fluoroquinolone is totally reversed in the presence of trace amount of antibiotics. Our results

suggest that low-level antibiotics exposure in human and animal environments contributed to the expansion of both European-ST80 and USA300 lineages in community setting. This surge was likely driven by antibiotic (ab)use promoting the accumulation of antibiotics as environmental pollutants. The current results provide a novel link between effective population size increase of a pathogen and a selective advantage conferred by antibiotic resistance.

INTRODUCTION

Staphylococcus aureus remains one of the most common causative agents of both nosocomial and community-acquired infections. It colonizes asymptomatically about one third of the human population and may cause infections with outcomes ranging from mild to life-threatening (Lowy, 1998). Until the mid-1990's, methicillin-resistant *S. aureus* (MRSA) infections were reported almost exclusively from hospital settings and most hospital-associated MRSA (HA-MRSA) diseases resulted from a limited number of successful clones (Thurlow *et al.*, 2012). These HA-MRSA, which remained confined to healthcare settings, were exposed to a high antibiotic pressure among patients with frequent immunity impairment and/or invasive devices such as urinary/vascular catheters or mechanical ventilation (Chavez and Decker, 2008). Therefore, HA-MRSA were likely under strong positive selection within these healthcare-associated niches where the acquisition of resistance to multiple antibiotic families provided them with a major competitive advantage despite their impaired fitness. However, in the beginning of the 2000's, MRSA infections began to be reported in healthy individuals without known risk factors or apparent connections to healthcare institutions (Chambers, 2001),

(Vandenesch *et al.*, 2003). These community-acquired (CA)-MRSA strains had genetic backgrounds distinct from the traditional HA-MRSA strains with specific lineages predominating in different continents such as the Sequence Type 8 (ST8) SCCmecIVa (standing for staphylococcal cassette chromosome encoding methicillin resistance gene of type IVa) pulsotype USA300 in the USA (abbreviated to “USA300” below), the ST80 SCCmecIV in Europe, North Africa and the Middle East (hereinafter referred to as “EU-ST80”), and the ST30 SCCmecIV in Oceania (Mediavilla *et al.*, 2012). Some genetic features of these CA-MRSA were postulated to be major determinants of their selective advantages against HA-MRSA in community settings (David and Daum, 2010). Fitness impairment associated with the SCCmec mobile element is a well-described example: large SCCmec elements shared by HA-MRSA have higher fitness cost compared to small SCCmec of CA-MRSA (Ma *et al.*, 2002); thus, under the lightened antibiotic pressure encountered outside healthcare settings HA-MRSA are outcompeted by CA-MRSA. Successful community spread of CA-MRSA has also been allegedly associated with ecological factors such as modifications of colonisation niches. This was illustrated by the hypothesis of a deleterious impact of anti-pneumococcal vaccines on nasal microbiota facilitating CA-MRSA colonization (Regev-Yochay *et al.*, 2006). Finally, the observation that CA-MRSA had apparently increased virulence for human (Li *et al.*, 2010) notably in skin infection, suggested that higher bacterial load associated with increased severity of cutaneous infections could promote dissemination between humans.

Regarding the population dynamics of CA-MRSA, recent phylogenetic studies, conducted on the USA300 (Glaser *et al.*, 2016) and the EU-ST80 (Stegger *et al.*, 2014) lineages, proposed two

84 Bayesian evolutionary models inferring their population size through time among hundreds of
85 isolates sampled from 1980's to 2000's. Those phylogenetic analyses strongly suggested that in
86 the transition from an MSSA lineage to a successful CA-MRSA clone, the USA300 lineage first
87 became resistant to multiple antibiotics, acquired the arginine catabolic mobile element (ACME)
88 which encodes factors promoting skin colonization and infection (Thurlow *et al.*, 2013)(Planet *et*
89 *al.*, 2013), and subsequently acquired resistance to fluoroquinolones (Challagundla MBIO 2018)
90 (Uhlmann *et al.*, 2015). These two steps were associated with two successive phases of sharp
91 demographic expansion of what is known as the USA300 North-American (NA) lineage as
92 opposed to the Latin-American Variant (LV) which does not harbor ACME (Glaser *et al.*, 2016).
93 This evolutionary scenario has been confirmed recently on an independent set of strains
94 revealing, in addition, the European origin of the USA300 lineage (StrauB *et al* PNAS 2017). A
95 similar study, performed on the EU-ST80 epidemic CA-MRSA lineage, depicted a clone derived
96 from a Panton-Valentine (PVL)-positive methicillin-susceptible *S. aureus* (MSSA) ancestor from
97 sub-Saharan Africa that dramatically expanded in the early 1990's once out of West Africa, upon
98 acquisition of the SCCmec element, the plasmid-encoded fusidic-acid resistance (*fusB*) and four
99 canonical SNPs including a non-synonymous mutation in the accessory gene regulator C (*agrC*)
100 (Stegger *et al.*, 2014), a major virulence regulator in *S. aureus* (Reynolds and Wigneshweraraj,
101 2011). However, for both the USA300 and the EU-ST80 lineage it remains to be demonstrated
102 that the identified genetic events, which correlate with the demographic expansion, are causally
103 related with population size variations. In order to answer these questions, we explored fitness
104 and virulence factor expression of strains selected at various evolutionary and temporal stages

of the predicted population size inferred through Bayesian coalescence models.

MATERIALS & METHODS

Strain selection.

Infection-related strain selection among the CA-MRSA clones “USA300” and “EU-ST80” was determined by two previously published phylogenetic studies (Glaser *et al.*, 2016), (Stegger *et al.*, 2014). All isolates were stored at -20°C at the National Reference Center for Staphylococci (NRCS - HCL, Lyon), on cryobeads. Prior whole genome sequencing and Bayesian analysis of all strains enabled their assignment to an evolution phase of these clones; thus isolates of the two lineages were selected at various temporal steps of their inferred population dynamics as follows: for the CA-MRSA USA300 lineage and ancestors, ten clinical strains plus one reference strain were included (Fig. 1a & Table 1): (i) one ancestral strain of the ST8 lineage, susceptible to methicillin and lacking ACME (Ancestral ST8 MSSA), (ii) one strain corresponding to the most recent common ancestor of the USA300 clone, being resistant to methicillin but lacking the ACME sequence (Basal USA300 MRSA), (iii) four strains from the early expansion phase characterized by ACME and SCC*mec* acquisition (Derived USA300 MRSA 1, 2, 3 & 4), (iv) four strains from the most recent evolutionary phase subsequent to fluoroquinolones resistance acquisition (Derived USA300 MRSA 5, 6, 7 & 8), (v) one USA300 reference strain and its ACME single deletion mutant (SF8300ax) and ACME-SSC*mec* double deletion mutant (SF8300aex), both previously constructed by allelic replacement (Diep 2008). For the EU-ST80 lineage, eleven

clinical isolates plus one reference strain were selected (Fig. 1b & Table 1): (i) five strains from the basal clade with a high genetic proximity with their hypothetical common MSSA ancestor from Sub-Saharan Western Africa (Basal MSSA 1, 2, 3, 4 & 5), (ii) two MRSA strains from the derived clade isolated from a patient from Maghreb (Derived MRSA 1 & 2), (iii) two MRSA strains from the derived clade isolated on patients from Europe (Derived MRSA 3 & 4), (iv) two MRSA strains from the derived clade and associated with the stabilization/decline phase of the lineage (Derived MRSA 5 & 6); and (v) one EU-ST80 lineage reference strain HT20020209 (Fig.1 & Table 1).

Construction of a EU-ST80 agrC mutant.

The *agrC* locus of one basal MSSA of the ST80 lineage (Basal MSSA 3) (Fig. 1b & Table 1) was mutated by allelic replacement to confer the sequence carried by isolates of the derived clade (isoleucine instead of leucine at position 184). This mutation was obtained by using pMAD (Arnaud *et al.*, 2004). Two *agrC* DNA fragments flanking the *agrC* target region were amplified from a wild type strain using *agrC*2912/*agrC*555 and *agrC*544/*agrC*4238 primers respectively (Table S2). DNA fragments were then blunt-ended by *ScaI* and *PvuII* restriction enzymes, before being ligated and amplified using external primers *agrC*2912/*agrC*4238. The resulting DNA fragment corresponding to an *agrC* encoding sequence for the mutated amino-acid I184 was restricted by *XhoI* and *PvuII* and cloned in pMAD linearized by *SaII* and *SmaI*. The resulting plasmid, pLUG1166, was electroporated into RN4220, and then into Basal MSSA 3. Transformants were grown at non-permissive temperature (42°C), to select for cells with chromosome-integrated plasmid by homologous recombination. Successful double crossover

mutants were subsequently selected on X-gal agar plates after single colony culture at 30°C for 10 generations. PCR amplifications and sequencing were used to confirm the mutation of *agrC* in the resulting strain LUG2417 designated “Lab mutated basal MSSA” (Table 1).

RNA extraction from S. aureus.

Brain-Heart Infusion broth (BHI) was inoculated with an overnight culture to an initial OD_{600nm} of 0.05 and grown up on aerated Erlenmeyer flask to the end of exponential phase (6h) at 37°C under agitation (200 rpm). One milliliter of bacterial suspension was harvested and concentration adjusted to an OD_{600nm} = 1.0. Bacteria were washed in 10 mM Tris buffer and treated with lysostaphin and β-mercaptoethanol. RNAs were extracted with the RNeasy Plus Mini Kit® (Qiagen), quantified by spectrophotometry and stored at -80°C. This process was repeated on three different days for biological replicates.

RNA quantification by real-time PCR.

A random-primers based reverse transcription of 1µg of RNA was performed with the A3500 Reverse Transcription System Kit (Promega), followed by quantitative real-time PCR on cDNA using the FastStart Essential DNA Green Master kit (Roche) and the LightCycler® Nano (Roche). As previously described (Li *et al.*, 2010), we targeted five virulence genes (*RNAIII*, *lukS-PV*, *hla*, *hlgC*, *psmA*) and the housekeeping gene *gyrB* for normalization. Gene expression levels were compared between our clinical isolates and against the reference strains (SF8300-WT for the USA300 clone and HT20020209 for the EU-ST80 clone); levels were expressed as n-fold differences relative to reference strains or an isolate from another evolutionary phase. These

qRT-PCR were performed as technical triplicates (three RNA quantification per RNA sample), on RNA obtained from three biological replicates (three independent cultures and extractions per strain).

Biofilm production assay.

Each isolate was incubated overnight on blood agar (Columbia) at 35°C under ambient air. Three colonies were transferred into 9mL of BHI and incubated with agitation (200 rpm) overnight at 35°C under ambient air. Bacterial suspensions were then placed in a 96-well plate and incubated at 35°C under ambient air for 24h and 48h, respectively. Biofilm production was assessed by spectrophotometry after well drying and crystal violet fixation. *S. aureus* laboratory strains SH1000 (a frequently used biofilm-producing control for in vitro biofilm models) was added as a positive control (Horsburgh *et al.*, 2002), (O'Neill, 2010), *S. epidermidis* ATCC12228 (a biofilm-negative strain, non-producer) (Zhang *et al.*, 2003) and *S. carnosus* TM300 (biofilm-negative isolate constructed by intercellular adhesion (*ica*)-deletion) (Rosenstein *et al.*, 2009) as negative control. Biofilm experiments were performed as technical replicates (three wells per strain) and biological replicates (three independent plate series).

MIC determination.

In order to adjust their concentrations in selective media and broth used for sub-inhibitory antibiotic pressure, MIC of second line antibiotics were measured by E-tests on Mueller-Hinton agar, and interpreted according to EUCAST specifications (EUCAST/CA-SFM v2.0, 2015 July).

Crude doubling time.

Isolates growth curves were determined from BHI cultures incubated in 96-well plates for 24 hours at 37°C with continuous optical density monitoring at 600nm (Tecan Infinite® 200 PRO). Each strain was inoculated in three independent wells (technical replicate), and the experiment was repeated on three different days (biological replicate). Doubling times were calculated by graphical method with the Log-transformed optical density data of the exponential growth phase.

Competitive fitness.

Each strain to be tested in a competitive pair was adjusted to an $OD_{600nm} = 1$, then 3 mL of a $1/100$ dilution in BHI of each strain was mixed in a glass tube. For some experiments ofloxacin, ceftriaxone, or fusidic acid were added at final concentrations corresponding to $1/4$ to $1/100$ of the susceptible strain's MIC. Tubes were incubated at 35°C in aerobic atmosphere under agitation (200 rpm) for 22 +/- 2h, and 50 µL were transferred daily for 21 days to a fresh tube containing 3 mL of BHI. The proportion of each strain in the competitive mix was monitored daily with differential colony count based on selective agar inoculated with a calibrated amount of competitive mix (Spiral System® - Interscience) followed by aerobic incubation for 24h at 37°C. For MSSA vs MRSA couples, we used the ChromAgar® medium (i2A, France) allowing for growth of both strains (total count) and the ChromID-MRSA® medium (bioMérieux, France) for MRSA colony count. For the USA300 clone, where all isolates were MRSA, we used second line antibiotics resistance for strain discrimination. Therefore, differential colony counts were performed with simultaneous inoculation of a brain-heart agar (BHA) and a BHA with ofloxacin (2 µg/mL, i.e. x5 above sensitive strain MIC, x6 below resistant strain MIC). Similarly, for MRSA

vs MRSA pairs belonging to the EU-ST80 lineage, a combination of BHA and BHA with tetracycline (1 µg/mL, x8 above sensitive strain MIC, x8 below resistant strain MIC) was used. Strain quantifications calculated from colony counts on selective agar were confirmed by quantitative PCR targeting discriminant genes (*mecA* for MSSA versus MRSA, *grlA* for fluoroquinolones sensitive versus fluoroquinolones resistant, *tetK* for tetracycline sensitive versus tetracycline resistant, or *arcA*-ACME for ACME negative versus ACME positive strains) carried by one of the strains in the competitive pair. This approach was used to rule out a growth inhibition bias on selective medium. This was also the only strain quantification method usable for the EU-ST80 Basal MSSA 1 in competition with its *agrC* derivative obtained by allelic replacement. Strain proportions were determined with a L184I-specific set of primers (Table S2). All the PCRs were performed at days 0, 7, 14 and 21. Continuous competitive cultures were performed on three independent series (biological triplicates), each colony count or qPCR was performed on three technical triplicates. For all strains pairs tested, one of the strains was eventually reduced to a trace level, so no statistical test was required for strains proportions comparison.

Statistical analysis

Results are mostly presented as box plots in figures, and expressed as medians with 95% confidence interval (CI). Results from figures 2, 3, 4, 5, 6 and 7 are presented in box-plots and expressed as medians with 95% CI obtained from three technical replicates and three independent biological replicates. All statistical comparisons were performed through non-parametric Mann-Whitney test (p-value < 0,05).. In competition experiments in which one strain

was eventually reduced at undetectable level, no statistical test was required for proportions comparisons. All data were processed with Graphpad(r) PRISM 7.

RESULTS

Growth rate along the phylogeny.

Previous studies showed that CA-MRSA grew significantly faster than HA-MRSA, a property that may be a prerequisite for CA-MRSA, in the absence of antibiotic pressure, to achieve successful colonization of humans by outcompeting the numerous bacterial species in the human environment outside the hospital setting (Okuma *et al.*, 2002). We thus tested whether growth rate assessed by doubling-time varied between isolates of USA300 and EU-ST80 CA-MRSA lineages selected at various temporal steps of their Bayesian demography (Fig. 1 and Table 1). Whilst the ancestral strain of ST8 (a) had short doubling time (ca. 23'), the basal strain of the USA300 lineage carrying SCCmec (strain b) had altered fitness with doubling time of ca 30' (Fig. 2a). Early derived isolates of USA300 carrying SCCmec and ACME (strain c and d) displayed shortened doubling time (Fig. 2a). Within the derived clade corresponding to the epidemic phase, crude fitness appeared to be fading as we observed significant increase of doubling time along the phylogeny as shown by intraclade comparisons (Mann-Whitney test, $P = 0.029$ for all comparisons) (Fig. 2a). Each doubling time rise appeared to be related to a new acquisition of antibiotics resistance, namely aminoglycosides and macrolides, then fluoroquinolones, followed by tetracyclines (Fig. 2a and Table 1). The opposite impact of SCCmec and ACME on doubling

time was further confirmed by comparing the reference strain SF8300 (strain k, phylogenetically close to strain e and f, see Fig. 1) with its ACME deletion mutant (strain l), the later showing lengthened doubling time (Fig. 2a), whilst additional deletion of *SCCmec* (strain m, double ACME-*SCCmec* mutant) reduced the doubling time to an intermediate level (Fig. 2a). Among the twelve strains belonging to the EU-ST80 CA-MRSA lineage that were tested, the shortest doubling times were observed for the “basal clade” isolates (interclade comparison, Mann-Whitney test, $P = 0.029$) (Fig. 2b). Within each clade (basal and derived), we observed a decreasing crude fitness along the phylogeny as shown by increasing of doubling times (intraclade comparisons, Mann-Whitney test, $P = 0.029$ for all comparisons) (Fig. 2b). Like for USA300 strains, antibiotics resistance appeared to be a major determining factor of doubling time lengthening as shown by interclade comparison (Basal MSSA vs Derived MRSA, Mann-Whitney test, $P = 0.029$), and by intraclade comparisons revealing longer doubling times associated with new acquisition of antibiotics resistance, namely tetracyclines within the basal clade of MSSA strains (strain c, e and f); whereas in the derived clade, fitness impairments resulted from the consecutive acquisition of resistance to beta-lactams, fusidic acid, aminoglycosides, and finally tetracyclines and macrolides for the most recent isolates (Mann-Whitney test, $P = 0.029$ for all comparisons) (Fig. 2b and Table 1). At this stage, as epidemic strains (from derived clades) displayed the longest doubling times, we concluded that crude *in vitro* fitness did not explain the evolutionary dynamic of the two lineages. We therefore investigated other features related to host interaction and antibiotic pressure that could explain the demography of both lineages.

269

270 *Expression of core-genome encoded virulence factors along the lineages' evolutionary history.*

271 Previous studies revealed that overexpression of core-genome encoded virulence factors was a
272 common feature of CA-MRSA, a characteristic that has been proposed to contribute to the
273 expansion of these lineages (Li *et al.*, 2010). We therefore tested whether variations in the
274 expression of core genome encoded virulence factors along the Bayesian demographic models
275 could be observed (Fig. 1). To this end, RT-PCRs targeting virulence factors of the core- (α -toxin,
276 PSM α , γ -toxin) and accessory-genome (*LukS-PV*), as well as the major regulator (*agr-RNAIII*),
277 were performed after *in vitro* post-exponential growth as previously described (Li *et al.*, 2010).
278 Among the USA300 CA-MRSA isolates, despite an outlier strain with no measurable expression
279 of *hla*, no major variations were detected in expression levels of the targeted virulence factors
280 (above the accepted 2-fold level generally considered as a minimum biological relevant variation
281 in RT-PCR approaches) between the strains representing the various steps of the demography
282 (Fig. 3a). This lack of significant differences was observed by either using an ancestral strain
283 (Basal USA300 1) or the reference strain SF8300 as comparators (Fig. 3a). For the EU-ST80
284 lineage, most of the targeted virulence factors studied showed variation in expression below -
285 or close to - two-fold, between ancestral and derived isolates with the exceptions of i) *psma*
286 increasing by 3 - 3.5-fold in two isolates from the evolutionary-derived clade (designated
287 derived MRSA 2 and 5), and ii) *lukS-PV* increasing by a factor of 2.8-fold in one isolate (derived
288 MRSA 3) (Fig. 3b). In addition, expression of RNAIII, the *agr*-related regulatory RNA, was slightly

increased among isolates of the derived clade (reaching a 2.1-fold increase for one strain) compared to the basal clade (Fig. 3b).

All the EU-ST80 isolates from the derived clade harbor an L184I mutation in the extracellular loop of the AgrC receptor (Stegger *et al.*, 2014), a mutation that may have a functional impact on Agr signalling and expression of *agr*-RNAIII. To further investigate this point, an ancestral ST80 (AgrC L184) was engineered by allelic replacement to carry the L184I substitution and was then tested (as “Lab Mutated Basal MSSA”) for quantification of RNAIII and virulence factor expression. Compared to wild type (L184), the mutated (L184I) isogenic derivative (Lab Mutated Basal MSSA) showed a slight enhancement of RNAIII expression, but below the level of 2 (Fig. 3b). This mutation had no significant impact on virulence gene expression, except a mild 2.2-fold increase in *psmA* expression (Fig. 3b).

Biofilm production.

The detection of a slight difference in RNAIII production associated with the *agrC* mutation in the EU-ST80 lineage prompted us to test whether it could translate into differences in biofilm production. After 48 hours of growth, basal MSSA strains (c and e) displayed a higher production of biofilm compared to derived MRSA strains (l and k) carrying the L184I AgrC substitution ($P = 0.0002$) (Fig. 4). The role of AgrC L184I substitution in this phenotypic difference was confirmed by comparing strain c, a basal MSSA strain (AgrC L184) with its isogenic derivative, strain d (L184I), the latter showing a significant reduction of biofilm production ($P < 0.0001$). Importantly, the AgrC L184I mutation had no significant impact on crude fitness since doubling

times of the mutated strain (d) and its wild type parental strain (c) were similar (Fig. 2b). Therefore, differences observed in biofilm production were not due to growth variations but rather actual differences in biofilm production.

Competitive fitness along the phylogeny.

Doubling times comparisons used for crude fitness assessment highlighted fitness modulations along the phylogeny that did not match the Bayesian models inferred for both clones (Fig. 1). To better address this issue, we conducted a competitive fitness experiment in more stringent conditions based on continuous co-cultures for 21 days with isolates belonging to each phase of these lineages' evolution. Competitive strains pairs were designed in order to assess each evolutionary breakpoint identified in their inferred Bayesian phylogenetic models (Glaser *et al.*, 2016), (Stegger *et al.*, 2014), (Tables 1 & S1). Moreover, since our assessment of crude doubling times identified the acquisition of antibiotics resistance as a major determinant of fitness alteration, we tested the impact of sub-inhibitory concentrations of antibiotics on competitive fitness of these isolates. Within the USA300 lineage, the acquisition of ACME was associated with an increased fitness: during continuous competitive culture, ACME-positive MRSA strains outcompeted ACME-negative MRSA strains (Fig. 5a). Similarly, the laboratory engineered deletion mutant of ACME in SF8300 background was outcompeted by the wild type strain (Fig. 5c?). This confirmed the results obtained by crude fitness assessment where shorter doubling-times were obtained with ACME-positive strains compared to ACME-negative ones (Fig. 2a). However, this fitness enhancement was progressively abolished along the phylogeny with the

acquisition of fluoroquinolone (FQ) resistance; competitive fitness dropped even below the level observed prior to ACME acquisition: FQ-resistant ACME-positive strain was outcompeted by both FQ-susceptible ACME-positive or -negative strains (Fig. 5b & c). Same results were obtained with a competition between the FQ-resistant strain (Derived USA300 5) and another FQ-susceptible isolate (Derived USA300 4) (data not shown). Altogether these results indicate that ACME enhances fitness but is insufficient to compensate for the fitness cost of FQ resistance.

To assess whether the fitness cost of resistance could be reversed in the presence of trace amounts of antibiotics that could be present in the environment (Okuma *et al.*, 2002), (Gothwal and Shashidhar, 2015), competitive cultures were performed at various sub-inhibitory concentrations of antibiotics. The antibiotics chosen were those for which resistance acquisition correlate with noticeable variation in effective population size of the lineages (beta-lactams, fusidic acid for EU-ST80, and fluoroquinolones for USA300). Strikingly, even extremely low FQ concentration ($1/_{100}$ of the FQ-susceptible strain's MIC, 0.0038 $\mu\text{g/mL}$) was sufficient to confer a strong selective advantage of FQ-resistant ACME-positive strain toward FQ-susceptible ACME-positive strain (Fig. 6). These competitive cultures were also performed on the deletion mutant for ACME, and ACME-SCCmec double mutant, versus the parental strain SF8300 in the absence or presence of beta-lactam antibiotic. The results..... (fig xx). Similar analyses performed on the EU-ST80 strains also confirmed the results obtained during doubling times assessment suggesting that the major factor ruling the fitness downfall along the phylogeny was not the AgrC L184I but the acquisition of SCCmec/*fusB* and further extended antibiotics resistance. In

competitive culture assays, the laboratory engineered *agrC* mutation did not translate into fitness impairment after 21 days of competitive culture with its wild type progenitor (Fig. 7a), whilst competitive culture of the clinical strains confirmed the strong fitness reduction of the derived MRSA isolates compared to the ancestral MSSA in favor of a fitness cost of antibiotics acquisition (the most premature being *SCCmec* and *fusB*) in the absence of antibiotics (Fig. 7b). Similar results were obtained with Basal MSSA 4 versus Derived MRSA 3 (data not shown). The same competition performed in the presence of sub-inhibitory concentration of beta-lactam or fusidic acid totally reversed the result with a strong advantage of the MRSA even at extremely low concentrations ($1/100$ of MSSA Ceftriaxone MIC, 0.03 $\mu\text{g/mL}$, and $1/100$ of MSSA fusidic acid MIC, 0.0009 $\mu\text{g/mL}$) (Fig. 7c & d). The same results were obtained with antibiotics concentrations of $1/16$ and $1/32$ of their MICs and with the couple Basal MSSA 4 versus Derived MRSA 3 (data not shown).

DISCUSSION

Polyphyletic CA-MRSA emergence and spread at the end of the 20th century (Vandenesch *et al.*, 2003), (Tristan *et al.*, 2007), remains a challenging issue. As pointed out by A-C. Uhlemann, “our understanding of how a clone [such as USA300 or EU-ST80] became established as an endemic pathogen within communities remains limited” (Planet *et al.*, 2013). Increased expression of core-genome encoded virulence factors has been shown to be a common feature of CA-MRSA (Li *et al.*, 2010); we have investigated whether such characteristics varied along the longitudinal

372 short-term evolution of CA-MRSA. However, by assessing transcription of virulence factors
373 previously described as overexpressed among CA-MRSA lineages (Li *et al.*, 2010), we could
374 detect only minor variation (ca. 1.5-fold increase) in virulence factor expression between
375 USA300 strains when comparing ancestral and derived isolates of the North American clone
376 (Fig. 3a); we cannot rule out however that, at the population level, these minor increases in
377 virulence factor expression enhanced the success of the lineage, for instance by increasing
378 cutaneous infection rate (the most common infections caused by *S.aureus*) and thus human-to-
379 human transmission by skin contact as observed in prisons, sport team or men-having-sex-with-
380 men (Planet *et al.*, 2015). Within this USA300 clone, 20 SNPs were identified for being under
381 positive selection along successful evolution of this lineage (Glaser *et al.*, 2016); however, all the
382 derived isolates assessed for doubling time carried these 20 SNPs. Therefore, despite being
383 under positive selection they could not be reliable determinants of crude fitness evolution of
384 the derived clade isolates as competitive fitness impairment observed along the phylogeny
385 could not be explained by these genetic variations. Thus, the major variable feature of USA300
386 along the demography was the acquisition of ACME which is a now well-characterized mobile
387 genetic element (MGE) acquired from *S. epidermidis* by horizontal gene transfer (Diep *et al.*,
388 2006), (Pi *et al.*, 2009), (Uhlemann *et al.*, 2014). Its multiple functions in resistance to acidic pH
389 which enhances skin colonization, and as a factor promoting resistance to skin innate-immune
390 defences (Thurlow *et al.*, 2012) makes it a very plausible contributor of the USA300 expansion
391 (Planet, 2017). This is further strengthened by our findings of a shorter doubling-time of strains
392 carrying ACME (Fig. 2a) both when comparing strains along the phylogeny (such as strain b

393 lacking ACME and strains c and d that carry it) and laboratory deletion mutants of ACME
394 (SF8300 and derivative) (Fig. 2a). In the case of EU-ST80, analysis of virulence factor expression
395 along the demographical steps of the lineage showed that two derived isolates had a two-fold
396 increase in *psmA* expression and another one had a 2.5-fold increase in PVL expression when
397 compared to the ancestral isolates. As previously described (Stegger *et al.*, 2014), isolates of the
398 basal and derived clades of this lineage were discriminated by four canonical SNPs. One was
399 located in a non-coding region, two were synonymous SNPs, and one was a non-synonymous
400 SNP located in *agrC*, the major virulence factor regulator involved in quorum sensing and
401 biofilm production. We focused our attention on this SNP located in the *agrC* gene, because of
402 its potential association to fitness and colonization ability. This SNP resulted in a L184I amino
403 acid change in the extracellular loop of the AgrC receptor (Stegger *et al.*, 2014) shared by all EU-
404 ST80 isolates belonging to the derived clade. To investigate this point further, an ancestral ST80
405 (AgrC L184) was engineered by allelic replacement to carry the L184I substitution. Despite a
406 slight increase of doubling time compared to its parental strain, the L184I change in AgrC did
407 not translate into significant crude fitness variation (Mann-Whitney test, $P = 0.343$). Assessment
408 of virulence factor expression revealed a two-fold increase of *psmA* in the Lab Mutated basal
409 MSSA compared to its parental wild-type strain (Fig. 3b). AgrC L184I could therefore have a
410 moderate impact on virulence. We further detected a strong and significant decrease in biofilm
411 production associated with the AgrC L184I mutation (Fig. 4). Assessing which of these
412 phenotypes (slight increase in PSM α or PVL, strong decrease in biofilm) was under selection
413 remains speculative because they could be strongly dependent on the ecosystem in which

414 selection has occurred. However, little is known regarding these ecological conditions since the
415 current model for CA-MRSA ST80 lineage expansion places the acquisition of the AgrC L184I
416 mutation in the early 1990s in strains originating from Sub-Saharan Western Africa,
417 concomitantly with the acquisition of SCCmecIV and *fusB* (Stegger *et al.*, 2014). Alternatively,
418 the AgrC L184I substitution might be neutral, belonging to canonical mutations following the
419 acquisition of SCCmecIV and *fusB* or a consequence of genetic drift. Importantly, antibiotic
420 resistances were associated with demographic expansion of EU-ST80 (acquisition of SCCmecIV
421 and *fusB*) and also North American USA300 (acquisition of SCCmec and fluoroquinolone
422 resistance) (Fig. 1) (Stegger *et al.*, 2014), (Glaser *et al.*, 2016). These resistance acquisitions were
423 associated with a significant fitness cost as indicated by both extended doubling-time of the
424 derived isolates (Fig. 2) and by the results of competition experiments where derived isolates
425 were outcompeted by their basal counterparts (Fig. 5-7). These observations were in
426 accordance with the classical fitness costs associated with de novo antibiotic resistance,
427 specifically those selected at high antibiotic concentration (Martinez, 2009), (Andersson and
428 Hughes, 2014). Conversely, they did not match the Bayesian evolutionary models of these
429 lineages as strains belonging to the epidemic phase (derived clade) displayed the lowest *in vitro*
430 competitive fitness, with each step of fitness decrease being associated with new acquisition of
431 antibiotic resistance (Fig. 2, Table 1). However, the most striking observation was that extremely
432 low concentrations of antibiotics (those for which resistance acquisition correspond to
433 demographic expansion of the two lineages), totally reversed this fitness cost. This in keeping
434 with recent demonstration of a close correspondence between the estimated growth rates of a

435 lineage of MRSA and population-level prescription rates of β -lactam antibiotics (Volz bioRxiv
436 2017). Since both USA300 and EU-ST80 likely emerged in low income populations (Vandenesch
437 *et al.*, 2003), (Martinez, 2009), (Planet, 2017) where antibiotic exposure was not believed to be
438 substantial, the role of antibiotic selective pressure was not initially considered to be the major
439 trait under positive selection. However, increasing number of reports reveals the escalation of
440 antibiotic as environmental pollutants originating from hospital wastewater, bulk drug producer
441 wastewater and unused antibiotics dumped in landfills in countries without solid take-back
442 programs (Naimi *et al.*, 2003), (Thurlow *et al.*, 2012), (Larsson, 2014), (Gothwal and Shashidhar,
443 2015), (See *et al.*, 2017). From these sources, in which antibiotics such as fluoroquinolones can
444 reach concentrations ranging from 3 ng/L to 240 μ g/L (Van Doorslaer *et al.*, 2014), antibiotics
445 are disseminated in various environmental matrices such as surface water, soil, sediments, and
446 eventually living organism including livestock (Van Doorslaer *et al.*, 2014). Hence, community
447 settings, even in remote populations, can be exposed to low-level concentrations of various
448 antibiotics that could have promoted the expansion of CA-MRSA at least by enriching for
449 resistant bacteria (Andersson and Hughes, 2014), if not selecting for de novo resistance, the
450 latter being typically associated with no fitness cost (Gullberg *et al.*, 2011), (Andersson and
451 Hughes, 2014), (Westhoff *et al.*, 2017). Here, we demonstrate with competition experiments –
452 with the limitations inherent to every in vitro models- that the biological cost of antibiotic
453 resistance (to beta-lactams, fusidic-acid and fluoroquinolones) is entirely reversed in the
454 presence of trace amounts of antibiotics. Previous studies based on multidrug resistant plasmids
455 showed that, for specific combinations of drugs, each new compound added, lowered the

minimal selective concentration of the others (Gullberg *et al.*, 2014). However antibiotic resistance acquisitions (both by horizontal transfer of resistance genes and by mutations) are the genetic events that best match the variation of the demography in both lineages (Fig. 1a & b). Altogether, our findings support a model of antibiotic use, misuse and pollution as a major driving force for the emergence and expansion of CA-MRSA. In conclusion, CA-MRSA dynamics appear to be ruled by a complex interplay between resistance, virulence and fitness cost in which the contribution of anthropogenic activities is substantial.

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471 interpretation, or the decision to submit the work for publication.

472

473 **CONFLICT OF INTEREST**

474 The authors declare no conflict of interest.

475

476 Supplementary information is available at the ISME Journal's website

477

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FIGURE LEGEND

Figure 1: Bayesian demography of USA300 and EU-ST80 lineages.

Bayesian skyline plot indicating population size changes in the USA300 **(a)** and EU-ST80 **(b)** lineages over time with a relaxed molecular clock. The shaded area represents the 95% confidence interval. Strain selection and their designation are indicated by colored thumbnails. Adapted from Glaser *et al.* (2016) and Stegger *et al.* 2014.

Figure 2: Doubling times of USA300 and EU-ST80 strains.

USA300 **(a)** or EU-ST80 **(b)** isolates were cultured in BHI incubated on 96-wells plates for 24 hours at 37°C with continuous optical density monitoring at 600nm (Tecan Infinite® 200 PRO). Doubling times were calculated by graphical method after Log transformation of data from the exponential growth phase. The color codes for each strain correspond to those in Fig. 1. (*: $P = 0.029$). Experiments were performed on three independent series (biological replicates), and optical densities were measured on three wells for each strain (technical replicates).

Figure 3: Expression of virulence related genes among USA300 and EU-ST80 strains.

Expression of virulence factor- and regulatory-genes were assessed by qRT-PCR among USA300 isolates **(a)** and EU-ST80 isolates **(b)** of various temporal phases of the demographic expansion. Results are expressed as fold change in comparison to the most ancestral strain of the lineage (plain) or to the reference strain of the lineage (striped). Experiments were performed on three independent series (biological replicates), and three RNA quantifications were done for each RNA sample (technical replicates).

Figure 4: Biofilm production assay for EU-ST80 strains.

(a) Biofilm production was assessed by crystal violet stain on strains of the EU-ST80 lineage belonging to ancestral clade (Basal MSSA 3 and 4) or derived clade (Derived MRSA 3 and 5), the latter carrying the *mecA* gene and expressing an AgrC L184I variant. *S. aureus* SH1000 was used as positive control for biofilm production and *S.*

carneus TM300 and *S. epidermidis* ATCC12228 were negative controls. **(b)** Comparison in biofilm production by crystal violet from EU-ST80 Basal MSSA 3 and its isogenic derivative expressing an AgrC L184I variant (Lab Mutated Basal MSSA). The color codes for each strain correspond to those in Fig. 1. (*: $P = 0.015$; **: $P = 0.002$). Experiments were performed on three independent series (biological replicates), and biofilm production was quantified on three wells for each strain (technical replicates).

Figure 5: Impact of ACME in competitive fitness of USA300.

(a) ACME-negative and -positive strains, both MRSA with no associated antibiotics resistance were co-cultivated for 21 days in BHI with daily subculture in fresh medium. The proportion of each strain was monitored at day 1, 7, 14 and 21 with qPCR targeting *arcA*-ACME. **(b)** ACME-positive strains, one Fluoroquinolone (FQ)-susceptible (Derived USA300 3) and one FQ-resistant (Derived USA300 5) were co-cultivated for 21 days in BHI with daily subculture in fresh medium. The proportion of each strain was monitored daily with differential colony count based on selective agar inoculated with a calibrated amount of competitive mix. Same results were obtained with a competition between the FQ-resistant strain (Derived USA300 5) and another FQ-susceptible isolate (Derived USA300 4), data not shown. **(c)** ACME-positive FQ-resistant strain (Derived USA300 5) and ACME-negative strain FQ-susceptible (Basal USA300 1) were co-cultivated for 21 days and assessed as in **(b)**. Competitive cultures were performed on three independent series (biological replicates), and each colony count or qPCR was repeated three times (technical replicates).

Figure 6: Potential impact of FQ resistance in competitive fitness of USA300.

(a) Fluoroquinolone (FQ)-susceptible and -resistant strains of USA300, both ACME-positive, were co-cultivated for 21 days in BHI without antibiotics, or **(b)** containing ofloxacin at $\frac{1}{16}$ of FQ MIC of the susceptible strain (0.024 $\mu\text{g/mL}$), **(c)** $\frac{1}{32}$ MIC (0.012 $\mu\text{g/mL}$) or **(d)** $\frac{1}{100}$ MIC (0.003 $\mu\text{g/mL}$) with daily subculture in fresh medium. The proportion of each strain was monitored daily with differential colony count based on selective agar inoculated with a calibrated amount of competitive mix. Competitive cultures were performed on three independent series (biological replicates), and each colony count or qPCR was repeated three times (technical replicates).

Figure 7: Potential impact of *mecA/fusB* acquisition and *agrC* mutation on competitive fitness of EU-ST80.

(a) The *mecA/fusB*-negative (Basal MSSA 3) AgrC L184 was co-cultivated with its *agrC* derivative (Lab Mutated Basal MSSA) carrying the AgrC L184I mutation. **(b)** A *mecA/fusB*-negative (Basal MSSA 3) AgrC wild type (L184) strain and

a *mecA/fusB*-positive (Derived MRSA 5) AgrC L84I strain were then co-cultivated for 21 days in BHI without antibiotics; same results were obtained with Basal MSSA 4 versus Derived MRSA 3 (data not shown). **(c) & (d)** The Basal MSSA 3 strain was co-cultivated with the Derived MRSA 5 strain for 21 days in BHI containing ceftriaxone or fusidic acid at $1/100$ of MIC for the MSSA/*fusB*^{neg} strain (0.03 µg/mL or 0.0009 µg/mL respectively) with daily subculture in fresh medium. The proportion of each strain was monitored daily with differential colony count based on selective agar inoculated with a calibrated amount of competitive mix for **(b), (c), (d)** or by quantitative PCR due to the lack of discriminant antibiotic resistance marker for **(a)**. The same results were obtained with antibiotics concentrations of $1/16$ and $1/32$ of their MICs and with the couple Basal MSSA 4 versus Derived MRSA 3 (data not shown). Competitive cultures were performed on three independent series (biological replicates), and each colony count or qPCR was repeated three times (technical replicates).

Table 1: Relevant characteristics of strains

Strains list with their ID's (as used in the manuscript and figures), NRCS Number (as used in reference publications), relevant characteristics, and reference publications.

Table S1: Strains pairs used for competitive fitness study

Strains pairs listed according to their lineage assignment, with discriminant parameters ("Striking difference").

Table S2: Primers used for qPCR and RT-qPCR

Primers list including target genes used for resistance-based strain discrimination (*mecA*, *tetK*, *griA*), phylogenetic clade discrimination (*agrC*, *arcA*-ACME), virulence factor expression assay (*RNAIII*, *hla*, *hlgC*, *lukS-PV*, *PSMα*), engineering of the Lab Mutated basal MSSA (*agrC2912*, 555, 4238, 544), and standardization (*gyr*).

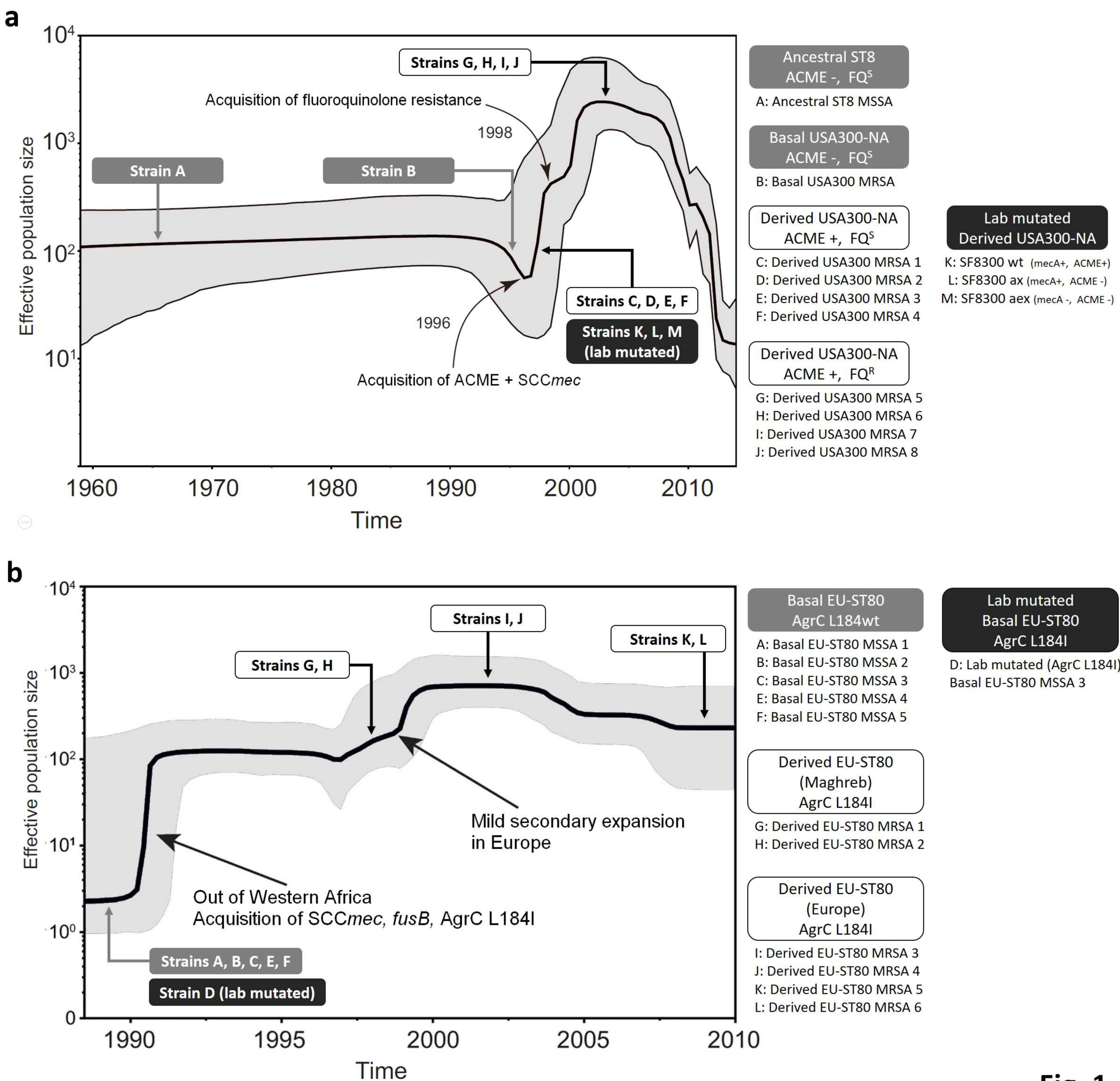


Fig. 1

1 **Table 1:** Relevant characteristics of strains

STRAIN DESIGNATION (label in figures)	Ref. ID	RELEVANT CHARACTERISTICS	REFERENCES
USA300 strains			
Ancestral ST8 MSSA (A)	ERS092996	<i>mecA</i> (-), ACME (-), [R = P, F]	Uhlmann et al., 2015
Basal USA300 MRSA (B)	ERS092816	<i>mecA</i> +, ACME (-), [R = P, Oxa]	Uhlmann et al., 2015
Derived USA300 MRSA 1 (C)	ST2012-0558	<i>mecA</i> +, ACME+, [R = P, Oxa]	Glaser et al., 2016
Derived USA300 MRSA 2 (D)	ST2012-1514	<i>mecA</i> +, ACME+, [R = P, Oxa]	Glaser et al., 2016
Derived USA300 MRSA 3 (E)	ST2013-0343	<i>mecA</i> +, ACME+, [R = P, Oxa, K, E]	Glaser et al., 2016
Derived USA300 MRSA 4 (F)	ST2011-2484	<i>mecA</i> +, ACME+, [R = P, Oxa, K, E]	Glaser et al., 2016
Derived USA300 MRSA 5 (G)	ST2011-1414	<i>mecA</i> +, ACME+, [R = P, Oxa, K, E, O]	Glaser et al., 2016
Derived USA300 MRSA 6 (H)	ST2013-0068	<i>mecA</i> +, ACME+, [R = P, Oxa, K, E, O]	Glaser et al., 2016
Derived USA300 MRSA 7 (I)	ST2013-1284	<i>mecA</i> +, ACME+, [R = P, Oxa, K, E, O, T]	Glaser et al., 2016
Derived USA300 MRSA 8 (J)	ST2013-1763	<i>mecA</i> +, ACME+, [R = P, Oxa, K, E, O, T]	Glaser et al., 2016
SF8300wt (K)	LUG2295 USA300 ref strain	<i>mecA</i> +, ACME+, [R = P, Oxa, E, C, T, Cip, Mup]	Diep et al., 2008
SF8300ax (L)	LUG2977 ACME deletion mutant	<i>mecA</i> +, Δ-ACME, [R = P, Oxa, E, C, T, Cip, Mup]	Diep et al., 2008
SF8300aex (M)	LUG2979 ACME SCC <i>mec</i> deletion mutant	Δ- <i>mecA</i> , Δ-ACME, [R = P, E, C, T, Cip, Mup]	Diep et al., 2008
EU-ST80 strains			
Basal EU-ST80 MSSA 1 (A)	HT2002-0042	<i>mecA</i> (-), AgrC L184 [R = P]	Stegger et al., 2014
Basal EU-ST80 MSSA 2 (B)	HT2006-0859	<i>mecA</i> (-), AgrC L184 [R = P]	Stegger et al., 2014
Basal EU-ST80 MSSA 3 (C)	HT2003-0006	<i>mecA</i> (-), AgrC L184, [R = P, T]	Stegger et al., 2014
Lab Mutated Basal EU-ST80 MSSA 3 (D)	LUG2417	<i>mecA</i> (-), AgrC L184I , [R = P, T]	This study
Basal EU-ST80 MSSA 4 (E)	HT2004-1302	<i>mecA</i> (-), AgrC L184, [R = P, T]	Stegger et al., 2014
Basal EU-ST80 MSSA 5 (F)	HT2005-0374	<i>mecA</i> (-), AgrC L184, [R = P, T]	Stegger et al., 2014
Derived EU-ST80 MRSA 1 (G)	ST2007-1277	<i>mecA</i> +, AgrC L184I , [R = P, Oxa]	Stegger et al., 2014
Derived EU-ST80 MRSA 2 (H)	ST2007-1273	<i>mecA</i> +, AgrC L184I , [R = P, Oxa]	Stegger et al., 2014
Derived EU-ST80 MRSA 3 (I)	ST2005-0508	<i>mecA</i> +, AgrC L184I , [R = P, Oxa, K, F]	Stegger et al., 2014
Derived EU-ST80 MRSA 4 (J)	ST2009-0942	<i>mecA</i> +, AgrC L184I , [R = P, Oxa, K, F, T]	Stegger et al., 2014
Derived EU-ST80 MRSA 5 (K)	HT2007-0258	<i>mecA</i> +, AgrC L184I , [R = P, Oxa, K, F, T, E, C]	Stegger et al., 2014
Derived EU-ST80 MRSA 6 (L)	ST2007-1047	<i>mecA</i> +, AgrC L184I , [R = P, Oxa, K, F, T, E, C]	Stegger et al., 2014

2
3 ACME: Arginine Catabolism Mobile Element – AgrC: Accessory gene regulator - C: Clindamycin - Cip: Ciprofloxacin -
4 E: Erythromycin - F: Fusidic acid - I: Isoleucine - K: Kanamycin - L: Leucine – Mup: Mupirocin - O: Ofloxacin - Oxa:
5 Oxacillin - P : Penicillin - R: resistant – Ref. ID: strain designation used in reference articles – SCC: Staphylococcal
6 chromosomal cassette - T: Tetracycline

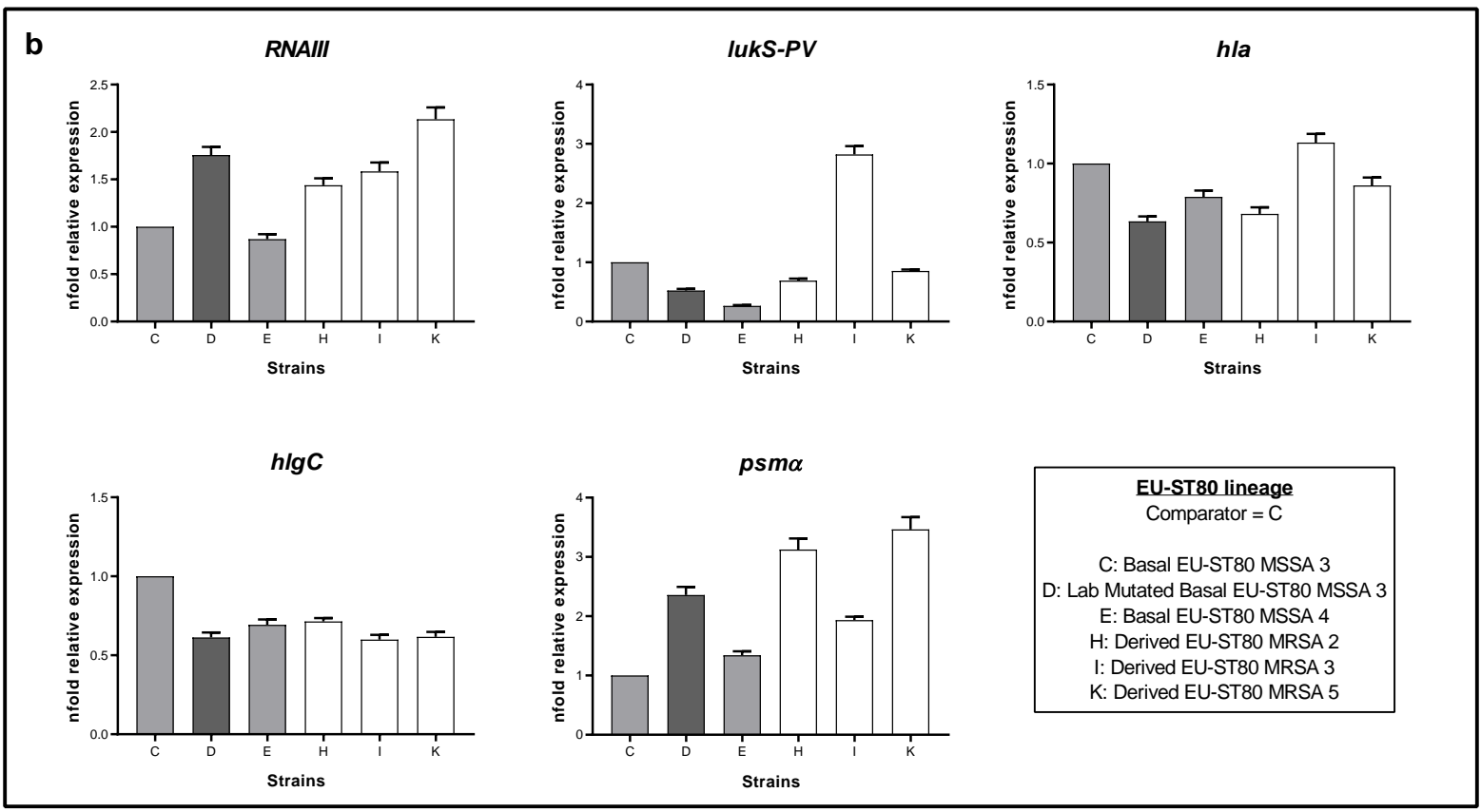
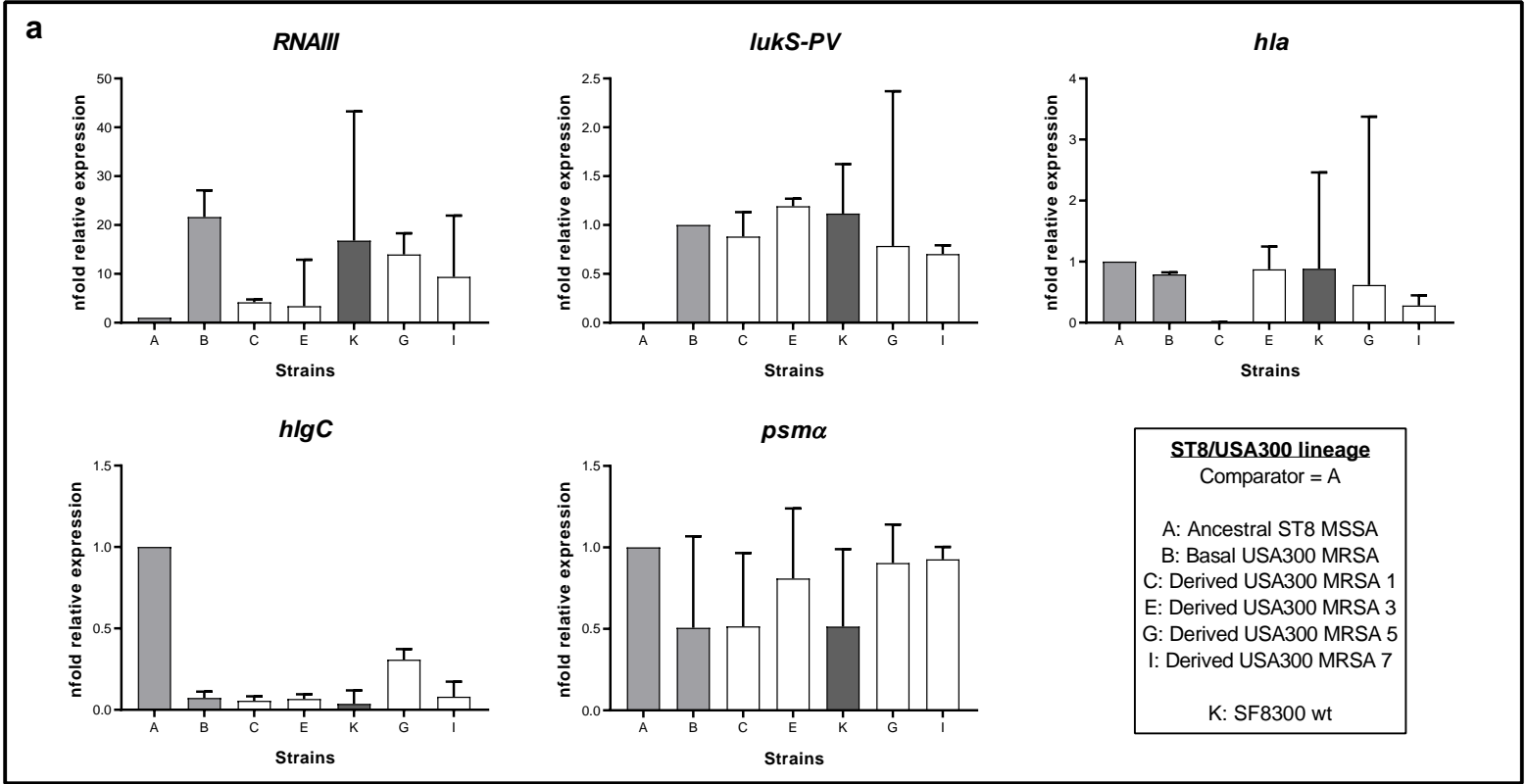


Fig. 3

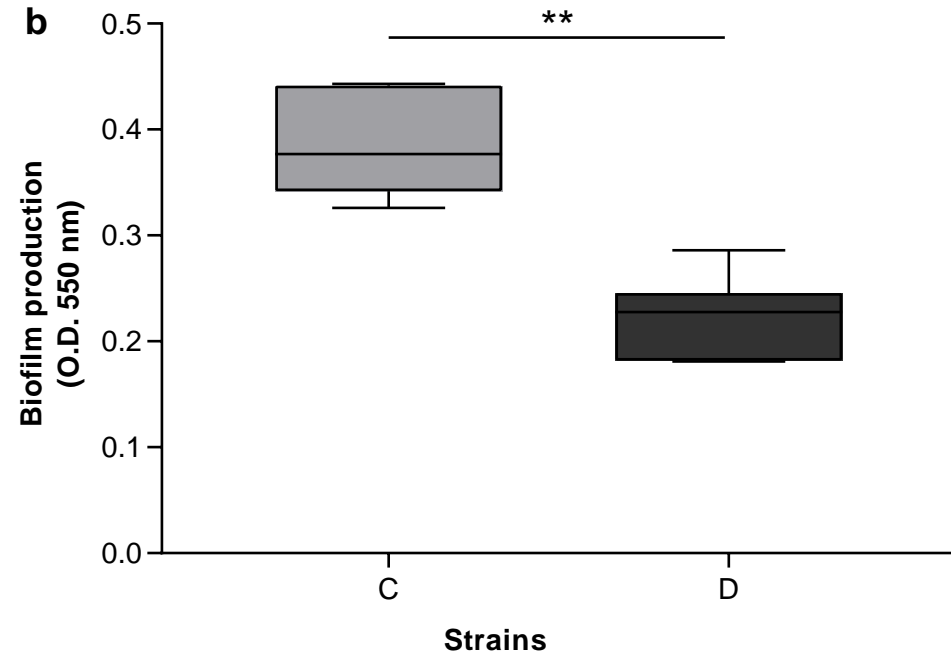
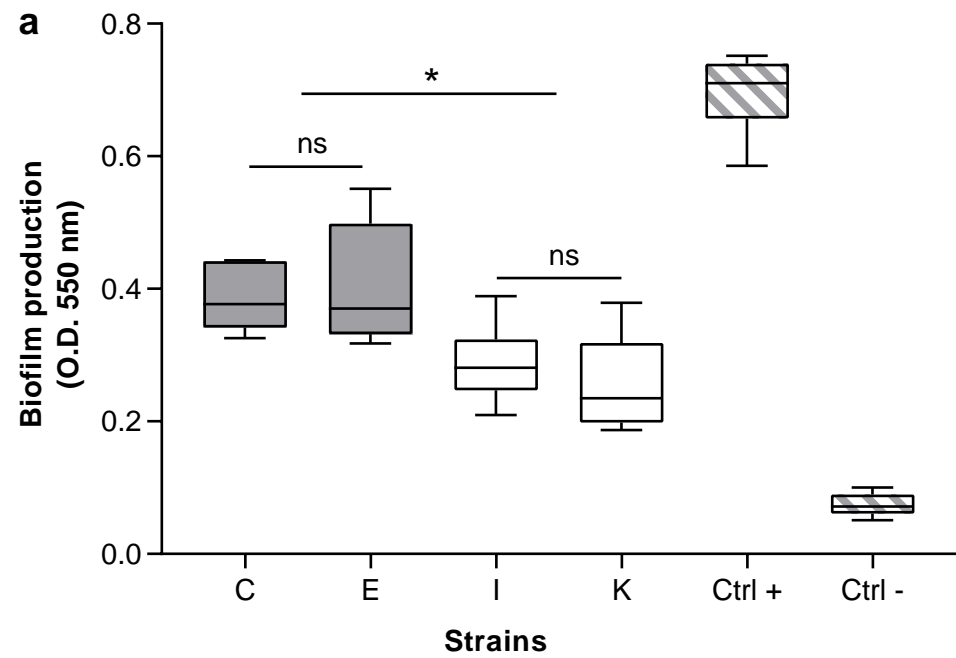


Fig. 4

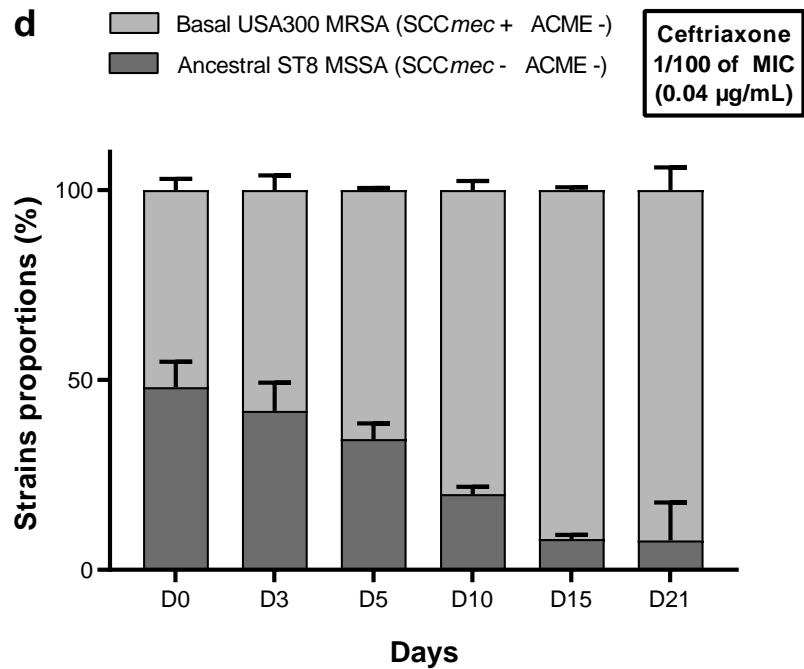
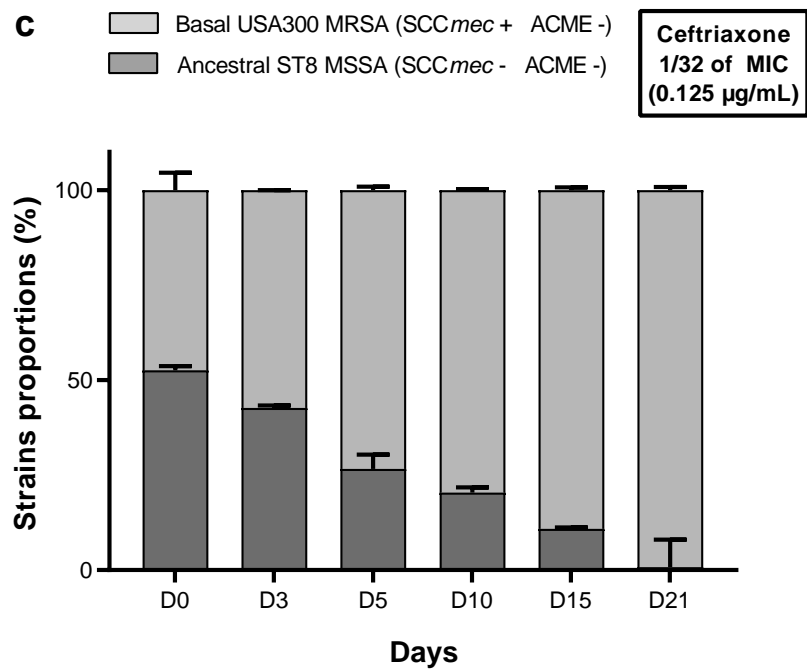
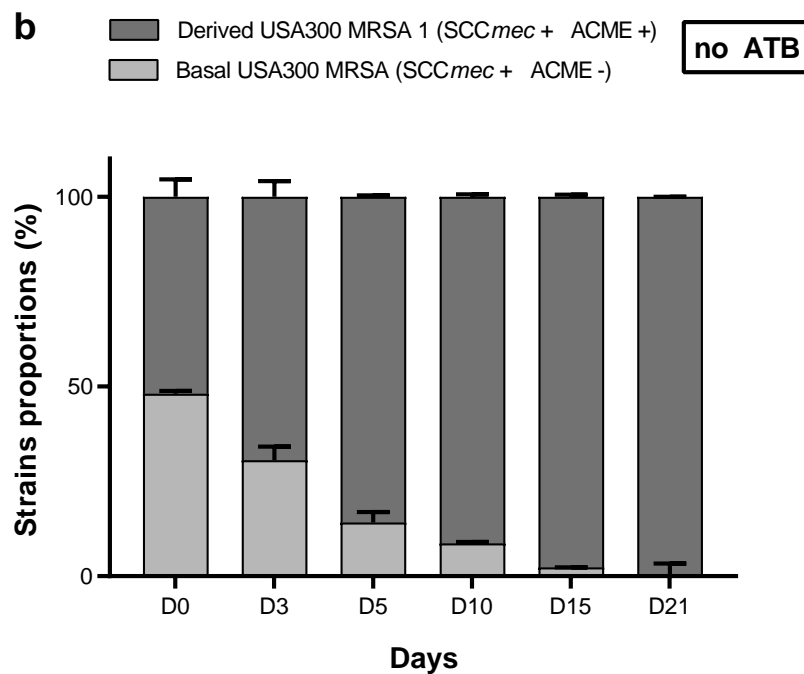
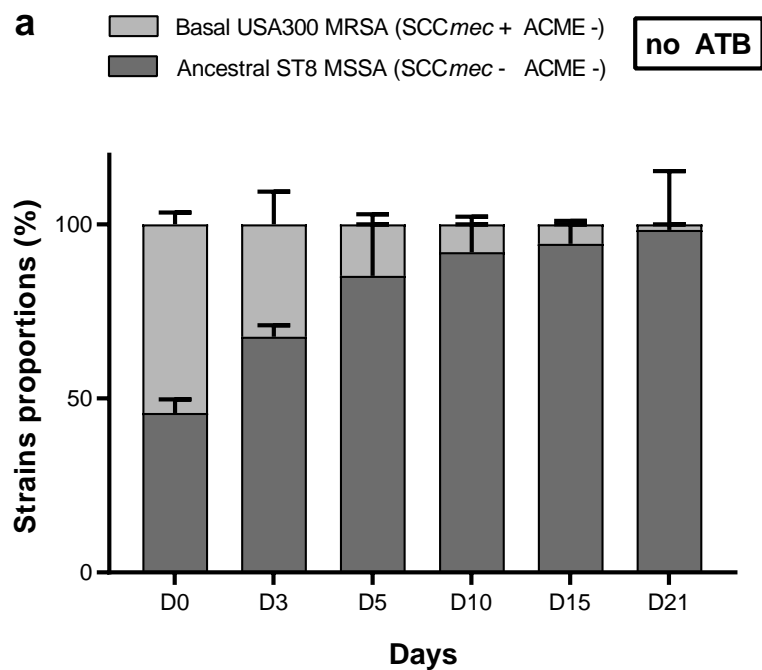


Fig. 5

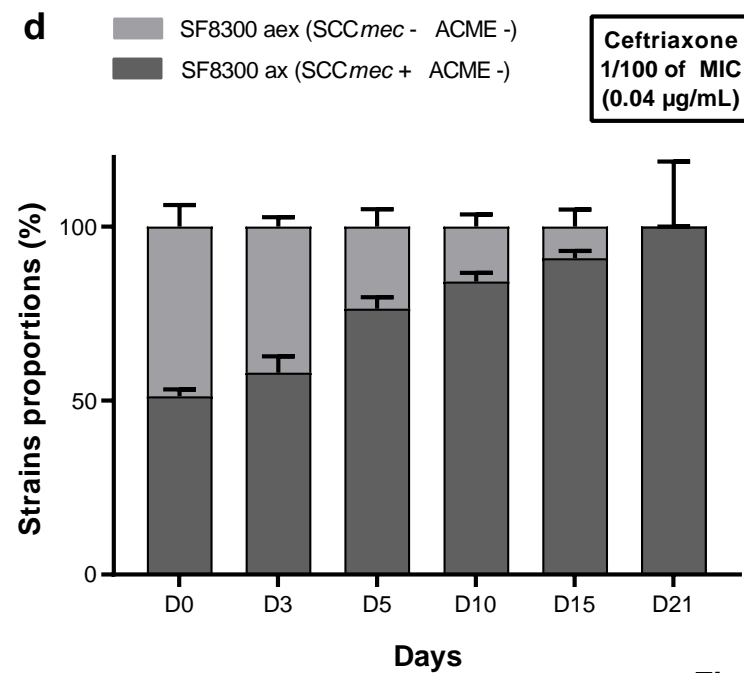
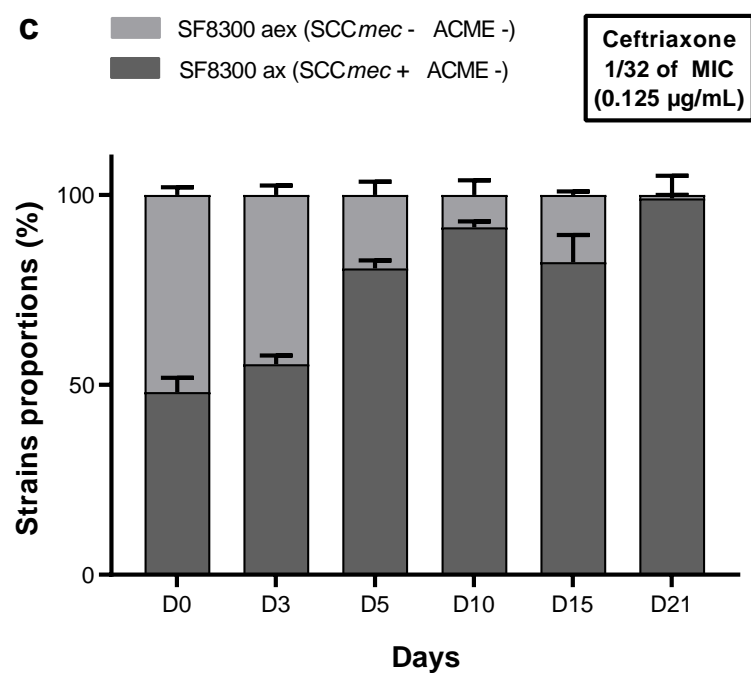
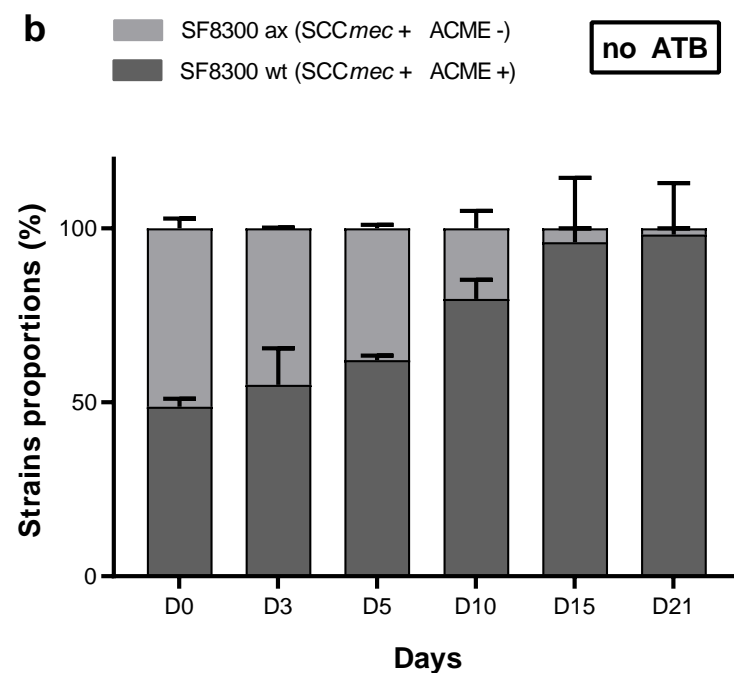
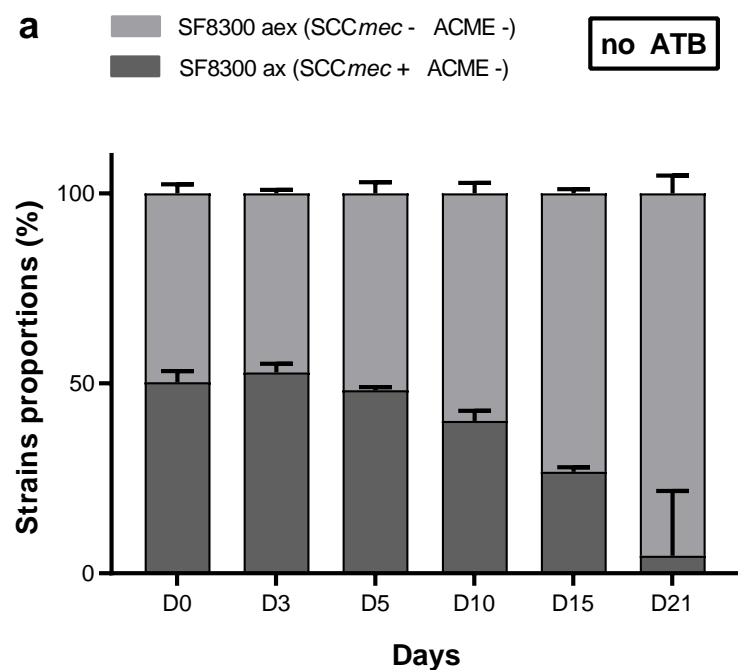


Fig. 6

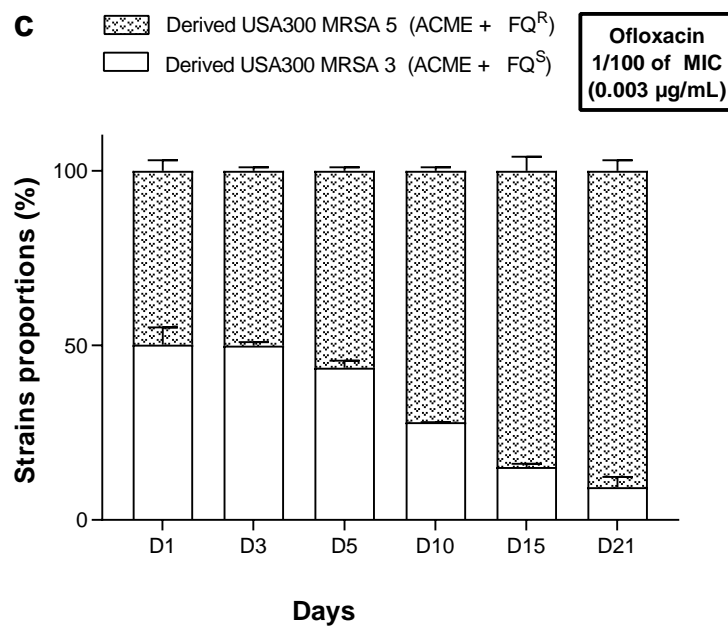
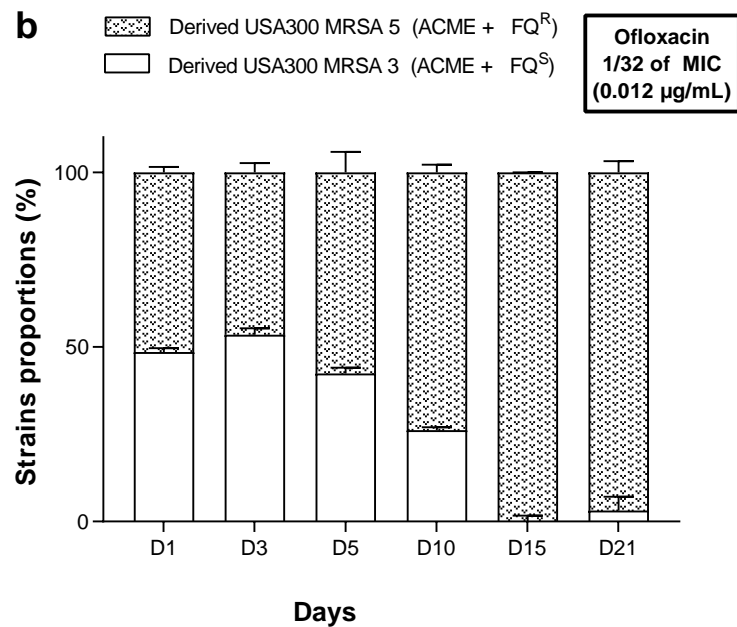
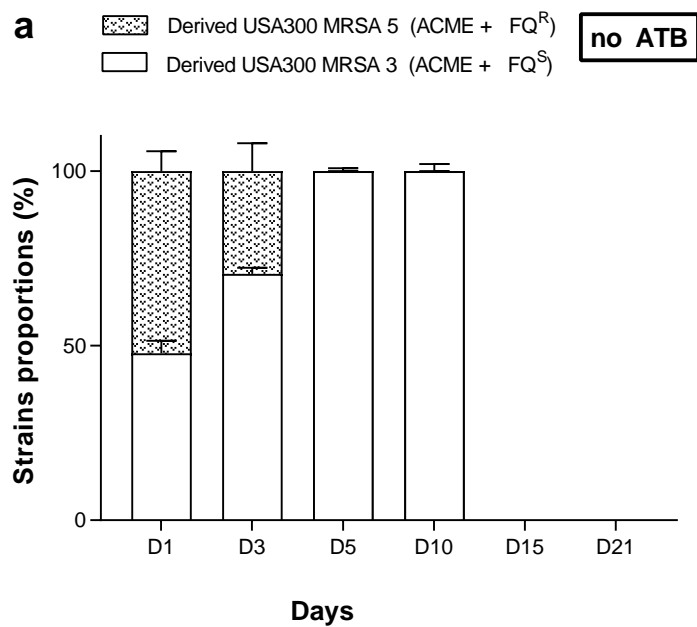


Fig. 7

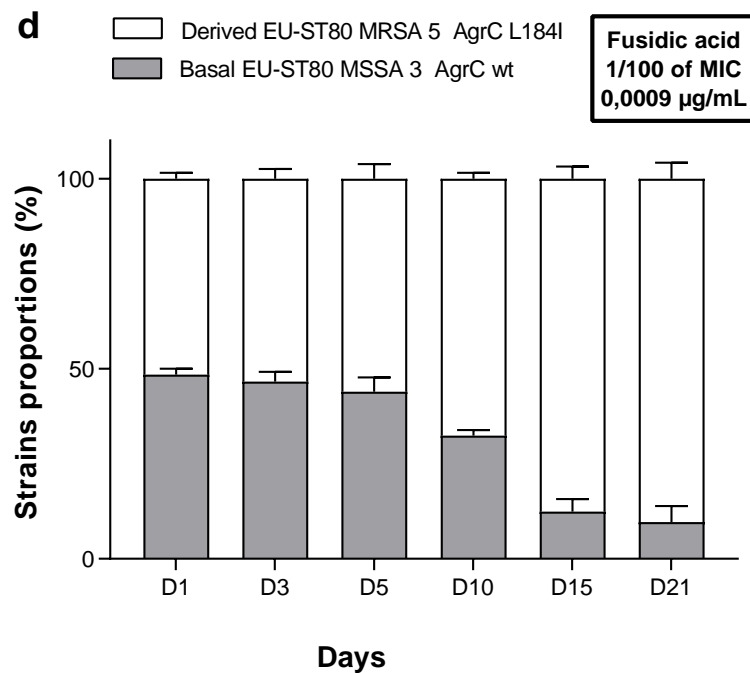
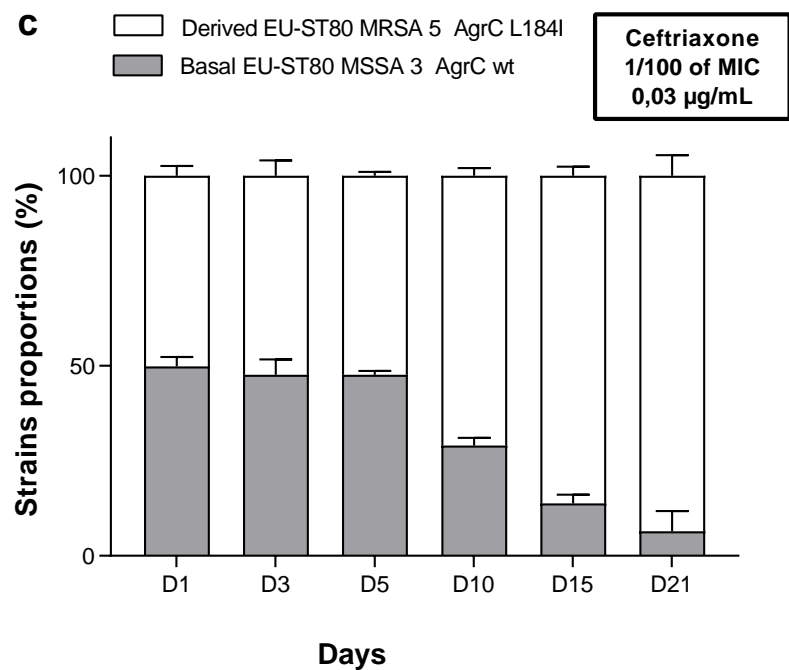
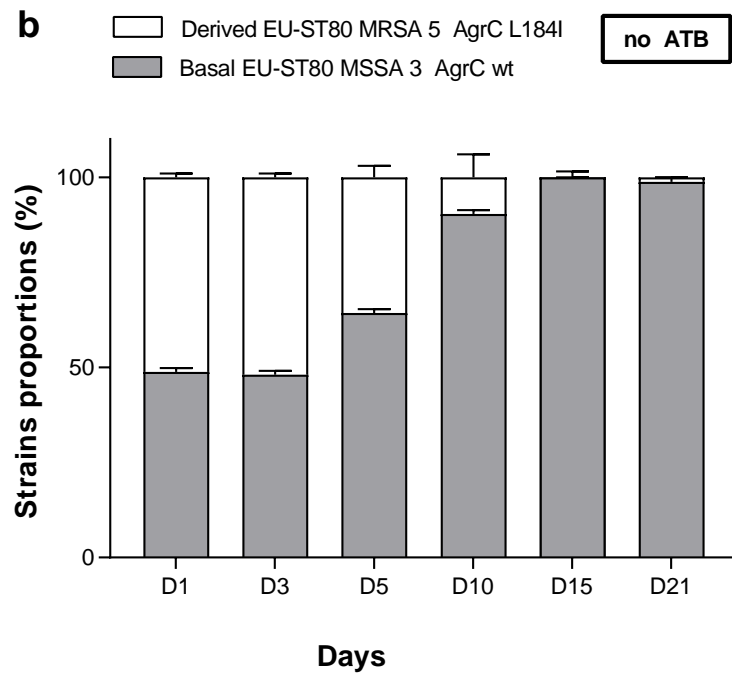
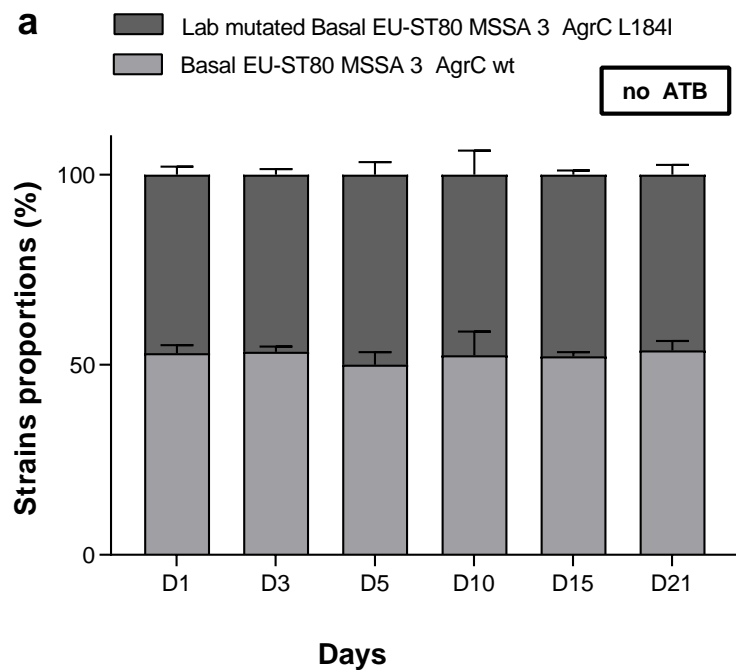


Fig. 8

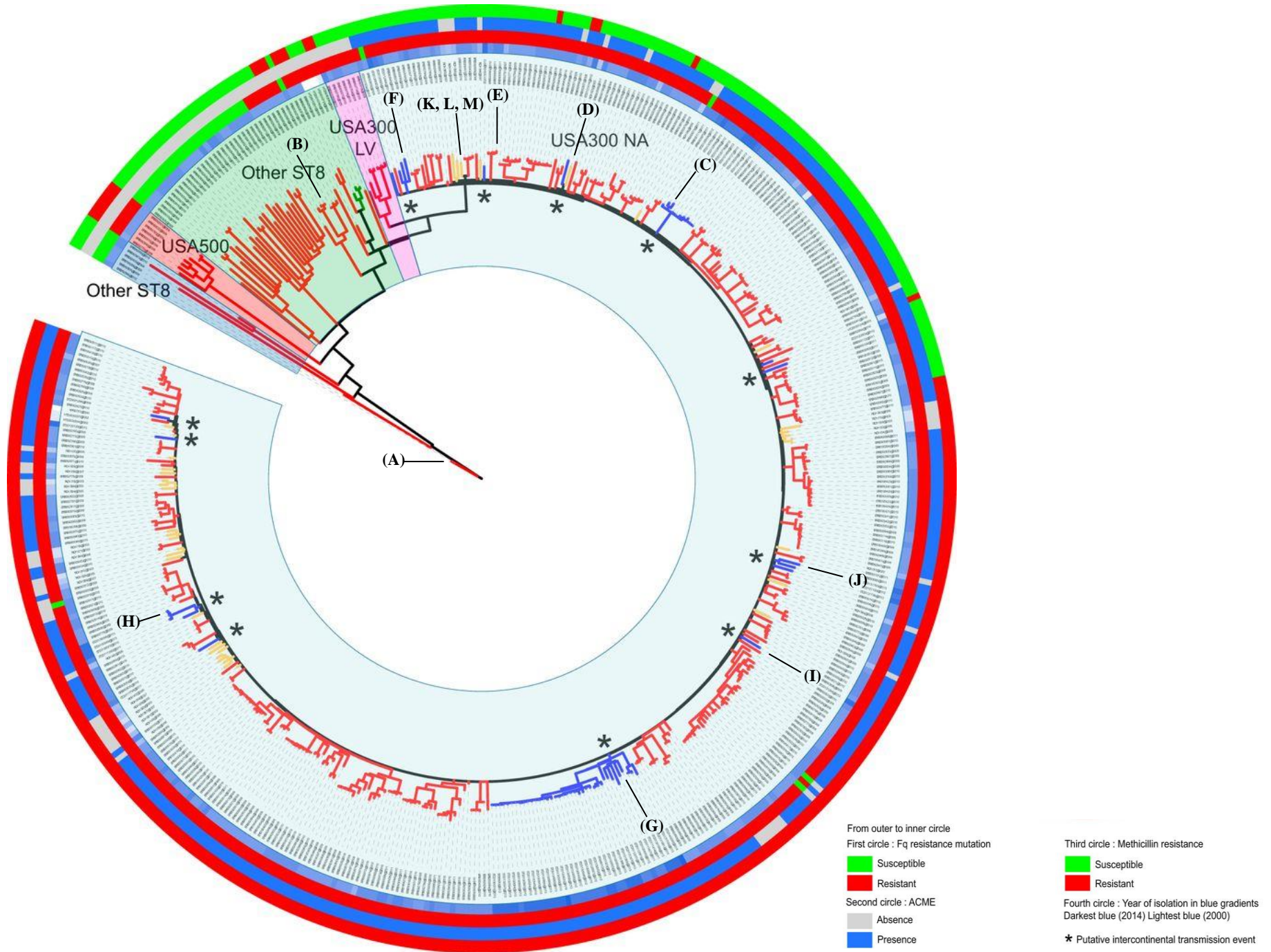
Table S1: Strains pairs used for competitive fitness study

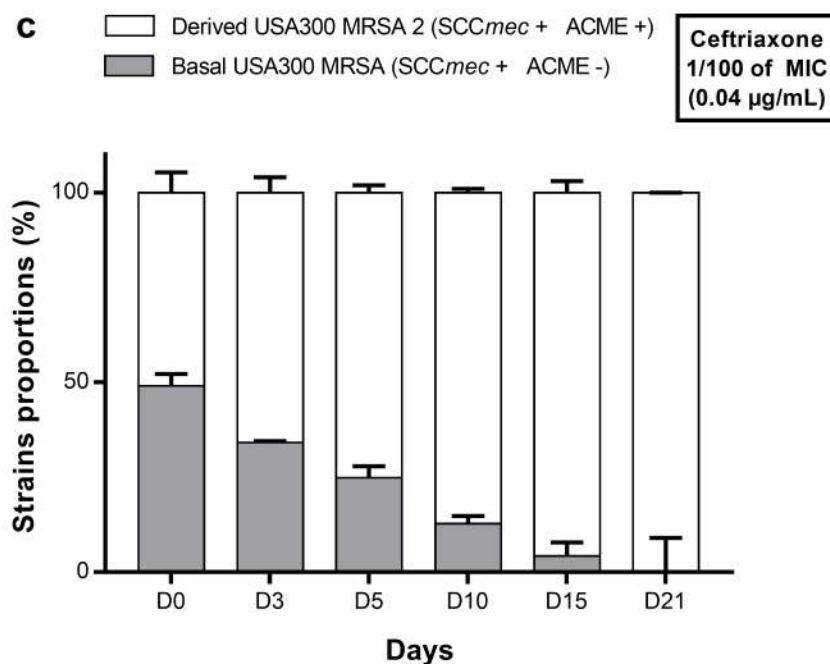
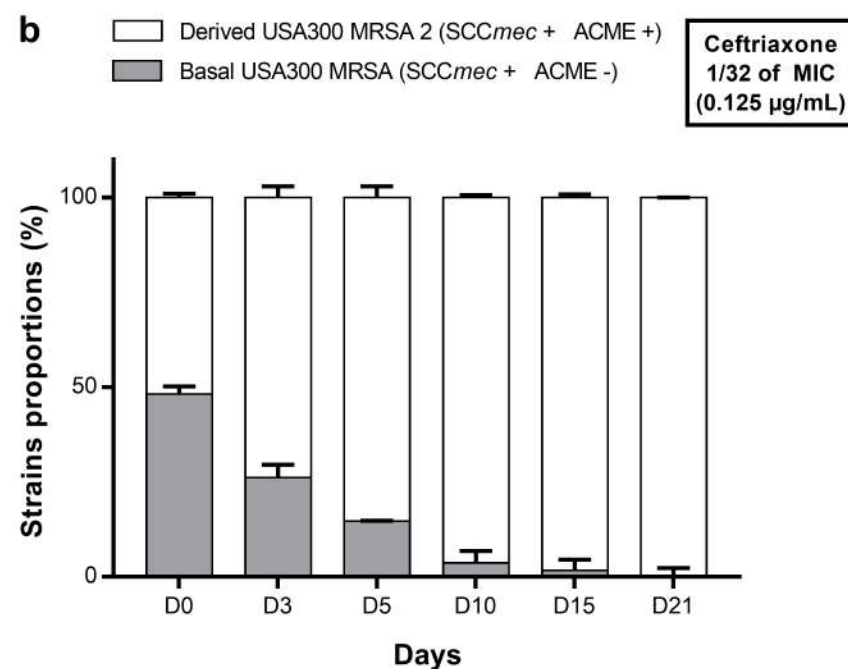
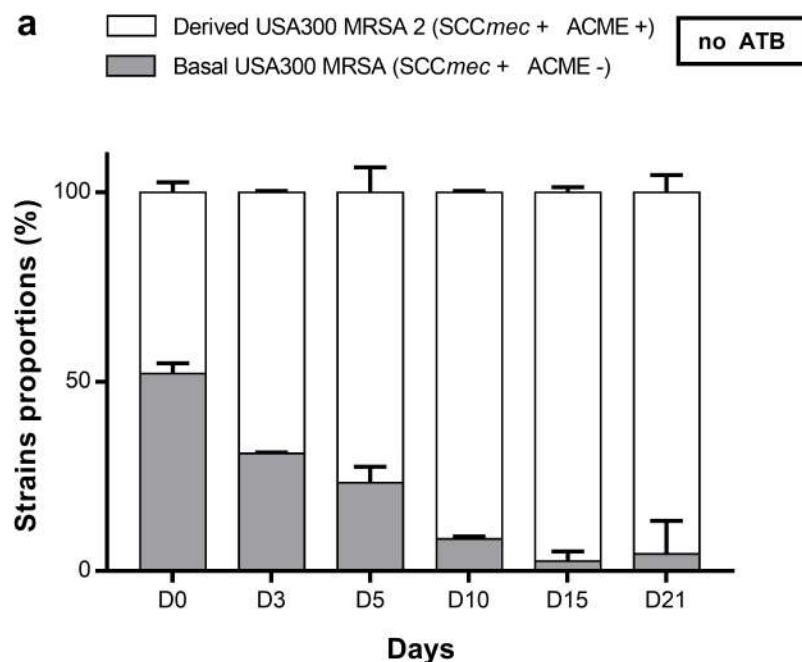
Strain 1	Strain 2	Striking difference
<u>USA300</u>		
Ancestral ST8 MSSA (A)	Basal USA300 MRSA (B)	SCC <i>mec</i> acquisition
SF8300ax (L)	SF8300aex (M)	SCC <i>mec</i> acquisition
Basal USA300 MRSA (B)	Derived USA300 MRSA 1 (C)	ACME acquisition
Basal USA300 MRSA (B)	Derived USA300 MRSA 2 (D)	ACME acquisition
SF8300wt (K)	SF8300ax (L)	ACME acquisition
Ancestral ST8 MSSA (A)	Derived USA300 MRSA 1 (C)	SCC <i>mec</i> and ACME acquisition
Basal USA300 MRSA (B)	Derived USA300 MRSA 5 (G)	ACME and FQ resistance acquisition
Derived USA300 MRSA 1 (C)	Derived USA300 MRSA 5 (G)	FQ resistance acquisition
Derived USA300 MRSA 3 (E)	Derived USA300 MRSA 5 (G)	FQ resistance acquisition
Derived USA300 MRSA 4 (F)	Derived USA300 MRSA 5 (G)	FQ resistance acquisition
<u>EU-ST80</u>		
Basal EU-ST80 MSSA 4 (E)	Derived EU-ST80 MRSA 3 (I)	<i>mecA</i> and AgrC L184I mutation
Lab Mutated Basal EU-ST80 MSSA 3 (D)	Basal EU-ST80 MSSA 3 (C)	AgrC L184I mutation effect
Lab Mutated Basal EU-ST80 MSSA3 (D)	Derived EU-ST80 MRSA 3 (I)	<i>mecA</i> acquisition
Lab Mutated Basal EU-ST80 MSSA 3 (D)	Derived EU-ST80 MRSA 5 (K)	<i>mecA</i> acquisition
Derived EU-ST80 MRSA 2 (H)	Derived EU-ST80 MRSA 3 (I)	Expansion toward Europe
Basal EU-ST80 MSSA 3 (C)	Derived EU-ST80 MRSA 5 (K)	Overall evolution

Table S2: Primers used for qPCR and RT-qPCR

TARGET	Forward primer (5'-3')	Reverse primer (5'-3')
<i>gyr</i>	CGGTGGCGACTTTGATCTAGCG	GTCAGTTTATACAACGGTGGCTGTGC
<i>mecA</i>	TGGCAATATTAACGCACCTCAC	AGTTCTGCAGTACCGGATTTGC
<i>agrC</i> (wild type)	CAGTAATATAATAATTCCCAAGAATAAAAG	CAATTTCTTATGCACTTGCC
<i>agrC</i> (mutated)	CAGTAATATAATAATTCCCAAGAATAAAAT	CAATTTCTTATGCACTTGCC
<i>agrC2912</i>	CTACTCGAGAAAGTGTGATAGTAAG	
<i>agrC555</i>		TAAAGTACTATACTGTTTGATTGTTGATGC
<i>agrC4238</i>	ATCAGATCTGCTAGTTGTTAATAATTTTC	
<i>agrC544</i>		TATCAGCTGCTATTCTTAGGAATTA
		TTATATTACTGAGTATC
<i>tetK</i>	CTGCTGCATTCCCTTCACTG	CTTCACCTAAAGCTACAATTGATCC
<i>griA</i> (wild type)	CAATATCATCCACATGGAGACTC	CTGGCGGATCATTATCGATAC
<i>griA</i> (mutated)	CAATATCATCCACATGGAGACTA	CTGGCGGATCATTATCGATAC
<i>arcA-ACME</i>	AAGTTGCACAAGAAGAGCATGAC	CTCGCGTACTTCTGGCTC
<i>RNAIII</i>	GGAAGGAGTGATTTCAATGG	GGGATGGCTTAACTCATAC
<i>hla</i>	AATAACTGTAGCGAAGTCTGGTGAAA	GCAGCAGATAACTTCCTTGATCCT
<i>hlgC</i>	CAATCAGCCCCATCACTCGG	TCGCTTTGACGCCCCATAAA
<i>LukS-PV</i>	GCTGCAACATTGTCGTTAGGAA	GACTACCTCAGCGCCATCAC
<i>psma</i>	TATCAAAAGCTTAATCGAACAATTC	CCCCTTCAAATAAGATGTTTCATATC

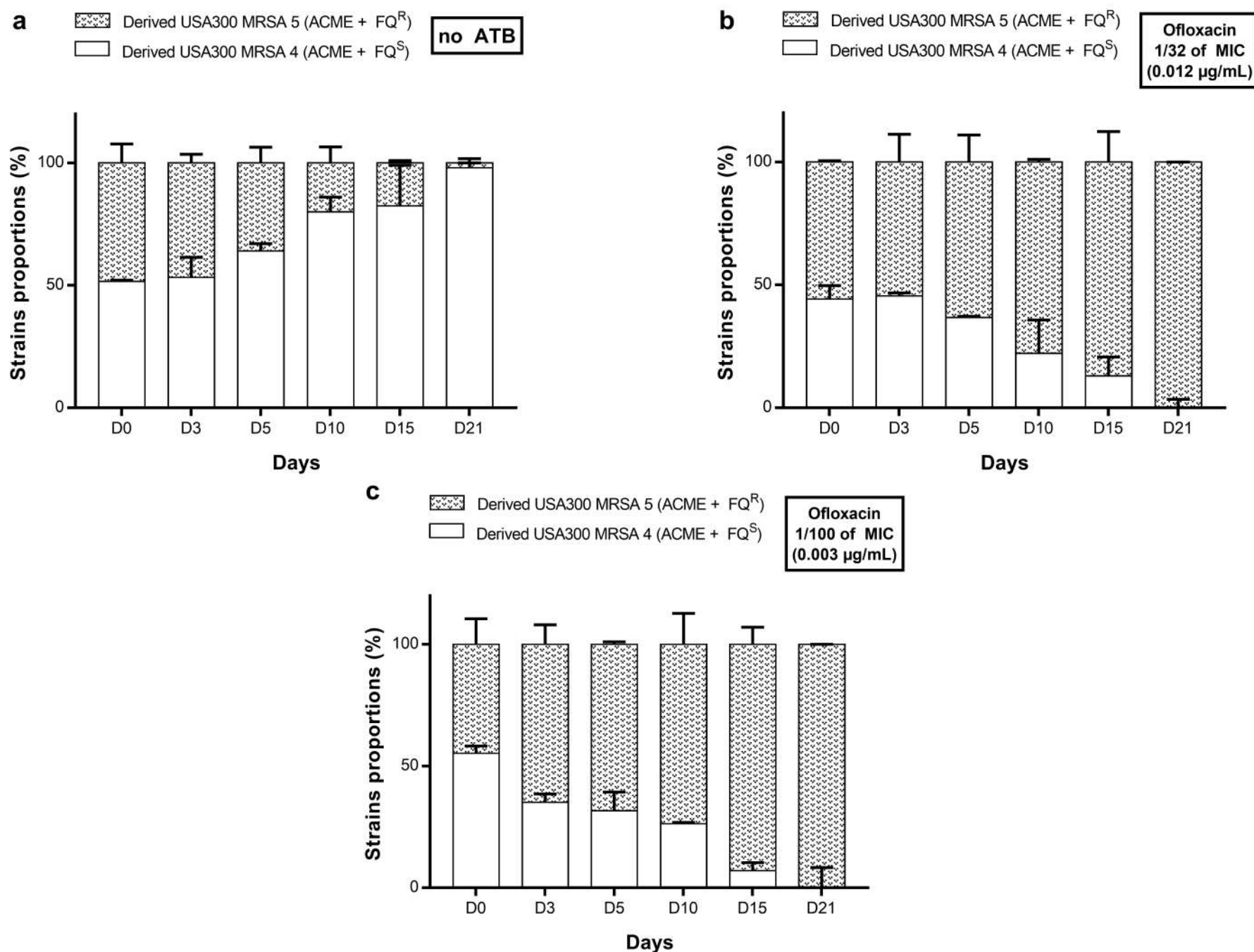
Sup. Fig.1a. Phylogenetic reconstruction of ST8 and its derived USA300 lineage, adapted from Glaser *et al.*, mBio 2016; doi:10.1128/mBio.02183-15. The letters in bracket refer to the strain designation of Fig. 1 and Table 1





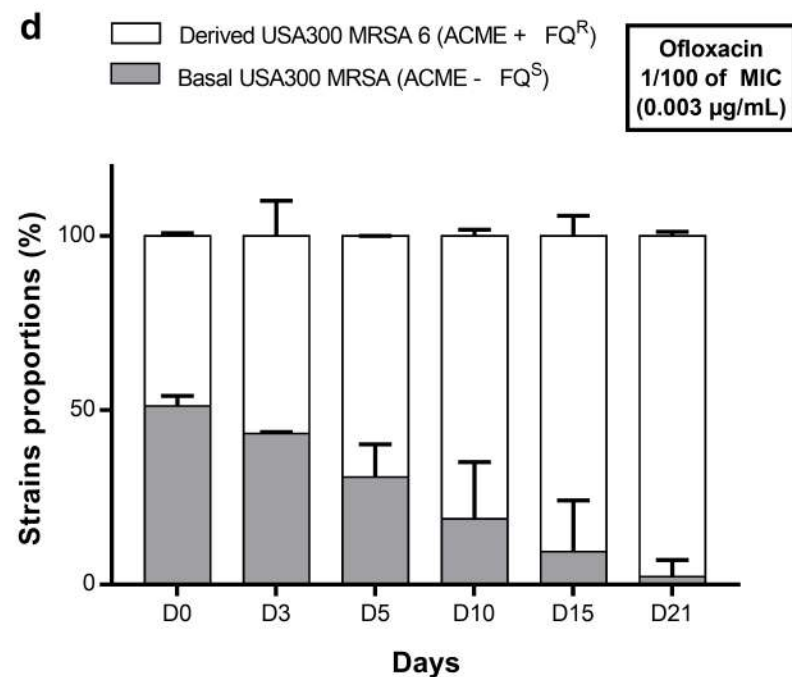
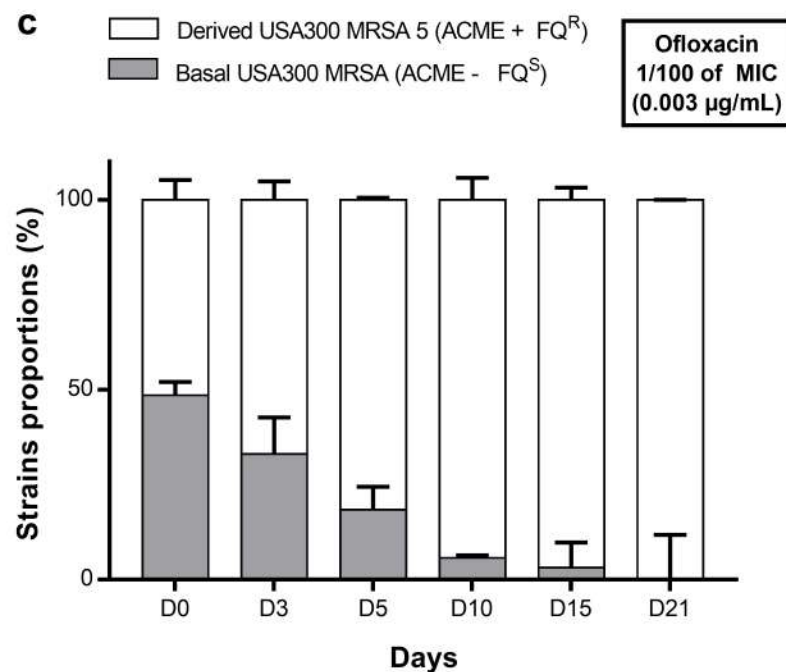
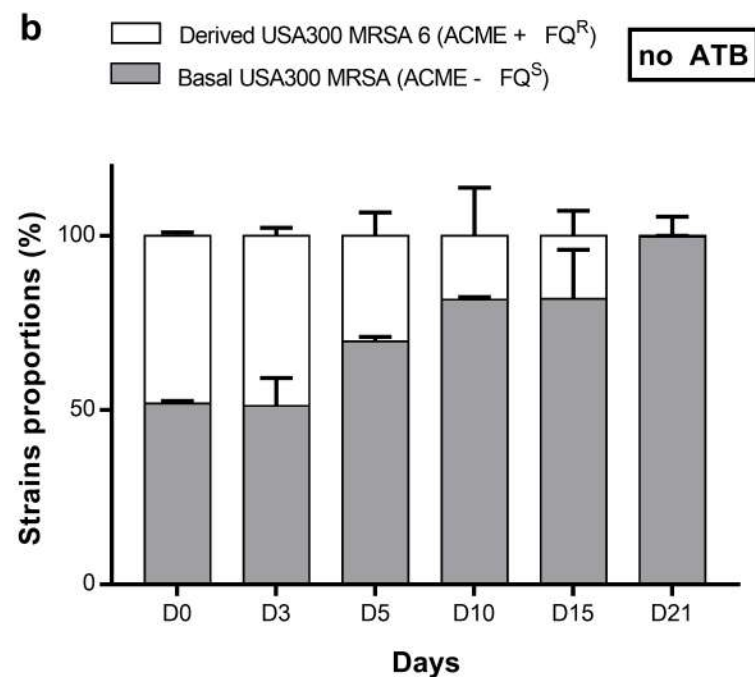
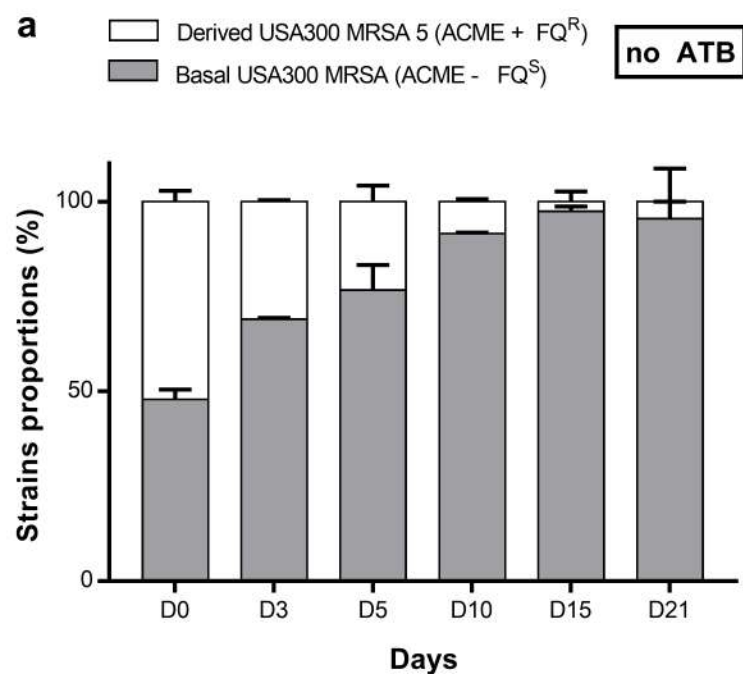
Supplementary figure 2: Impact of *mecA* and *ACME* in competitive fitness of USA300.

Basal USA300 MRSA and Derived USA300 MRSA 2 were co-cultivated for 21 days in BHI with daily subculture in fresh medium without antibiotic (a), or (b) containing ceftriaxone at 1/32 of ceftriaxone MIC of the susceptible strain (0.125 µg/mL), or (c) 1/100 MIC (0.04 µg/mL). The proportion of each strain was monitored at day 0, 3, 5, 10, 15 and 21 with qPCR targeting *arcA*-ACME. Competitive cultures were performed on three independent series (biological replicates), and each colony count or qPCR was repeated three times (technical replicates). Error bars represent 95% confidence interval



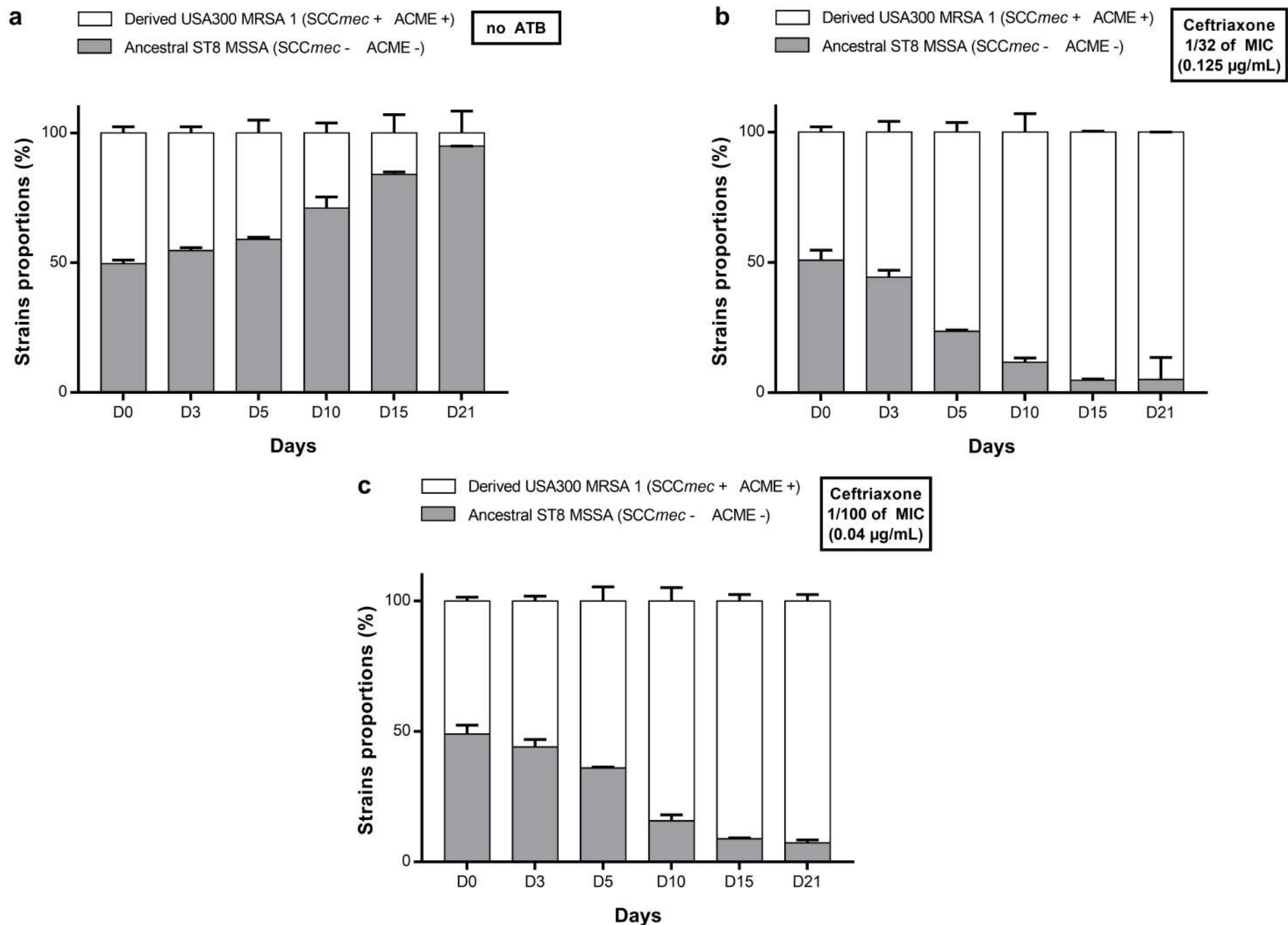
Supplementary figure 3: Potential impact of FQ resistance in competitive fitness of USA300.

(a) Fluoroquinolone (FQ)-susceptible Derived USA300 MRSA 4 and FQ-resistant Derived USA300 MRSA 5, both ACME-positive, were co-cultivated for 21 days in BHI without antibiotics, or (b) containing ofloxacin at 1/32 of FQ MIC of the susceptible strain (0.012 µg/mL), or (c) 1/100 MIC (0.003 µg/mL) with daily subculture in fresh medium. The proportion of each strain was monitored at day 0, 3, 5, 10, 15 and 21 with differential colony count based on selective agar inoculated with a calibrated amount of competitive mix. Competitive cultures were performed on three independent series (biological replicates), and each colony count or qPCR was repeated three times (technical replicates). Error bars represent 95% confidence interval.



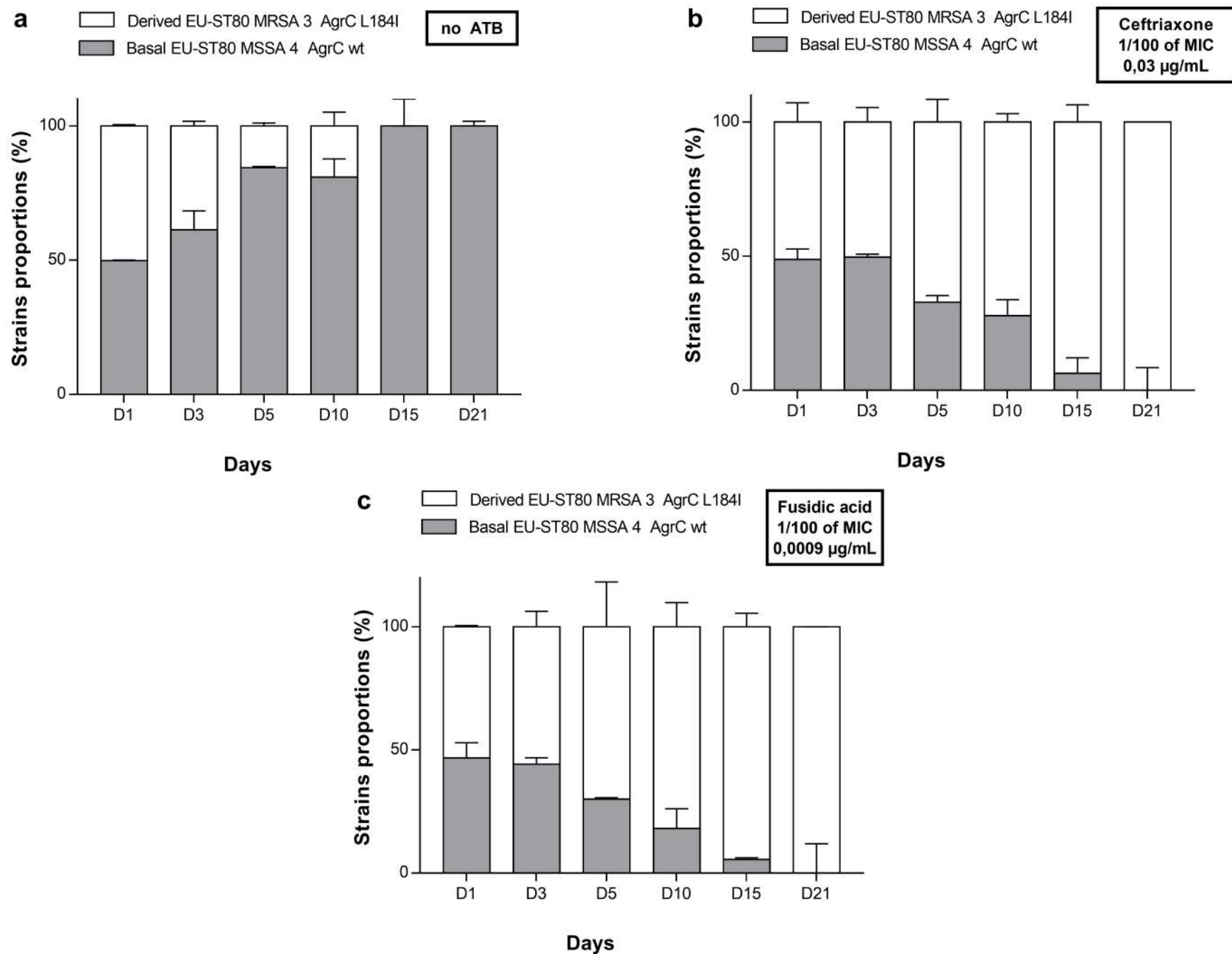
Supplementary figure 4: Potential impact of FQ resistance in competitive fitness of USA300.

Fluoroquinolone (FQ)-susceptible ACME-negative Basal USA300 MRSA and FQ-resistant ACME-positive Derived USA300 MRSA 5 and 6, were co-cultivated for 21 days in BHI without antibiotics (**a and b**), or (**c and d**) containing ofloxacin at up to 1/100 of FQ MIC of the susceptible strain (0.003 µg/ml) with daily subculture in fresh medium. The proportion of each strain was monitored at day 0, 3, 5, 10, 15 and 21 with qPCR targeting *arcA*-ACME. Competitive cultures were performed on three independent series (biological replicates), and each colony count or qPCR was repeated three times (technical replicates). Error bars = 95% confidence interval.



Supplementary figure 5: Impact of *mecA* and *ACME* in competitive fitness of USA300.

(a) Ancestral ST8 MSSA and Derived USA300 MRSA 1 were co-cultivated for 21 days in BHI with daily subculture in fresh medium without antibiotics, or (b) containing ceftriaxone at 1/32 of ceftriaxone MIC of the susceptible strain (0.125 µg/mL), or (c) 1/100 MIC (0.04 µg/mL). The proportion of each strain was monitored at day 0, 3, 5, 10, 15 and 21 with qPCR targeting *mecA*. Competitive cultures were performed on three independent series (biological replicates), and each colony count or qPCR was repeated three times (technical replicates). Error bars = 95% confidence interval.



Supplementary figure 6: Potential impact of *mecA/fusB* acquisition and *agrC* mutation on competitive fitness of EU-ST80.

(a) Basal EU-ST80 MSSA 4 *agrC* wild-type was co-cultivated with Derived EU-ST80 MRSA 3 carrying the AgrC L184I mutation, for 21 days in BHI without antibiotics or **(b)** containing ceftriaxone or **(c)** fusidic acid at 1/100 of MIC for the MSSA (0.03 µg/mL or 0.0009 µg/mL respectively) with daily subculture in fresh medium. The proportion of each strain was monitored at day 0, 3, 5, 10, 15 and 21 with differential colony count based on selective agar inoculated with a calibrated amount of competitive mix. The same results were obtained with antibiotics concentrations of 1/16 and 1/32 of their MICs (data not shown). Competitive cultures were performed on three independent series (biological replicates), and each colony count or qPCR was repeated three times (technical replicates). Error bars = 95% confidence interval.