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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ABSTRACT

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

40 the expansion of both European-ST80 and USA300 lineages in community setting. This surge
41 was likely driven by antibiotic (ab)use promoting the accumulation of antibiotics as
42 environmental pollutants. The current results provide a novel link between effective population
43 size increase of a pathogen and a selective advantage conferred by antibiotic resistance.
44
45 **INTRODUCTION**

46 *Staphylococcus aureus* remains one of the most common causative agents of both nosocomial
47 and community-acquired infections. It colonizes asymptomatically about one third of the human
48 population and may cause infections with outcomes ranging from mild to life-threatening
49 (Lowy, 1998). Until the mid-1990's, methicillin-resistant *S. aureus* (MRSA) infections were
50 reported almost exclusively from hospital settings and most hospital-associated MRSA (HA-
51 MRSA) diseases resulted from a limited number of successful clones (Thurlow *et al.*, 2012).
52 These HA-MRSA, which remained confined to healthcare settings, were exposed to a high
53 antibiotic pressure among patients with frequent immunity impairment and/or invasive devices
54 such as urinary/vascular catheters or mechanical ventilation (Chavez and Decker, 2008).
55 Therefore, HA-MRSA were likely under strong positive selection within these healthcare-
56 associated niches where the acquisition of resistance to multiple antibiotic families provided
57 them with a major competitive advantage despite their impaired fitness. However, in the
58 beginning of 2000's, MRSA infections began to be reported in healthy individuals without known
59 risk factors or apparent connections to healthcare institutions (Chambers, 2001), (Vandenesch
60 *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) SCC*mec*IVa (standing for staphylococcal cassette chromosome encoding methicillin resistance gene of type IVa) pulsotype USA300 in the USA (abbreviated to "USA300" below), the ST80 SCC*mec*IV in Europe, North Africa and the Middle East (hereinafter referred to as "EU-ST80"), and the ST30 SCC*mec*IV 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 SSC*mec* mobile element is a well-described example; large SCC*mec* elements shared by HA-MRSA induced a stronger fitness decrease compared to small SCC*mec* of CA-MRSA (Ma *et al.*, 2002), the latter being therefore promoted under lightened antibiotic pressure outside of healthcare settings. 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 Bayesian evolutionary models inferring their population size through time among hundreds of isolates sampled from 1980's to 2000's. Those phylogenetic analyses strongly suggested that in

the transition from an MSSA lineage to a successful CA-MRSA clone, the USA300 lineage first became resistant to multiple antibiotics, acquired the arginine catabolic mobile element (ACME) which encodes factors promoting skin colonization and infection (Thurlow *et al.*, 2013), and subsequently acquired resistance to fluoroquinolones (Planet *et al.*, 2015). These two steps were associated with two successive phases of sharp demographic expansion of what is known as the USA300 North-American (NA) lineage as opposed to the Latin-American Variant (LV) which does not harbor ACME (Glaser *et al.*, 2016). A similar study, performed on the EU-ST80 epidemic CA-MRSA lineage, depicted a clone derived from a Panton-Valentine (PVL)-positive methicillin-susceptible *S. aureus* (MSSA) ancestor from sub-Saharan Africa that dramatically expanded in the early 1990's once out of West Africa, upon acquisition of the SCC*mec* element, the plasmid-encoded fusidic-acid resistance (*fus*B) and four canonical SNPs including a non-synonymous mutation in the accessory gene regulator C (*agrC*) (Stegger *et al.*, 2014), a major virulence regulator in *S. aureus* (Reynolds and Wigneshweraraj, 2011). However, for both the USA300 and the EU-ST80 lineage it remains to be demonstrated that the identified genetic events, which correlate with the demographic expansion, are causally related with population size variations. In order to answer these questions, we explored fitness 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 phylogenic 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, ten clinical strains plus one reference strain were included (Fig. 1a & Table 1): (i) two strains corresponding to the most recent common ancestor of the USA300 clone, lacking the ACME sequence (Basal USA300 1 & 2), (ii) four strains from the early expansion phase characterized by ACME and SCC*mec* acquisition (Derived USA300 1, 2, 3 & 4), (iii) four strains from the most recent evolutionary phase subsequent to fluoroquinolones resistance acquisition (Derived USA300 5, 6, 7 & 8). 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); The competitive strain pairs are summarized in Table S1. Reference strain

for the USA300 and EU-ST80 lineages were SF8300-LUG2295 and HT20020209-LUG1799 respectively (Table 1).

Construction of 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 agrC2912/agrC555 and agrC544/agrC4238 primers respectively (Table S2). DNA fragments were then blunt-ended by *Sca*I and *Pvu*II restriction enzymes, before being ligated and amplified using external primers agrC2912/agrC4238. The resulting DNA fragment corresponding to an *agrC* encoding sequence for the mutated amino-acid I184 was restricted by *Xho*I and *Pvu*II and cloned in pMAD linearized by *Sal*I and *Sma*I. 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

143 under agitation (200 rpm). One milliliter of bacterial suspension was harvested and
144 concentration adjusted to an OD_{600nm} = 1.0. Bacteria were washed in 10 mM Tris buffer and
145 treated with lysostaphin and β-mercaptoethanol. RNAs were extracted with the RNeasy Plus
146 Mini Kit® (Qiagen), quantified by spectrophotometry and stored at -80°C. This process was
147 repeated on three different days for biological replicates.

148 *RNA quantification by real-time PCR.*
149 A random-primers based reverse transcription of 1µg of RNA was performed with the A3500
150 Reverse Transcription System Kit (Promega), followed by quantitative real-time PCR on cDNA
151 using the FastStart Essential DNA Green Master kit (Roche) and the LightCycler® Nano (Roche).
152 As previously described (Li *et al.*, 2010), we targeted five virulence genes (*RNAIII, lukS-PV, hla,*
153 *hlgC, psmα*) and the housekeeping gene *gyrB* for normalization. Gene expression levels were
154 compared between our clinical isolates and against the reference strains (SF8300-LUG2295 for
155 the USA300 clone and HT20020209-LUG1799 for the EU-ST80 clone); levels were expressed as
156 n-fold differences relative to reference strains or an isolate from another evolutionary phase.
157 These qRT-PCR were performed as technical triplicates (three RNA quantification per RNA
158 sample), on RNA obtained from three biological replicates (three independent cultures and
159 extractions per strain).

160 *Biofilm production assay.*
161 Each isolate was incubated overnight on blood agar (Columbia) at 35°C under ambient air. Three
162 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, 24 and 48h respectively. Biofilm production was assessed by spectrophotometry after well drying and crystal violet fixation. *S. aureus* laboratory strains SH1000 was added as a positive control (Horsburgh *et al.*, 2002), (O'Neill, 2010), *S. epidermidis* ATCC12228 (Zhang *et al.*, 2003) and *S. carnosus* TM300 (Rosenstein *et al.*, 2009) as negative controls of adhesion. 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 according to EUCAST specifications.

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 ofloxacine, ceftriaxone, or fusidic acid were added at final concentrations corresponding to ¼ 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°. 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 *agr*C 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.

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). The experiment performed on ten USA300 isolates pointed out the impact of ACME acquisition on doubling time shortening (Derived USA300 1 or 2 versus Basal USA300 1 or 2, Mann-Whitney test, $P = 0.029$) (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). 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; 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.

244

245 *Expression of core-genome encoded virulence factors along the lineages' evolutionary history*.
246 Previous studies revealed that overexpression of core-genome encoded virulence factors was a
247 common feature of CA-MRSA, a characteristic that has been proposed to contribute to the
248 expansion of these lineages (Li *et al.*, 2010). We therefore tested whether variations in the
249 expression of core genome encoded virulence factors along the Bayesian demographic models
250 could be observed (Fig. 1). To this end, RT-PCRs targeting virulence factors of the core- (α-toxin,
251 PSMα, γ-toxin) and accessory-genome (*LukSF-PV*), as well as the major regulator (*agr*-RNAIII),
252 were performed after *in vitro* post-exponential growth as previously described (Li *et al.*, 2010).
253 Among the USA300 CA-MRSA isolates, despite an outlier strain with no measurable expression
254 of *hla*, no major variations were detected in expression levels of the targeted virulence factors
255 (above the accepted 2-fold level generally considered as a minimum biological relevant variation
256 in RT-PCR approaches) between the strains representing the various steps of the demography
257 (Fig. 3a). This lack of significant differences was observed by either using an ancestral strain
258 (Basal USA300 1) or the reference strain SF8300 as comparators (Fig. 3a). For the EU-ST80
259 lineage, most of the targeted virulence factors studied showed variation in expression below -
260 or close to - two-fold, between ancestral and derived isolates with the exceptions of i) *psmα*
261 increasing by 3 - 3.5-fold in two isolates from the evolutionary-derived clade (designated
262 derived MRSA 2 and 5), and ii) *luk*SF-PV increasing by a factor of 2.8-fold in one isolate (derived
263 MRSA 3) (Fig. 3b). In addition, expression of RNAIII, the *agr*-related regulatory RNA, was slightly

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

266 All the EU-ST80 isolates from the derived clade harbor an L184I mutation in the extracellular
267 loop of the AgrC receptor (Stegger *et al.*, 2014), a mutation that may have a functional impact
268 on Agr signalling and expression of *agr*-RNAIII. To further investigate this point, an ancestral
269 ST80 (AgrC L184) was engineered by allelic replacement to carry the L184I substitution and was
270 then tested (as "Lab Mutated Basal MSSA") for quantification of RNAIII and virulence factor
271 expression. Compared to wild type (L184), the mutated (L184I) isogenic derivative (Lab Mutated
272 Basal MSSA) showed a slight enhancement of RNAIII expression, but below the level of 2 (Fig.
273 3b). This mutation had no significant impact on virulence gene expression, except a mild 2.2-fold
274 increase in *psm*α expression (Fig. 3b).
275
276 *Biofilm production.*

277 The detection of a slight difference in RNAIII production associated with the *agrC* mutation in
278 the EU-ST80 lineage prompted us to test whether it could translate into differences in biofilm
279 production. After 48 hours of growth, ancestral MSSA strains displayed a higher production of
280 biofilm compared to derived MRSA strains carrying the L184I AgrC substitution ($P = 0.0002$) (Fig.
281 4). The role of AgrC L184I substitution in this phenotypic difference was confirmed by
282 comparing the ancestral ST80 (AgrC L184) with its isogenic derivative (L184I), the latter showing
283 a significant reduction of biofilm production ($P < 0.0001$). Importantly, the AgrC L184I mutation
284 had no impact on crude fitness since doubling times of Lab Mutated Basal MSSA and its wild

type parental strain 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 phylogenic 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). 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 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 µg/mL) was sufficient to confer a strong selective advantage of FQ-resistant ACME-positive strain toward FQ-susceptible ACME-positive strain (Fig. 6). 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 SCC*mec/fusB* and further extended antibiotics resistance. In competitive culture assays, the laboratory engineered *agr*C 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 SCC*mec* and *fus*B) 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 µg/mL, and $^1/_{100}$ of MSSA fusidic acid MIC, 0.0009 µg/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" (Uhlemann *et al.*, 2014). 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 short-term evolution of CA-MRSA. However, by assessing transcription of virulence factors previously described as overexpressed among CA-MRSA lineages (Li *et al.*, 2010), we could detect only minor variation (ca. 1.5-fold increase) in virulence factor expression between USA300 strains when comparing ancestral and derived isolates of the North American clone (Fig. 3a); we cannot rule out however that, at the population level, these minor increases in virulence factor expression enhanced the success of the lineage, for instance by increasing

cutaneous infection rate (the most common infections caused by *S.aureus*) and thus human-to-human transmission by skin contact as observed in prisons, sport team or men-having-sex-with-men (Planet *et al.*, 2015). Within this USA300 clone, 20 SNPs were identified for being under positive selection along successful evolution of this lineage (Glaser *et al.*, 2016); however, all the derived isolates assessed for doubling time carried these 20 SNPs. Therefore, despite being under positive selection they could not be reliable determinants of crude fitness evolution of the derived clade isolates as competitive fitness impairment observed along the phylogeny could not be explained by these genetic variations. Thus, the major variable feature of USA300 along the demography was the acquisition of ACME which is a now well-characterized mobile genetic element (MGE) acquired from *S. epidermidis* by horizontal gene transfer (Diep *et al.*, 2006), (Pi *et al.*, 2009), (Uhlemann *et al.*, 2014). Its multiple functions in resistance to acidic pH which enhances skin colonization, and as a factor promoting resistance to skin innate-immune defences (Thurlow *et al.*, 2012) makes it a very plausible contributor of the USA300 expansion (Planet, 2017). This is further strengthened by our findings of a shorter doubling-time of strains carrying ACME (Fig. 2a). In the case of EU-ST80, analysis of virulence factor expression along the demographical steps of the lineage showed that two derived isolates had a two-fold increase in *psm*α expression and another one had a 2.5-fold increase in PVL expression when compared to the ancestral isolates. As previously described (Stegger *et al.*, 2014), isolates of the basal and derived clades of this lineage were discriminated by four canonical SNPs. One was located in a non-coding region, two were synonymous SNPs, and one was a non-synonymous SNP located in *agrC*, the major virulence factor regulator involved in quorum sensing and biofilm production.

We focused our attention on this SNP located in the *agrC* gene, because of its potential association to fitness and colonization ability. This SNP resulted in a L184I amino acid change in the extracellular loop of the AgrC receptor (Stegger *et al.*, 2014) shared by all EU-ST80 isolates belonging to the derived clade. To investigate this point further, an ancestral ST80 (AgrC L184) was engineered by allelic replacement to carry the L184I substitution. Despite a slight increase of doubling time compared to its parental strain, the L184I change in AgrC did not translate into significant crude fitness variation (Mann-Whitney test, P = 0.343). Assessment of virulence factor expression revealed a two-fold increase of *psm*α in the Lab Mutated basal MSSA compared to its parental wild-type strain (Fig. 3b). AgrC L184I could therefore have a moderate impact on virulence. We further detected a strong and significant decrease in biofilm production associated with the AgrC L184I mutation (Fig. 4). Assessing which of these phenotypes (slight increase in PSMα or PVL, strong decrease in biofilm) was under selection remains speculative because they could be strongly dependent on the ecosystem in which selection has occurred. However, little is known regarding these ecological conditions since the current model for CA-MRSA ST80 lineage expansion places the acquisition of the AgrC L184I mutation in the early 1990s in strains originating from Sub-Saharan Western Africa, concomitantly with the acquisition of SCC*mec*IV and *fus*B (Stegger *et al.*, 2014). Alternatively, the AgrC L184I substitution might be a non-adaptive sequel – a genetic drift – parallel to the acquisition of SCC*mec*IV and *fusB*. Importantly, antibiotic resistances were associated with demographic expansion of EU-ST80 (acquisition of SCC*mec*IV and *fus*B) and also North American USA300 (acquisition of SCC*mec* and fluoroquinolone resistance) (Fig. 1) (Stegger *et al.*, 2014), (Glaser *et*

389 *al.*, 2016). These resistance acquisitions were associated with a significant fitness cost as
390 indicated by both extended doubling-time of the derived isolates (Fig. 2) and by the results of
391 competition experiments where derived isolates were outcompeted by their basal counterparts
392 (Fig. 5-7). These observations were in accordance with the classical fitness costs associated with
393 de novo antibiotic resistance, specifically those selected at high antibiotic concentration
394 (Martinez, 2009), (Andersson and Hughes, 2014). Conversely, they did not match the Bayesian
395 evolutionary models of these lineages as strains belonging to the epidemic phase (derived
396 clade) displayed the lowest *in vitro* competitive fitness, with each step of fitness decrease being
397 associated with new acquisition of antibiotic resistance (Fig. 2, Table 1). However, the most
398 striking observation was that extremely low concentrations of antibiotics (those for which
399 resistance acquisition correspond to demographic expansion of the two lineages), totally
400 reversed this fitness cost. Since both USA300 and EU-ST80 likely emerged in low income
401 populations(Vandenesch *et al.*, 2003), (Martinez, 2009), (Planet, 2017), the role of antibiotic
402 selective pressure was not initially considered to be the major trait under positive selection.
403 However, increasing number of reports reveals the escalation of antibiotic as environmental
404 pollutants originating from hospital wastewater, bulk drug producer wastewater and unused
405 antibiotics dumped in landfills in countries without solid take-back programs (Naimi *et al.*,
406 2003), (Thurlow *et al.*, 2012), (Larsson, 2014), (Gothwal and Shashidhar, 2015), (See *et al.*,
407 2017). From these sources, in which antibiotics such as fluoroquinolones can reach
408 concentrations ranging from 3 ng/L to 240 µg/L (Van Doorslaer *et al.*, 2014), antibiotics are
409 disseminated in various environmental matrices such as surface water, soil, sediments, and

410 eventually living organism including livestock (Van Doorslaer *et al.*, 2014). Hence, community
411 settings, even in remote populations, can be exposed to low-level concentrations of various
412 antibiotics that could have promoted the expansion of CA-MRSA at least by enriching for
413 resistant bacteria (Andersson and Hughes, 2014), if not selecting for de novo resistance, the
414 latter being typically associated with no fitness cost (Gullberg *et al.*, 2011), (Andersson and
415 Hughes, 2014), (Westhoff *et al.*, 2017). Here, we demonstrate with competition experiments
416 that the biological cost of antibiotic resistance (to beta-lactams, fusidic-acid and
417 fluoroquinolone) is entirely reversed in the presence of trace amounts of antibiotics. Previous
418 studies based on multidrug resistant plasmids showed that, for specific combinations of drugs,
419 each new compound added, lowered the minimal selective concentration of the others
420 (Gullberg *et al.*, 2014). However antibiotic resistance acquisitions (both by horizontal transfer of
421 resistance genes and by mutations) are the genetic events that best match the variation of the
422 demography in both lineages (Fig. 1a & b). Altogether, our findings support a model of antibiotic
423 use, misuse and pollution as a major driving force for the emergence and expansion of CA-
424 MRSA. In conclusion, CA-MRSA dynamics appear to be ruled by a complex interplay between
425 resistance, virulence and fitness cost in which the contribution of anthropogenic activities is
426 substantial.

427

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Supplementary information is available at the ISME Journal's website

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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 *mec*A gene and expressing an AgrC L184I variant. *S. aureus* SH1000 was used as positive control for biofilm production and *S.*

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

568

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

570 **(a)** ACME-negative and -positive strains, both MRSA with no associated antibiotics resistance were co-cultivated for
571 21 days in BHI with daily subculture in fresh medium. The proportion of each strain was monitored at day 1, 7, 14
572 and 21 with qPCR targeting *arcA*-ACME. **(b)** ACME-positive strains, one Fluoroquinolone (FQ)-susceptible (Derived
573 USA300 3) and one FQ-resistant (Derived USA300 5) were co-cultivated for 21 days in BHI with daily subculture in
574 fresh medium. The proportion of each strain was monitored daily with differential colony count based on selective
575 agar inoculated with a calibrated amount of competitive mix. Same results were obtained with a competition
576 between the FQ-resistant strain (Derived USA300 5) and another FQ-susceptible isolate (Derived USA300 4), data
577 not shown. **(c)** ACME-positive FQ-resistant strain (Derived USA300 5) and ACME-negative strain FQ-susceptible
578 (Basal USA300 1) were co-cultivated for 21 days and assessed as in **(b)**. Competitive cultures were performed on
579 three independent series (biological replicates), and each colony count or qPCR was repeated three times
580 (technical replicates).

581

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

583 **(a)** Fluoroquinolone (FQ)-susceptible and -resistant strains of USA300, both ACME-positive, were co-cultivated for
584 21 days in BHI without antibiotics, or **(b)** containing ofloxacin at $^1/_{16}$ of FQ MIC of the susceptible strain (0.024
585 µg/mL), **(c)** $^1/_{32}$ MIC (0.012 µg/mL) or **(d)** $^1/_{100}$ MIC (0.003 µg/ml) with daily subculture in fresh medium. The
586 proportion of each strain was monitored daily with differential colony count based on selective agar inoculated
587 with a calibrated amount of competitive mix. Competitive cultures were performed on three independent series
588 (biological replicates), and each colony count or qPCR was repeated three times (technical replicates).

589

590 **Figure 7: Potential impact of *mec*A/*fusB* acquisition and *agrC* mutation on competitive fitness**

591 **of EU-ST80.**

592 **(a)** The *mecA/fusB*-negative (Basal MSSA 3) AgrC L184 was co-cultivated with its *agrC* derivative (Lab Mutated Basal
593 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.

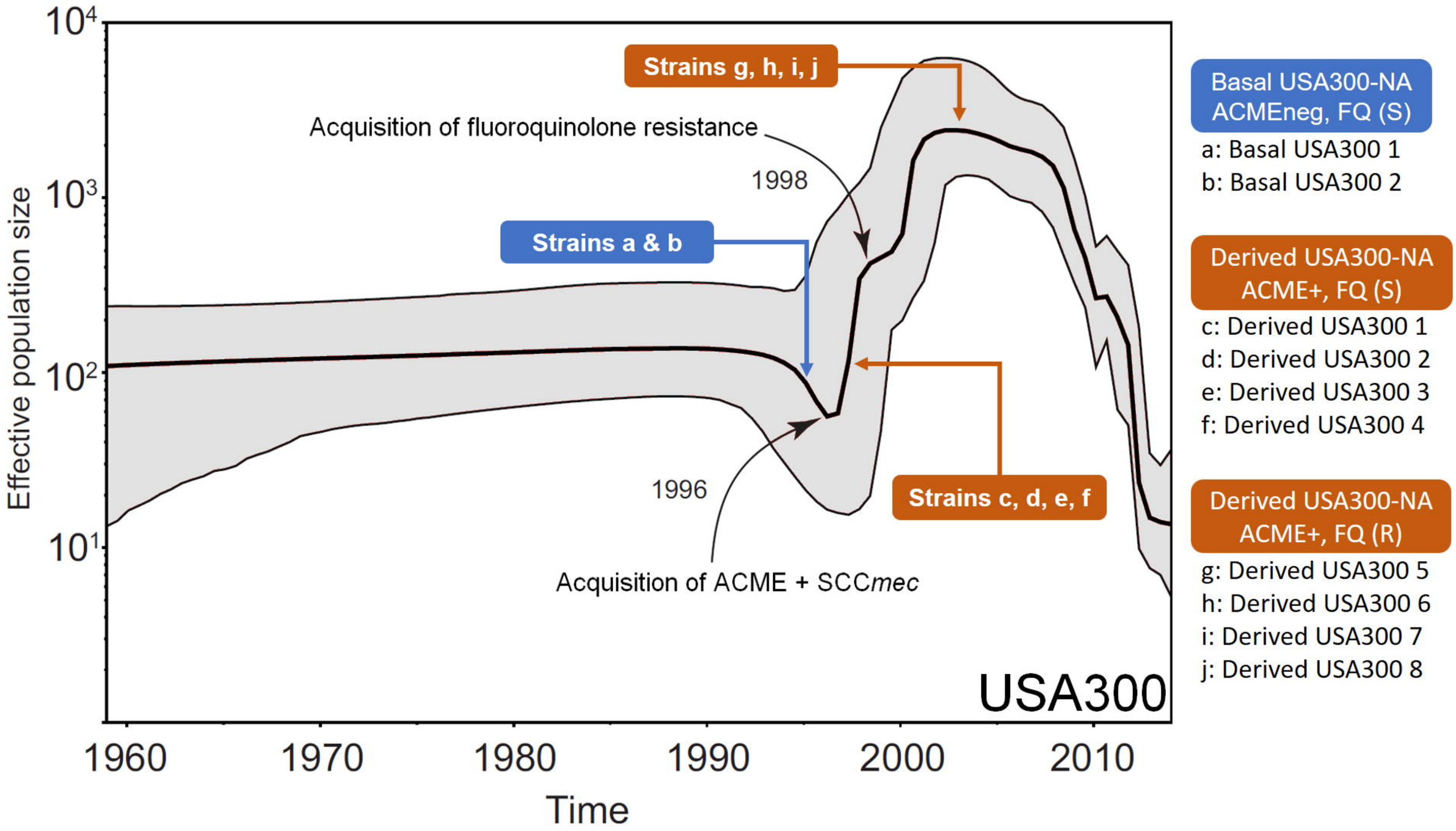
Table S1: Strains pairs used for competitive fitness study

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

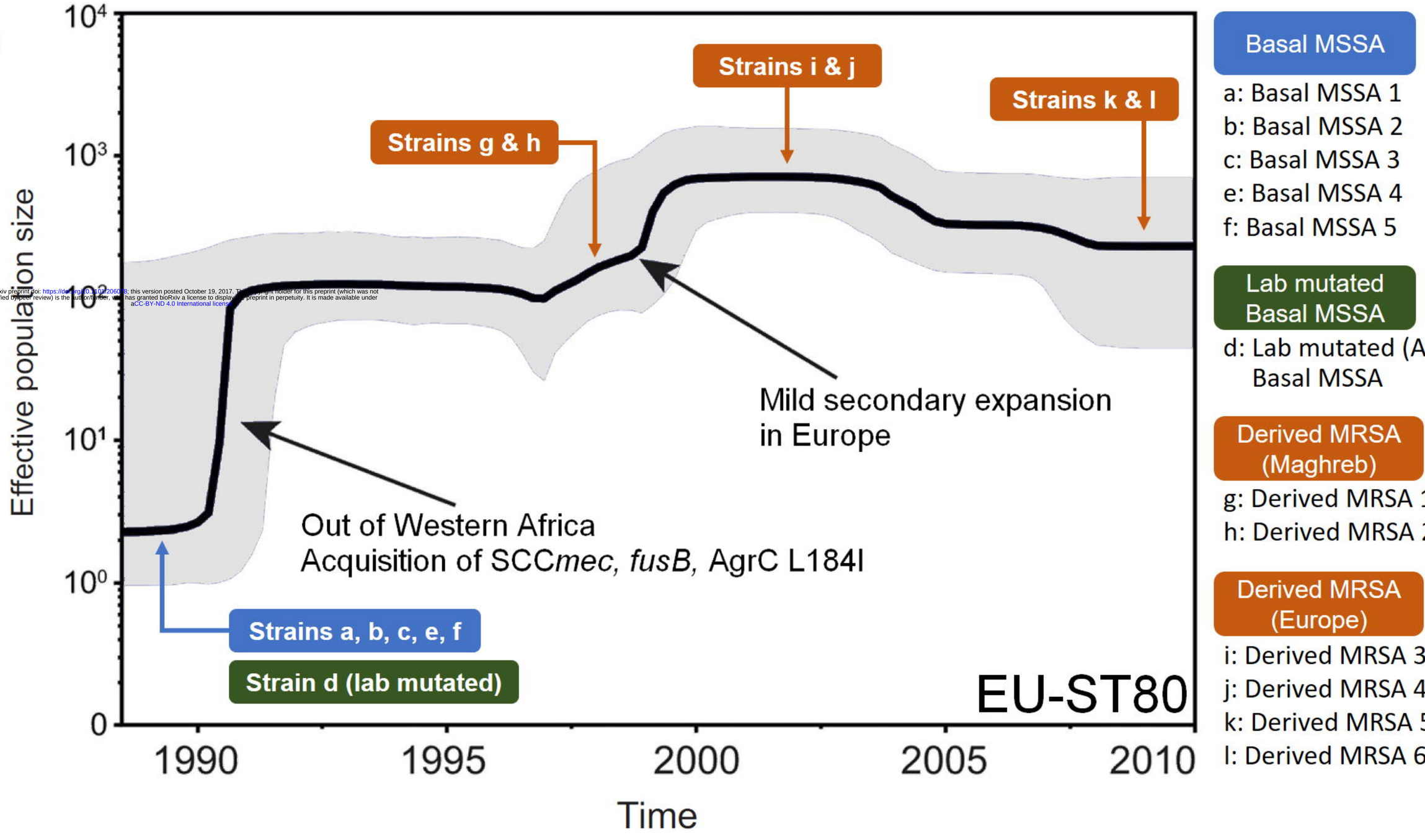
Table S2: Primers used for qPCR and RT-qPCR

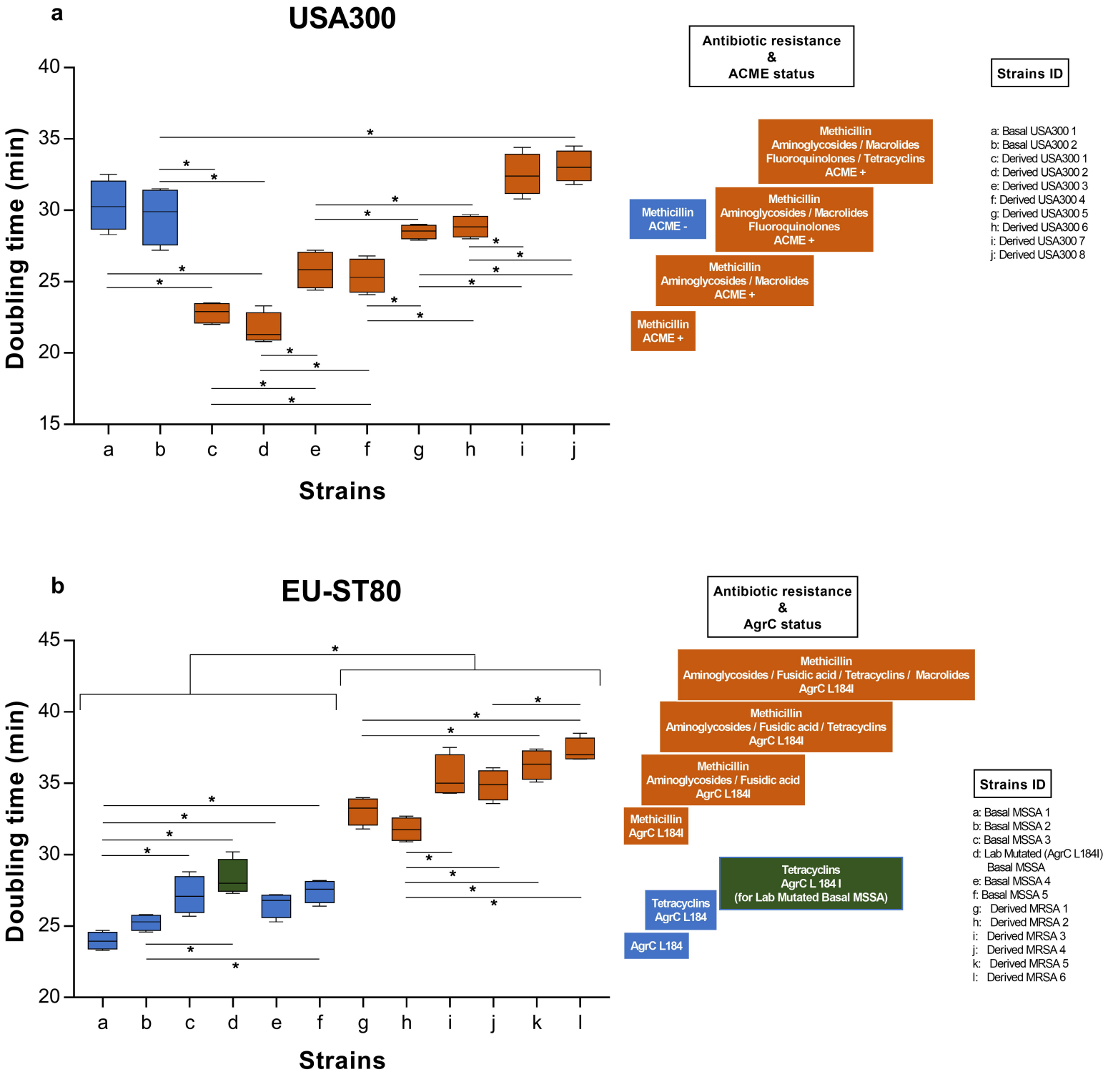
Primers list including target genes used for resistance-based strain discrimination (*mecA, tetK, grlA*), phylogenic clade discrimination (*agr*C, *arc*A-ACME), virulence factor expression assay (*RNAIII, hla, hlgC, lukS-PV, PSMα*), engineering of the Lab Mutated basal MSSA (*agrC*2912, 555, 4238, 544), and standardization (*gyr*).

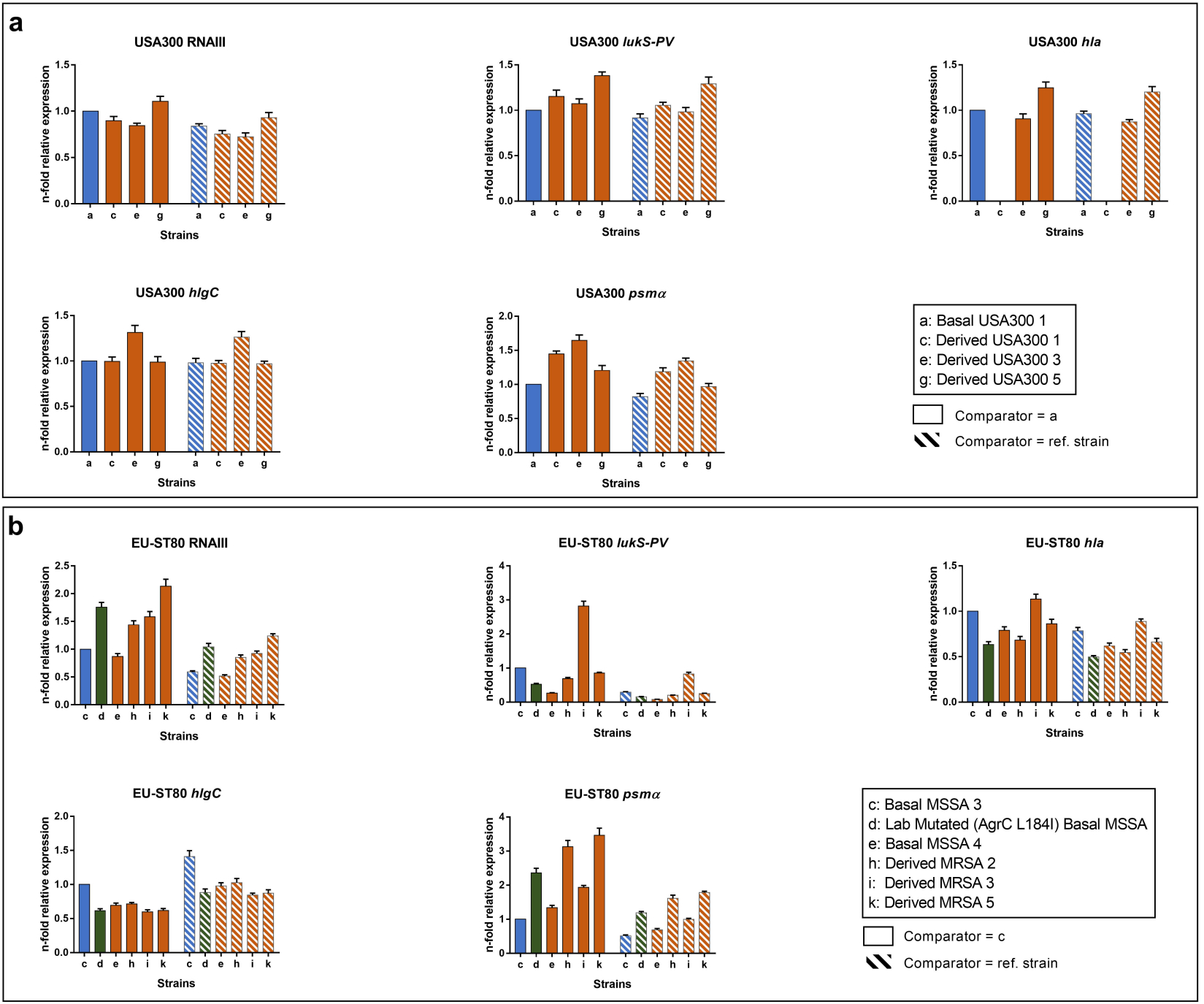
a

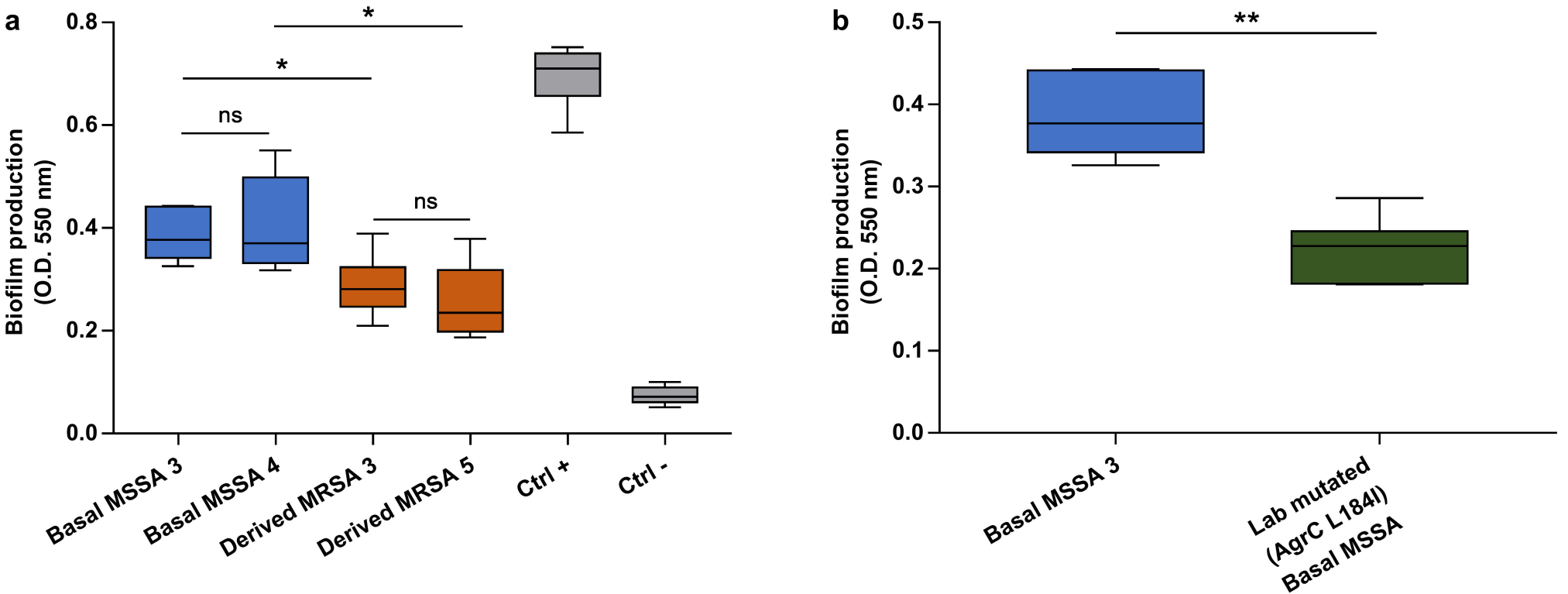


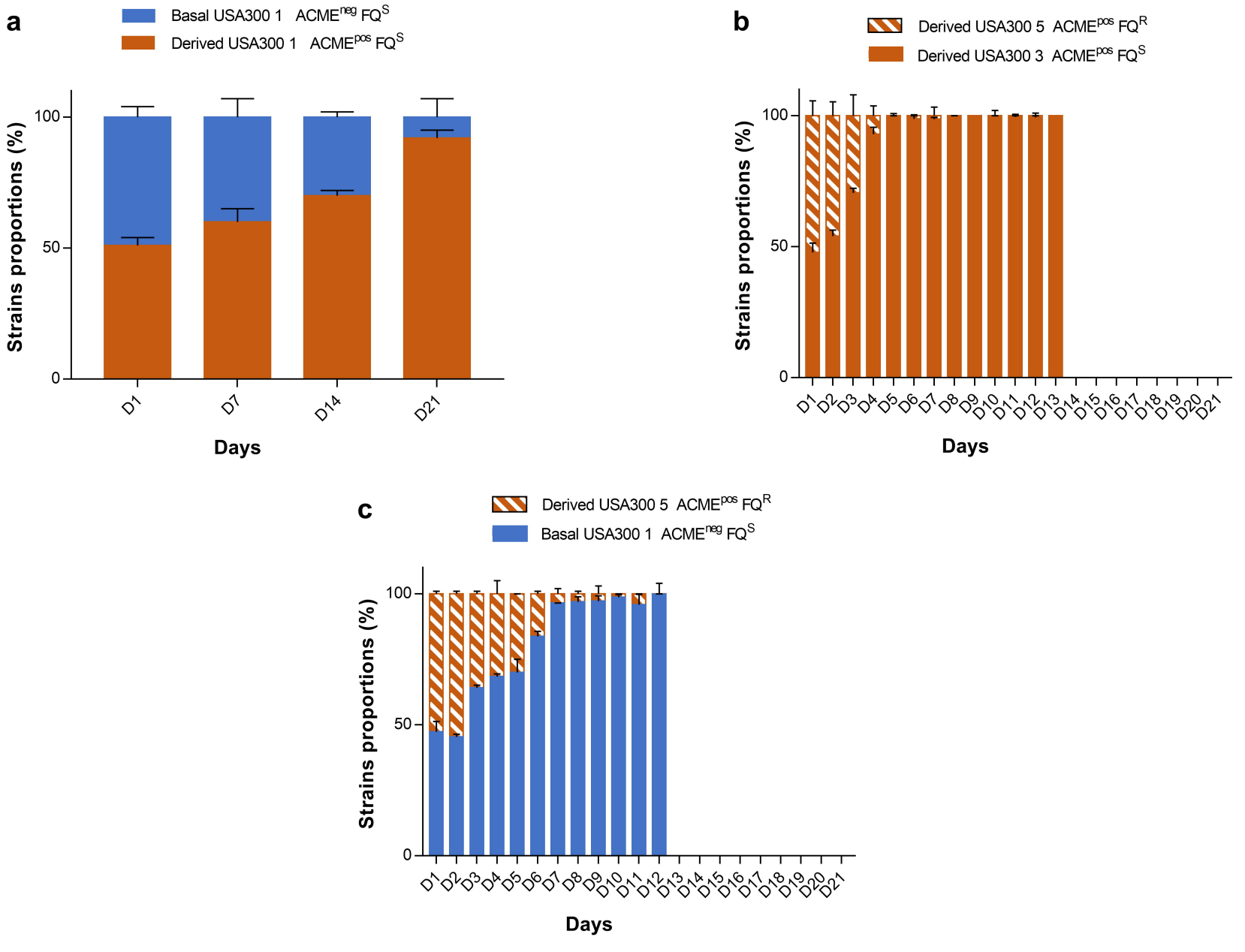
b

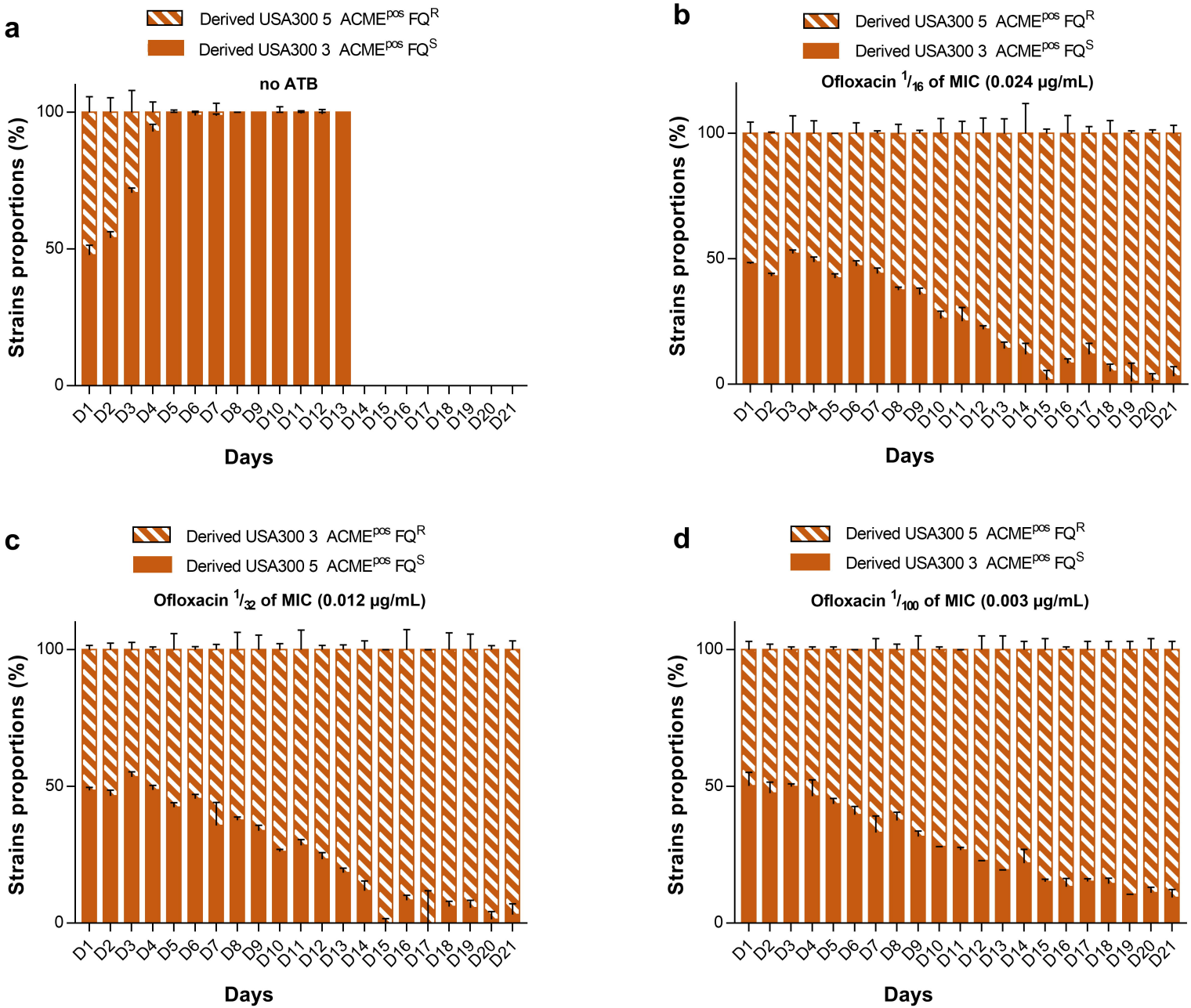



a
USA300
Doubling time (min)
40
35
30
25
20
15
a b c d e f g h i j
Strains
Antibiotic resistance & ACME status
Methicillin Aminoglycosides / Macrolides Fluoroquinolones / Tetracyclins ACME +
Methicillin ACME -
Methicillin Aminoglycosides / Macrolides Fluoroquinolones ACME +
Methicillin Aminoglycosides / Macrolides ACME +
Methicillin ACME +
Strains ID
a: Basal USA300 1
b: Basal USA300 2
c: Derived USA300 1
d: Derived USA300 2
e: Derived USA300 3
f: Derived USA300 4
g: Derived USA300 5
h: Derived USA300 6
i: Derived USA300 7
j: Derived USA300 8
b
EU-ST80
Doubling time (min)
45
40
35
30
25
20
a b c d e f g h i j k l
Strains
Antibiotic resistance & AgrC status
Methicillin Aminoglycosides / Fusidic acid / Tetracyclins / Macrolides AgrC L184I
Methicillin Aminoglycosides / Fusidic acid / Tetracyclins AgrC L184I
Methicillin Aminoglycosides / Fusidic acid AgrC L184I
Methicillin AgrC L184I
Tetracyclins AgrC L 184 I (for Lab Mutated Basal MSSA)
Tetracyclins AgrC L184
AgrC L184
Strains ID
a: Basal MSSA 1
b: Basal MSSA 2
c: Basal MSSA 3
d: Lab Mutated (AgrC L184I) Basal MSSA
e: Basal MSSA 4
f: Basal MSSA 5
g: Derived MRSA 1
h: Derived MRSA 2
i: Derived MRSA 3
j: Derived MRSA 4
k: Derived MRSA 5
l: Derived MRSA 6


a
USA300 RNAIII
USA300 lukS-PV
USA300 hla
USA300 hlgC
USA300 psmα
n-fold relative expression
Strains
a c e g a c e g
a: Basal USA300 1
c: Derived USA300 1
e: Derived USA300 3
g: Derived USA300 5
Comparator = a
Comparator = ref. strain
b
EU-ST80 RNAIII
EU-ST80 lukS-PV
EU-ST80 hla
EU-ST80 hlgC
EU-ST80 psmα
c d e h i k c d e h i k
c: Basal MSSA 3
d: Lab Mutated (AgrC L184I) Basal MSSA
e: Basal MSSA 4
h: Derived MRSA 2
i: Derived MRSA 3
k: Derived MRSA 5
Comparator = c
Comparator = ref. strain


a
Biofilm production (O.D. 550 nm)
0.0
0.2
0.4
0.6
0.8
*
*
ns
ns
Basal MSSA 3
Basal MSSA 4
Derived MRSA 3
Derived MRSA 5
Ctrl +
Ctrl -
b
Biofilm production (O.D. 550 nm)
0.0
0.1
0.2
0.3
0.4
0.5
**
Basal MSSA 3
Lab mutated (AgrC L184I) Basal MSSA


a
Basal USA300 1 ACME^neg FQ^S
Derived USA300 1 ACME^pos FQ^S
Strains proportions (%)
100
50
0
D1
D7
D14
D21
Days
b
Derived USA300 5 ACME^pos FQ^R
Derived USA300 3 ACME^pos FQ^S
Strains proportions (%)
100
50
0
D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21
Days
c
Derived USA300 5 ACME^pos FQ^R
Basal USA300 1 ACME^neg FQ^S
Strains proportions (%)
100
50
0
D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21
Days


a
Derived USA300 5 ACME^pos FQ^R
Derived USA300 3 ACME^pos FQ^S
no ATB
Strains proportions (%)
100
50
0
D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21
Days
b
Derived USA300 5 ACME^pos FQ^R
Derived USA300 3 ACME^pos FQ^S
Ofloxacin 1/16 of MIC (0.024 µg/mL)
Strains proportions (%)
100
50
0
D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21
Days
c
Derived USA300 3 ACME^pos FQ^R
Derived USA300 5 ACME^pos FQ^S
Ofloxacin 1/32 of MIC (0.012 µg/mL)
Strains proportions (%)
100
50
0
D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21
Days
d
Derived USA300 5 ACME^pos FQ^R
Derived USA300 3 ACME^pos FQ^S
Ofloxacin 1/100 of MIC (0.003 µg/mL)
Strains proportions (%)
100
50
0
D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21
Days

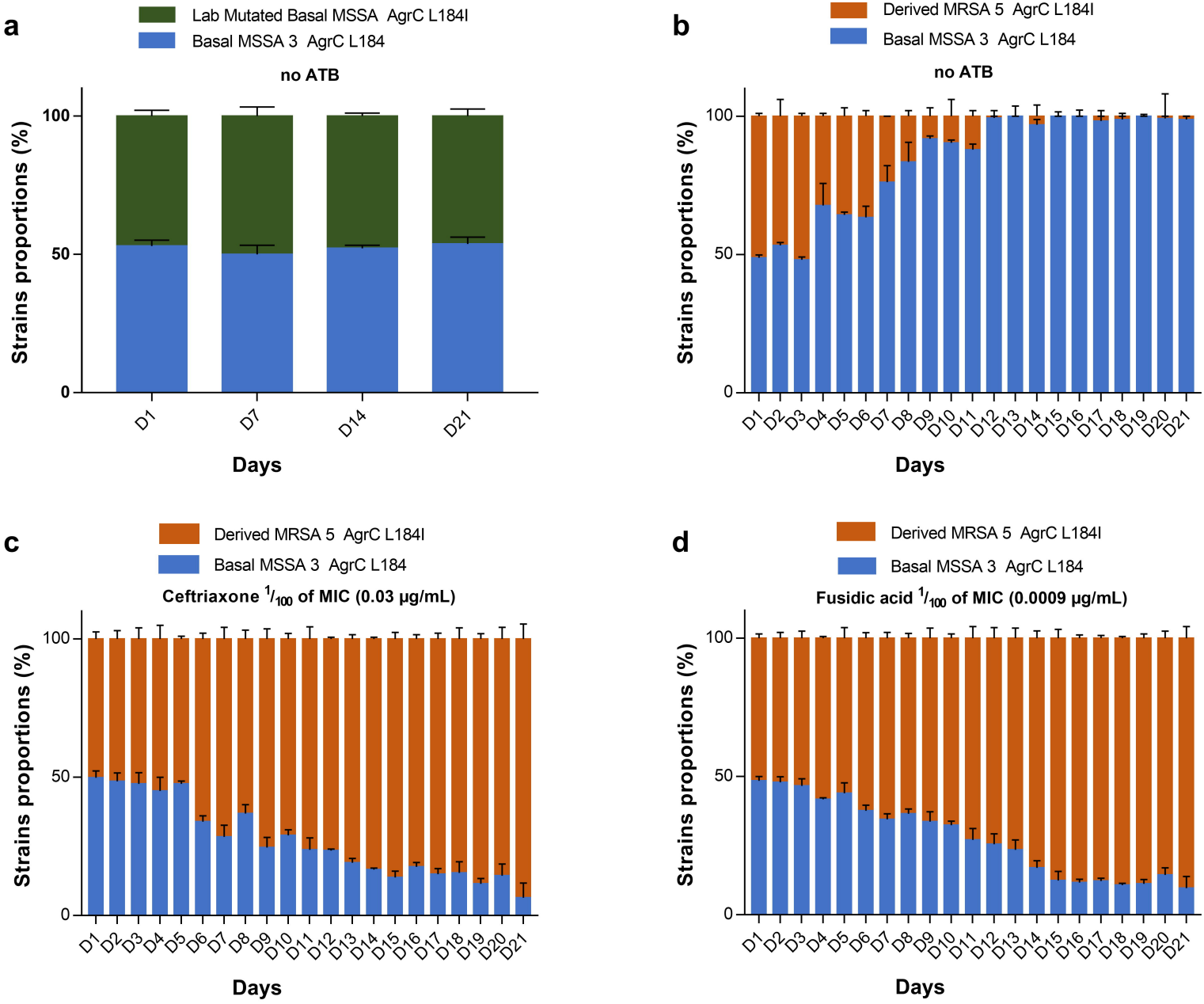

a
Lab Mutated Basal MSSA AgrC L184I
Basal MSSA 3 AgrC L184
no ATB
Strains proportions (%)
100
50
0
D1
D7
D14
D21
Days
b
Derived MRSA 5 AgrC L184I
Basal MSSA 3 AgrC L184
no ATB
Strains proportions (%)
100
50
0
D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21
Days
c
Derived MRSA 5 AgrC L184I
Basal MSSA 3 AgrC L184
Ceftriaxone 1/100 of MIC (0.03 µg/mL)
Strains proportions (%)
100
50
0
D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21
Days
d
Derived MRSA 5 AgrC L184I
Basal MSSA 3 AgrC L184
Fusidic acid 1/100 of MIC (0.0009 µg/mL)
Strains proportions (%)
100
50
0
D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21
Days

Table 1: Relevant characteristics of strains

STRAIN DESIGNATION	NRCS number	RELEVANT CHARACTERISTICS	REFERENCES
USA300 strains			
USA300 ref strain	SF8300-LUG2295	*mecA*, ACME, [R = Oxa, E, C, T, Cip, Mup]	Diep *et al*., 2008
Basal USA300 1	ST2003-0343	*mecA*, [R = Oxa]	This study
Basal USA300 2	ST2005-0026	*mecA*, [R = Oxa]	This study
Derived USA300 1	ST2012-0558	*mecA*, ACME, [R = Oxa]	Glaser *et al*., 2016
Derived USA300 2	ST2012-1514	*mecA*, ACME, [R = Oxa]	Glaser *et al*., 2016
Derived USA300 3	ST2013-0343	*mecA*, ACME, [R = Oxa,K,E]	Glaser *et al*., 2016
Derived USA300 4	ST2011-2484	*mecA*, ACME, [R = Oxa,K,E]	Glaser *et al*., 2016
Derived USA300 5	ST2011-1414	*mecA*, ACME, [R = Oxa,K,E,O]	Glaser *et al*., 2016
Derived USA300 6	ST2013-0068	*mecA*, ACME, [R = Oxa,K,E,O]	Glaser *et al*., 2016
Derived USA300 7	ST2013-1284	*mecA*, ACME, [R = Oxa,K,E,O, T]	Glaser *et al*., 2016
Derived USA300 8	ST2013-1763	*mecA*, ACME, [R = Oxa,K,E,O, T]	Glaser *et al*., 2016
EU-ST80 strains			
EU-ST80 ref strain	HT20020209-LUG1799	*mecA*, AgrC L184, [R = Oxa, K, T, F]	Perret *et al*.,2012
Basal MSSA 1	HT2002-0042	AgrC L184	Stegger *et al*., 2014
Basal MSSA 2	HT2006-0859	AgrC L184	Stegger *et al*., 2014
Basal MSSA 3	HT2003-0006	AgrC L184, [R = T]	Stegger *et al*., 2014
Lab Mutated Basal MSSA	LUG2417	**AgrC L184I**, [R = T]	This study
Basal MSSA 4	HT2004-1302	AgrC L184, [R = T]	Stegger *et al*., 2014
Basal MSSA 5	HT2005-0374	AgrC L184, [R = T]	Stegger *et al*., 2014
Derived MRSA 1	ST2007-1277	*mecA*, **AgrC L184I**, [R = Oxa]	Stegger *et al*., 2014
Derived MRSA 2	ST2007-1273	*mecA*, **AgrC L184I**, [R = Oxa]	Stegger *et al*., 2014
Derived MRSA 3	ST2005-0508	*mecA*, **AgrC L184I**, [R = Oxa,K,F]	Stegger *et al*., 2014
Derived MRSA 4	ST2009-0942	*mecA*, **AgrC L184I**, [R = Oxa,K,F,T]	Stegger *et al*., 2014
Derived MRSA 5	ST2007-0258	*mecA*, **AgrC L184I**, [R = Oxa,K,F,T,E,C]	Stegger *et al*., 2014
Derived MRSA 6	ST2007-1047	*mecA*, **AgrC L184I**, [R = Oxa,K,F,T,E,C]	Stegger *et al*., 2014

C: Clindamycin - Cip: Ciprofloxacin - E: Erythromycin - K: Kanamycin - NRCS: National Reference Center for Staphylococci - O: Ofloxacin - Oxa: Oxacillin - R: resistant - T: Tetracycline - F: Fusidic acid – I: Isoleucine – L: Leucine - NRCS: National Reference Center for Staphylococci - Oxa: Oxacillin - R: resistant