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# Development of homogeneous expression of resistance in methicillin-resistant *Staphylococcus aureus* clinical strains is functionally associated with a β-lactam-mediated SOS response

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***Objectives*: One of the main characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) from both hospitals and community is their heterogeneous expression of resistance. Recently, we reported new heterogeneous MRSA isolates phenotypically susceptible to oxacillin despite being *mecA* positive. These low-level *mecA*-mediated resistance MRSA strains are very heterogeneous in expression (HeR) and are likely to be clinically relevant since exposure of such isolates to β-lactams can result in high-level homotypic resistance (HoR). We hypothesized that HeR to HoR selection in these clinically relevant strains may be determined by the pre-existence of a hypermutable population that favours its selection in the presence of oxacillin.**

***Methods*: Using established procedures, SA13011 HeR to HoR selection was performed by using sub-inhibitory concentrations of oxacillin and examined for mutability. Real-time RT-PCR and transcriptional profiling by DNA microarray were used to compare gene expression between both populations and related genetically modified SA13011 strain.**

***Results*: We found that HeR/HoR selection by oxacillin was associated with increased mutation rate and oxacillin-mediated SOS response. We determined increased expression of both *mecA* and SOS response *lexA/recA* regulators. Mutational inactivation of *lexA* repressor resulted in a significant decrease in both mutation rate and oxacillin resistance in the HoR cells. Complementation of the *lexA* mutant strain restored oxacillin resistance to the high levels observed in the corresponding HoR wild-type strain.**

***Conclusions*: The present results support the notion that SOS response is mechanistically involved in generating mutations that, in addition to *mecA* induction, allow the selection of a highly oxacillin-resistant population.**

Keywords: oxacillin, mutation rate, *mecA*

## Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important nosocomial pathogens responsible for a wide range of severe infections[1] that also appear in community populations.[2,3] Methicillin resistance in *S. aureus* is mediated by the acquisition of a penicillin binding protein, PBP2a, which has decreased affinity for β-lactam antibiotics but can continue to cross-link the cell wall once the native PBPs (i.e. PBP1 to 4) have been inactivated.[4] PBP2a is encoded by the *mecA* gene, which is located on a 21–67 kb genomic island called the staphylococcal cassette chromosome *mec* (SCC*mec*).[5] Clinical resistance to oxacillin in *S. aureus* has been defined as strains having the *mecA* gene with an MIC of oxacillin >4 mg/L. However, due to their extreme heterogeneity, some clinical isolates have been described as *mecA* positive and oxacillin susceptible, with MICs of oxacillin of ≤2 mg/L.[6–8] The concept of heterogeneity arises from the fact that in some isolates resistant to methicillin or oxacillin, only a small proportion (≤0.1%) of the population expresses resistance to ≥10 mg/L of oxacillin (HeR), while in other isolates most of the population expresses high-level homotypic resistance (HoR). In previous observations, we have shown that conversion from the HeR to the HoR phenotype appeared to be due to the selection of a highly resistant mutant population that required both an

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intact *mecA* gene and an increase in the level of *mecA* transcription.[9] In fact, it was shown that the prevalence of the highly oxacillin-resistant subpopulation can be much higher than that seen for the spontaneous mutation of a single gene (e.g. $\sim 10^{-3}$ compared with $10^{-6}$–$10^{-9}$, respectively).[9] The nature of the mutational event(s) resulting in high-level oxacillin resistance has been only a matter of speculation up to this point.

Based on these observations we hypothesized that selection from HeR to HoR resistance in *mecA*-positive, oxacillin-susceptible MRSA clinical isolates may be determined by the pre-existence of a hypermutable population that favours its selection in the presence of oxacillin. One of the common mechanisms to elevate bacterial mutation rates is the acquisition of a hypermutable phenotype due to defects in methyl-direct mismatch repair (MMR).[10] A second mechanism involves the SOS system, which represents a global response to DNA damage that up-regulates genes involved in DNA repair and cell survival.[11] The SOS response is governed by LexA and RecA proteins.[11] LexA binds to operator sites of SOS-regulated genes, effectively repressing their expression and conversely, the presence of DNA lesions activates RecA, which promotes the autocatalytic cleavage of LexA at a specific Ala–Gly bond.[12] In the present study we demonstrate that: (i) selection from HeR to HoR in clinical *mecA*-positive, oxacillin-susceptible MRSA strains occurred after exposure to subinhibitory concentrations of β-lactam antibiotics; and (ii) this process involved, in addition to induced expression of *mecA*, the triggering of a β-lactam-mediated SOS response through *lexA/recA* regulators and increased mutation rate, supporting the notion that the SOS response is the mechanism responsible for the generation of mutations that allow the selection of a highly oxacillin-resistant population in low-level β-lactam-resistant clinical MRSA strains.

## Materials and methods

### *Bacterial strains and plasmids*

Primers and *S. aureus* strains and plasmids used in this study are listed in Table 1. Clinical oxacillin-susceptible, *mecA*-positive *S. aureus* strains were kindly provided by Drs F. Tenover, J. Swenson [Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA] and G. Archer [Virginia Commonwealth University (VCU), Richmond, VA, USA]. *S. aureus* SA13011 prototype strain used in this study was part of the above strain collection. All strains generated for this investigation were confirmed by PFGE of total genome SmaI digests as previously described.[13]

### *Materials and media*

Mueller–Hinton agar (MHA; BBL Microbiology Systems, Cockeysville, MD, USA), blood agar TSA (Difco Laboratories, Detroit, MI, USA) and Luria–Bertani broth (LB; USB corporation) were used for subculture and maintenance of *S. aureus* strains. All the antibiotics were purchased from Sigma–Aldrich.

### *Determination of antibiotic susceptibility and expression of oxacillin resistance*

MICs were determined by the broth microdilution method according to the guidelines of the CLSI.[14] Population analysis profiles were determined as previously described.[15]

### *Selection in broth from SA13011 heterotypic to homotypic resistance phenotype*

Selection of SA13011 from heterotypic (HeR) to the homotypic (HoR) resistance phenotype was performed as previously described,[9] using LB broth either with or without subinhibitory 0.5 mg/L oxacillin (Sigma) and grown at 37°C with shaking (180 rpm). Selection from HeR to HoR cells was verified by efficiency of plating (EOP) assays as described by Chambers *et al.*[16] except that methicillin was substituted for oxacillin.

### *Determination of mutation frequency*

Inoculated flasks were incubated at 37°C with shaking at 145 rpm; 100 μL aliquots were taken at various time intervals including 3, 6, 9, 27, 30 and 33 h during the SA13011 HeR (−oxacillin) to HoR (HeR + 0.5 mg/L oxacillin) selection process. Mutation frequencies for resistance to rifampicin were determined as previously described and expressed as the number of antibiotic-resistant mutants recovered as a fraction of the viable count.[17] Three independent cultures were sampled in triplicate to minimize error caused by inter- and intra-sample variation.

### *Analysis of gene expression by real-time RT-PCR and microarray transcriptional profiling*

RNA extraction and real-time PCR (RT-PCR) were performed as previously described.[18] Oligonucleotide primers are shown in Table 1. A microarray transcriptional profile was determined as previously described[18] by using a spotted DNA microarray (TIGR version 6 *S. aureus* slides) containing 4546 oligos (70mer) covering the genomes of *S. aureus* COL (2654 ORFs), N315 (2623 ORFs), Mu50 (2748 ORFs), MRSA 252 (2744 ORFs), methicillin-susceptible *S. aureus* (MSSA) 476 (2619 ORFs) and pLW043 (62 ORFs). Median signal intensity values, calculated from each set of in-slide replicates and flip-dye experiments, were used to calculate log 2 and *x*-fold changes in gene expression. Differential expression was defined as a change of >2-fold in transcript versus the comparator strain.

### *Cloning, transformation and DNA manipulation*

All restriction endonuclease digestions and ligations were performed in accordance with the manufacturer's (New England Biolabs, Beverly, MA, USA) specifications. Chromosomal DNA was prepared by using a Qiagen genomic DNA preparation kit according to the manufacturer's directions. PCR amplification of *mecA* and *mecA* promoter-operator sequences were performed with the corresponding primers previously described.[19] Sequencing of all PCR amplification products was performed by the Nucleic Acid Research Facility at VCU (Richmond, VA, USA).

### *Mutational insertion inactivation of lexA repressor and complementation*

PCR of the entire *lexA* gene (0.623 kb) plus upstream (0.6 kb) and downstream (0.915 kb) regions was carried out using primers LexA (Fl) and LexA (F2) (Table 1). The resulting 2.13 kb PCR fragment was cloned into pCR2.I (Invitrogen). Introduction of the G94E mutation at the *lexA* cleavage site was performed as described previously[20] with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using LM1 and LM2 primers (Table 1). The plasmid containing the mutation was sequenced and named

**Table 1.** Bacterial strains, plasmids and primers used in this study

| Strain number | *S. aureus* | Relevant genotype and phenotype [or sequence (5′–3′)] | Oxacillin MIC (mg/L) | Reference |
|---|---|---|---|---|
| 1 | 13011-HeR | oxacillin S-*mecA* (+) | 2 | this study |
| 2 | 13011-HoR | selected from SA13011-HeR + 0.5 mg/L OXA | ≥128 | this study |
| 3 | LMR1-HeR | SA13011-HeR + pAD2 | 2 | this study |
| 4 | LMR1-HoR | SA13011-HeR + pAD2 + 0.5 mg/L OXA | 8 | this study |
| 5 | LMR1-HeR-complemented | LMR1 pAD3 | 2 | this study |
| 6 | LMR1-HoR-complemented | LMR1 pAD3 + 0.5 mg/L OXA | ≥128 | this study |
| 7 | LMR1-HeR-empty vector | LMR1 pSK265 | 2 | this study |
| 8 | LMR1-HoR-empty-vector | LMR1 pSK265 + 0.5 mg/L OXA | 8 | this study |
| 9 | LMR2-HoR | SA13011-HeR + pAD4 + 0.5 mg/L OXA | 8 | this study |
| | RN4220 | restriction-deficient mutagenized RN450 (shuttle plasmid host) | | 9 |
| Plasmids | | | | |
| *E. coli* | | | | |
| TOPO PCR2.1 | | AMP-R, KAN-R | | Invitrogen |
| pAD1 | | PCR2.1 + 2.3 kb *lexA*(G94E), AMP-R, KAN-R | | this study |
| *S. aureus* | | | | |
| pSK950 | | thermosensitive shuttle vector | | 21 |
| pSK265 | | high-copy staphylococcal replicon | | 21 |
| pAD2 | | EcoRI fragment (pAD1 containing *lexA*(G94E) ligated at EcoRI site of pSK950 | | this study |
| pAD3 | | psK265 + 2 kb *lexA* (*lexA* + promoter) | | this study |
| pAD4 | | pMAD containing *lexA*(G94E) | | 22 |
| Primers | | | | |
| LexA (F1) | | GCACGTAGCGTATCAACAGCT | | |
| LexA (F2) | | AGAGAATTTAGCATATGGTC | | |
| LM1 | | GGTAAAGTCACAGCAGAAGTTCCTATTACC | | |
| LM2 | | GGTAATAGGAACTTCTGCTGTGACTTTACC | | |
| LM3 | | AGTGCATCTGAATTTCAAAAGGC | | |
| LM4 | | AGGAAACCTCCAATTAAACC | | |
| LexA (F3) | | GTATTACATTTAACTCAGCC | | |
| LexA (F4) | | ACATCTTGTGTATCTTCC | | |
| mecA-F | | GTTAGATTGGGATCATAGCGTCATT | | |
| mecA-R | | CGGGTTTGGTATATATTTTTATGCTT | | |
| recA-F | | GAAATCTTTCGGTAAAGGTGCC | | |
| recA-R | | CGCCTAATGCTTGAGCATATTC | | |

pAD1 (Table 1). The fragment containing the *lexA* mutated gene was then subcloned into the EcoRI site of the *Escherichia coli–S. aureus* shuttle, temperature-sensitive *S. aureus* replicon pSK950 vector.[21] The resulting construct (pAD2) was introduced into RN4220 by electroporation and transferred from RN4220 into SA13011 by generalized transduction with phage 80α.[9] The plasmid was integrated into the chromosome through homologous recombination at the non-permissive temperature (42°C). Colonies that no longer contained the plasmid were tested to confirm replacement by the presence of (G94E)-*lexA* by DNA sequence analysis using primers (LM3 and LM4), which are located outside on the chromosomal regions upstream and downstream of the cloned fragment. The resulting *lexA* mutant strain was named LMR1-HeR (Table 1). A second approach was used for the same purpose: the fragment containing the *lexA* gene was subcloned into the BamHI site of a modified version of shuttle plasmid pMAD, an *S. aureus* temperature-sensitive replicon.[22] Integration into the SA13011 chromosome was performed at (43°C) and by plating on TSA containing X-gal (5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside). White colonies, which no longer contain the plasmid pMAD, were tested to confirm the replacement; the corresponding strain was named LMR2 (Table 1).

Trans-complementation of *lexA* (LMR1-HeR-complemented) was performed by using a construct encompassing the complete *lexA* gene as well as the upstream region including the putative ribosomal binding site and promoter (primers LexA-F3 and LexA-F4; Table 1). The 2.0 kb PCR fragment products were purified using the QIAquick gel extraction kit, ligated into the ligase-independent cloning site of the PCR2.1-TOPO vector (Invitrogen) and transformed into chemically competent TOP10 *E. coli* (Invitrogen). A staphylococcal origin of replication was introduced by cloning plasmid pSK265 into the unique BamHI site on PCR 2.1-TOPO and the construct was moved into *S. aureus* RN4220 by electroporation.[9] Phage 80α was used to transduce the constructs from RN4220 into all other *S. aureus* strains used in this study.

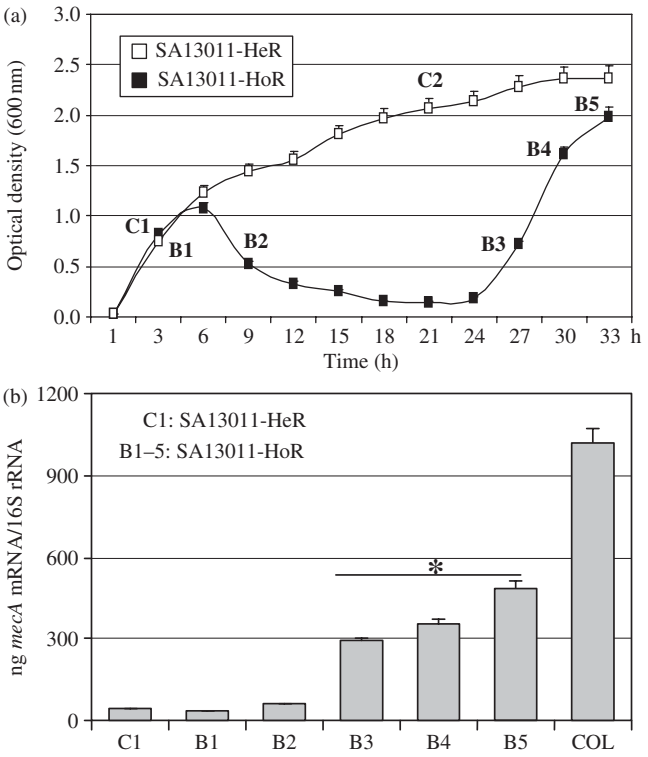


**Figure 1.** (a) Time-course analysis of SA13011 selection from heterotypic to homotypic oxacillin resistance as determined by optical density at 600 nm. Timepoints C1 and C2 correspond to SA13011-HeR grown in the absence of oxacillin; timepoints B1–B5, SA13011-HeR grown in the presence of 0.5 mg/L oxacillin leading to the selection of the homotypic counterpart derivative SA13011-HoR (B3–B5). (b) Determination of *mecA* expression levels by Taqman real-time RT-PCR in SA13011-HeR (C1) and SA13011-HoR obtained in the presence of 0.5 mg/L oxacillin (B1–B5). Quantities of *mecA* mRNA/16S rRNA in nanograms are shown on the vertical axis. *Transcription significantly greater than that seen in the absence of oxacillin [SA13011-HeR (C1)]; $P < 0.001$. COL, *S. aureus* COL used as a *mecA*-positive control. Three independent cultures were sampled in triplicate to minimize error caused by inter- and intra-sample variation.

## Results

### *Phenotypic and genotypic characteristics of selection HeR to HoR resistance phenotype in SA13011*

We have previously described MRSA isolates with an unusual oxacillin-resistant phenotype, which appeared as oxacillin susceptible although the isolates were *mecA* positive.[7] In the present study, we investigated SA13011-HeR, a representative strain that appeared phenotypically susceptible to β-lactams (disc diffusion inhibition zones: oxacillin, 19 mm; cefoxitin, 21 mm; MICs:[14] oxacillin, 2 mg/L; cefoxitin, 8 mg/L). Despite its apparent low level of resistance, SA13011 was tested for its ability to express a high homogeneous level. Selection from heterotypic to homotypic resistance phenotype was obtained by growing HeR cells in broth containing subinhibitory concentrations of oxacillin (0.5 mg/L). This concentration of oxacillin was sufficient to cause a drop to a cell density ($OD_{600}$) of 0.14, reflecting the killing of the HeR population and the selection of the most resistant cells (HoR) (Figure 1a), which occurred at this range of oxacillin concentration by 24–30 h of growth. The resistant phenotype of the nascent HoR population was clearly detectable by phenotypic methods including disc diffusion tests (6 mm) and MICs (128 mg/L) of oxacillin. Response to growth in oxacillin was evaluated by population analysis profile[23] showing that SA13011 with no known prior exposure to oxacillin (HeR) expressed a resistance profile resembling MSSA with no growth at concentrations of oxacillin between 1 and 2 mg/L; in contrast, after oxacillin exposure, $10^9$–$10^{10}$ cells grew at concentrations up to 64 mg/L and $10^4$ cells at concentrations up to ≥256 mg/L, indicating that after exposure to subinhibitory concentrations of oxacillin, SA13011 displayed a highly resistant phenotype (HoR; data not shown).

The identity of SA13011-HeR and the oxacillin-induced derivative SA13011-HoR was confirmed by PFGE and no differences or genomic rearrangements were observed (data not shown). Importantly, SA13011-HoR purified single colonies (timepoints B3–B5) remained stable and retained their high level of resistance in blood agar without oxacillin. HoR selection was also observed under similar conditions with other β-lactam antibiotics (cefoxitin, ampicillin, nafcillin and ceftazidime; data not shown). Analysis of *mecA* expression was performed by Taqman real-time RT-PCR using RNA samples corresponding to timepoints Cl, B1, B2, B3, B4 and B5 during the HeR–HoR selection process. A marked increase in *mecA* transcription was observed during growth/selection with oxacillin (0.5 mg/L), with

**Table 2.** Mutation rate analysis during HeR–HoR selection in SA13011-HeR/HoR and mutant *lexA* LMR1-HeR/HoR

| | 3 h | 6 h | 9 h | 27 h | 30 h | 33 h |
|---|---|---|---|---|---|---|
| SA13011-HeR/HoR | $2.3\times10^{-8}$ | $5.0\times10^{-8}$ | $6.1\times10^{-8}$ | $6.4\times10^{-5}$ | $3.0\times10^{-4}$ | $1.3\times10^{-4}$ |
| LMR1-HeR/HoR | $1.2\times10^{-8}$ | $2.5\times10^{-8}$ | $1.7\times10^{-8}$ | $5.2\times10^{-7}$ | $1.2\times10^{-6}$ | $7.0\times10^{-6}$ |

Samples were grown in LB broth and collected at different time intervals (3, 6, 9, 27, 30 and 33 h) during the HeR–HoR selection process and selected on MHA plates containing 100 mg/L rifampicin and MHA plates using serial dilutions to determine cfu/mL. Mutation frequencies were expressed as the number of antibiotic-resistant mutants recovered as a fraction of the viable count.

maximal expression at timepoints B3–B5, indicating that *mecA* transcription paralleled the rapid outgrowth of the homotypic resistance population (Figure 1b). Recently, extremely low-level oxacillin resistance in an MRSA strain was shown to be associated with changes in the *mecA* promoter sequence.[24] However, sequence analysis of the promoter-operator region of *mecA* in SA13011, performed as previously described,[19] showed no differences compared with the wild-type strain *S. aureus* N315 (A. E. Rosato, unpublished results), indicating that altered regulation of *mecA* does not account for the resistance phenotype observed in SA13011.

### *SA13011 HeR–HoR selection involves oxacillin-mediated increased mutation rates*

SA13011 HeR–HoR selection was also accompanied by increased resistance to rifampicin (HeR compared with HoR: disc diffusion results 32 mm compared with 10 mm, MIC 0.06–16 mg/L, respectively), which led us to hypothesize the existence of a hypermutable state that may result in an increased number of mutations in genes associated with antimicrobial resistance. To test this hypothesis, we determined the frequency of mutation during the HeR–HoR selection process. The results showed that mutations/bacteria/generation increased progressively along the selection, e.g. from $2.3\times10^{-8}$ to $6.4\times10^{-5}$ (SA13011-HeR compared with SA13011-HoR, respectively), representing an ~3 log increase (27 h; Table 2), consistent with the notion that β-lactam-mediated HeR–HoR selection was associated with the appearance of a hypermutable state.

To evaluate the mechanism(s) responsible for elevating the mutation rate, we first amplified and sequenced *mutS/mutL*. Deduced amino acid sequences of *mutSL* genes in both SA13011-HeR and SA13011-HoR strains appeared highly conserved and did not reveal differences when compared with the wild-type strain *S. aureus* N315 (data not shown). Although we could not exclude some potential involvement of the MMR system, these data seemed to indicate that alternative mechanisms may be playing a more relevant role. To investigate the SOS response, the effect of oxacillin on *recA* expression was analysed by real-time RT-PCR. RNA samples were prepared from cells collected at different timepoints during the HeR–HoR selection process. As shown in Figure 2(a), a progressive increase in the level of *recA* transcription was observed during growth/selection with 0.5 mg/L oxacillin, with maximal expression detected at timepoint B3 (exponential phase of growth), followed by a decline at timepoint B5 (stationary phase). RNA extracted from SA13011 exposed to mitomycin, a potent inducer of the bacterial SOS regulon,[25] was used as a positive control. To further investigate regulatory SOS pathways in relation to oxacillin resistance/homotypic phenotype, pairwise gene expression comparisons between SA13011-HeR and its homotypic derivative SA13011-HoR were performed by microarray analysis (Table 3). The results are based on a series

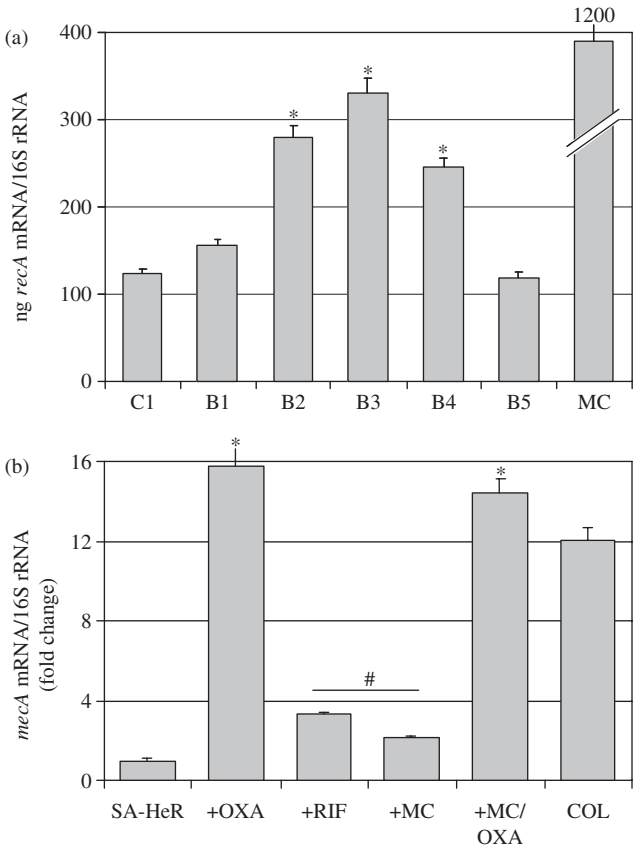


**Figure 2.** (a) Quantification of *recA* mRNA by Taqman real-time RT-PCR in SA13011 cells collected at the indicated timepoints as shown in Figure 1(a). Quantities of *recA* mRNA/16S rRNA in nanograms are shown on the vertical axis. *Transcription of *recA* in SA13011-HoR cells significantly higher than that seen in SA13011-HeR cells; $P<0.001$. MC, mitomycin. Three independent cultures were sampled in triplicate to minimize error caused by inter- and intra-sample variation. (b) *mecA* expression levels determined by real-time RT-PCR in SA13011-HeR (SA-HeR) and SA13011-HoR obtained in the presence of rifampicin (+RIF; 0.03 mg/L), mitomycin (+MC; 0.05 mg/L), mitomycin + oxacillin (+MC/OXA; 0.5 mg/L) and oxacillin (+OXA; 0.5 mg/L). COL, MRSA positive control of *mecA* expression. Relative values of specific *mecA* mRNA/16S rRNA are shown on the vertical axis. *Transcription significantly greater than SA13011-HeR, $P<0.001$; significantly lower than +OXA, $P<0.01$. Three independent cultures were sampled in triplicate to minimize error caused by inter- and intra-sample variation.

**Table 3.** Differential gene expression between SA13011-HeR and SA13011-HoR as determined by spotted DNA microarray

| Gene | Product or putative function | Fold change |
|---|---|---|
| *lexA* | SOS regulatory LexA protein | 2.25 |
| | DNA polymerase III α | 2.44 |
| *recA* | RecA protein | 2.69 |
| *recF* | recombination protein RecF | 1.5 |
| *dnaN* | DNA polymerase III subunit β | 2.82 |
| *dnaA* | chromosomal replication initiation protein | 2.95 |
| *gyrA* | DNA gyrase subunit A | 3.98 |
| *mecA* | penicillin binding protein 2 prime | 3.5 |
| *femA* | factor essential for expression of methicillin resistance | 2.8 |
| *fmt* | autolysis and methicillin resistance-related protein | 3.6 |
| *clfA* | clumping factor A | 3.2 |
| *lytM* | peptidoglycan hydrolase | 2.3 |
| *atl* | autolysin | 2.6 |
| *hexA* | DNA mismatch repair | −2.56 |
| *hexB* | DNA mismatch repair | −3.76 |
| *radC* | DNA repair protein | −2.28 |
| *radA* | DNA repair protein | −2.32 |

The data set was normalized by applying the LOWESS algorithm (block mode; smooth parameter, 0.33) and using TIGR MIDAS software (http://www.tigr.org/software/). Significant changes were identified with significance analysis of microarrays (SAM) software (http://www-stat.stanford.edu/tibs/SAM/index.html).

of statistical analyses (filtering) where ratios Cy3 and Cy5 are converted to log 2 values and cutoff is set at >1 (present) or below −1 (absent). Among the group of genes whose expression appeared modified during the selection process there was a group of genes directly involved in DNA metabolism, including *lexA* (repressor of SOS response), *recA* (activator), *recF* (recombination protein RecF), *dnaN* (DNA polymerase III subunit β) and *dnaA* (chromosomal replication initiation protein), all up-regulated, and *hexA* and *hexB* (DNA mismatch repair) and *radC* and *radA* (DNA repair), all down-regulated (Table 3). Other genes included cell wall-associated genes [*fmt* (SA0909_N315) and *femA* (SA1206_N315)], a gene encoding cell wall-anchored surface proteins (*clfA*) and autolysis genes [*atl* (SAV1052_Mu50) and *lytM* (SA0265_N315)], all of them up-regulated. As expected, *mecA* (SA0038_N315), the gene that encodes PBP2a, was up-regulated during the process of selection (Table 3). Together, these findings indicated that β-lactam-mediated HeR–HoR selection involved not only induction of *mecA* expression and cell wall-associated genes but also activation of the SOS response.

As mentioned above, SAl3011 HeR–HoR selection was associated with increased resistance to the RNA polymerase inhibitor rifampicin, which has been shown in *E. coli* to induce LexA cleavage and the SOS response.[26] To investigate whether rifampicin may promote SA13011 HeR–HoR selection as well, SA13011 HeR cells were exposed to subinhibitory concentrations of the antibiotic (0.03 mg/L). Rifampicin-mediated HeR–HoR selection was accompanied by increased MICs of oxacillin [i.e. from 2 mg/L (HeR) to 32 mg/L (HoR)]. Based on these observations, we investigated also the ease of HeR–HoR selection by mitomycin C (0.05 mg/L), a DNA damaging agent known to activate LexA-regulated genes.[27] As was the case with rifampicin, increased MICs of oxacillin were observed [i.e. 2 mg/L (HeR) and 32 mg/L (HoR)]; however, and similarly to rifampicin, these values were lower than those observed with oxacillin (≥128 mg/L). *mecA* expression analysis by real-time RT-PCR showed that either rifampicin and/or mitomycin C induced 2- to 4-fold changes in *mecA* mRNA levels during HeR–HoR growth/selection, in contrast to the 12-fold increase in *mecA* expression, observed with oxacillin (Figure 2b). However, for example, when the homotypic cells selected with mitomycin C were exposed to subinhibitory concentrations of oxacillin, the expression of *mecA* was strongly induced (Figure 2b) and a maximal level of resistance to oxacillin was reached (MICs ≥128 mg/L), supporting our hypothesis that HeR–HoR selection requires both an increased number of mutations (mediated by the SOS response) and induction of *mecA* expression, effects that are only completely achieved by exposure to β-lactam antibiotics.

### *Inactivation of lexA dramatically affects SAl3011 HeR–HoR selection*

To determine the precise role of the *recA/lexA*-mediated SOS response during the HeR–HoR selection process, we replaced the SA13011 endogenous *lexA* chromosomal copy with a modified (G94→E) non-cleavable form of the gene (strain LMRI-HeR, Table 1) by secondary homologous recombination. Sequence analysis of chromosomal DNA using primers located outside on the chromosomal regions upstream and downstream of the cloned fragment revealed that only the mutated *lexA* was present. To

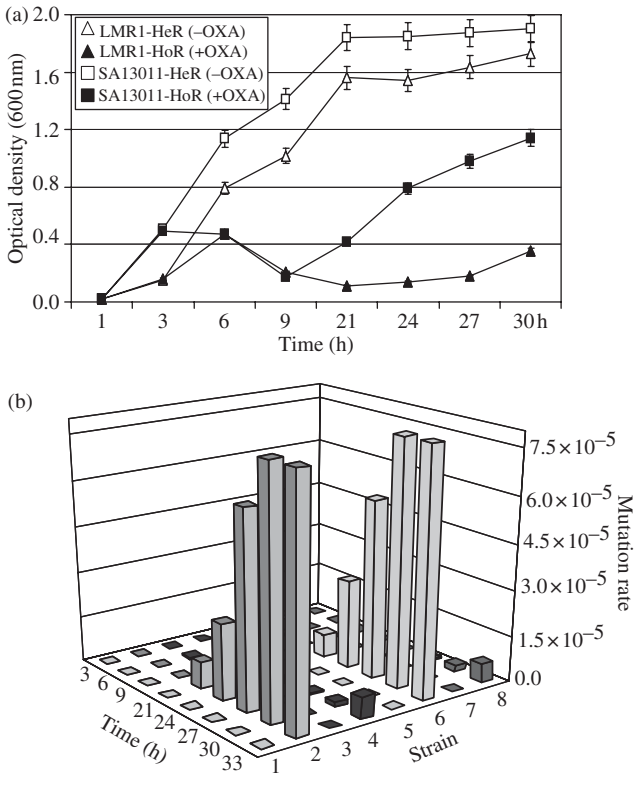


**Figure 3.** (a) Time-course analysis of SA13011 selection from heterotypic to homotypic oxacillin resistance as determined by optical density at 600 nm compared with the *lexA* mutant LMR1-HeR. The growth pattern was followed in the absence and presence of oxacillin (0.5 mg/L) at the indicated time intervals (3, 6, 9, 27, 30 and 33 h) during the HeR–HoR selection process. Three independent cultures were sampled in triplicate to minimize error caused by inter- and intra-sample variation. (b) Mutation rate analysis during HeR–HoR selection in SA13011 strains 1–8, described in Table 1. Mutation frequencies were expressed as the number of antibiotic-resistant mutants recovered as a fraction of the viable count. Three independent cultures were sampled in triplicate to minimize error caused by inter- and intra-sample variation.

establish the functional role that LexA may have either directly or indirectly in generation of mutations in heterogeneous expression of resistance, LMR1-HeR was tested for its capacity to induce HeR–HoR selection. Both the growth pattern, determined by measuring the optical density at 600 nm (Figure 3a), and the number of mutants generated by LMR1-HeR cells (Table 2) were followed in the absence and presence of oxacillin (0.5 mg/L) at the indicated time intervals (3, 6, 9, 27, 30 and 33 h) during the HeR–HoR selection process. Inactivated *lexA* mutant selected with 0.5 mg/L oxacillin (LMR1-HoR, strain 4; Table 1) showed a significant decrease in both parameters, i.e. growth (Figure 3a) and mutation rate [e.g. $2.3\times10^{-8}/6.4\times10^{-5}$ compared with $1.2\times10^{-8}/5.2\times10^{-7}$, timepoint 27 h, SA13011-HeR/HoR (strains 1 and 2) compared with LMR1-HeR/HoR (strains 3 and 4), respectively; Table 2]. Importantly, these changes were accompanied by a decreased level of resistance to oxacillin as indicated by an MIC of $\geq$128 mg/L (strain 2) compared with 8 mg/L (strain 4; Table 1); these results were also confirmed by oxacillin population analysis (data not shown).

Further evidence of the specific role of *lexA* was obtained by complementing LMR1-HeR *lexA* mutant strain with a cloned full-length *lexA* (LMR1-HeR-complemented, strain 5; Table 1). *lexA* complementation restored the growth of the *lexA* mutant strain in the presence of 0.5 mg/L oxacillin (LMR1-HoR-complemented, strain 6; Table 1) to a pattern comparable to that of the wild-type SA13011-HoR strain (strain 2; data not shown). Consistently, LMR1-HoR-complemented (strain 6) also displayed a progressively increased frequency of mutation along the selection from $1.4\times10^{-8}$ (strain 5) to $5.8\times10^{-5}$ (strain 6), an ~3 log increase (timepoint 27 h; Figure 3b). The *lexA* mutant harbouring the empty vector (LMR1-HoR-empty vector, strain 8) was comparable to the mutant *lexA* LMR1-HoR (strain 4) in both growth rate (not shown) and frequency of mutation ($5.2\times10^{-7}$ and $3.6\times10^{-7}$, strain 8 compared with strain 4, respectively, 27 h; Figure 3b). No significant changes were observed in either growth or mutation rate in strains growing in the absence of oxacillin (strains 1, 3, 5 and 7; Figure 3b).

Phenotypic analysis of these strains by determining oxacillin MICs showed also that trans-complementation of *lexA* (LMR1-HoR-complemented, strain 6) during HeR–HoR selection restored oxacillin resistance, i.e. from MIC 8 mg/L (strain 4) to MIC $\geq$128 mg/L (strain 6), values that were comparable to those observed in SA13011-HoR (strain 2; Table 1). Consistently, HeR–HoR selection in LMR1-HoR-empty vector (strain 8) displayed no differences with respect to the *lexA* mutant LMR1-HoR (strain 4; Table 1). The oxacillin resistance levels from the HeR–HoR selection were confirmed for SA13011 HeR–HoR, mutant *lexA* + 0.5 mg/L oxacillin (LMR1-HoR) and *lexA*-complemented + 0.5 mg/L oxacillin (LMR1-HoR-complemented) by swabbing a 0.5 McFarland inoculum in rectangular plates containing a 0–128 mg/L oxacillin gradient (Figure 4a). Similar results were obtained with the *lexA* mutant (LMR2) generated by using pMAD plasmid [Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. The homotypic population in the *lexA* mutant LMR1-HoR (lane 3) was less resistant to oxacillin than the corresponding SA13011-HoR (8 mg/L versus 256 mg/L, lanes 3 and 2, respectively) and was restored to wild-type levels (256 mg/L) after *lexA* complementation (LMR1-HoR-complemented, lane 4). *recA* expression was also verified following *lexA* complementation in RNA samples from cells collected during HeR–HoR selection at exponential phase of growth, i.e. $OD_{600}$ 0.6, range 3–6 h for cells growing without oxacillin (HeR, strains 1, 3, 5 and 7) and 21–24 h for nascent cells growing with 0.5 mg/L oxacillin (HoR, strains 2, 4, 6 and 8). As shown in Figure 4(b), *lexA* trans-complementation (LMR1-HoR-complemented, strain 6) resulted in increased *recA* transcription similarly to wild-type SA13011-HoR (strain 2); no changes in *recA* expression were observed in strain 8 (LMR1-HoR-empty vector) or strain 4 (LMR1-HoR).

## Discussion

The evolution of antibiotic resistance by accumulation of mutations during treatment is one of the most important factors contributing to therapy failure.[28] The main characteristic of methicillin resistance in *S. aureus*, either from hospital or community, is its heterogeneous expression of resistance.[9] This phenomenon has been explored in detail using laboratory strains[9] while no such studies have been conducted in the clinical counterparts. In the present work, we have analysed a

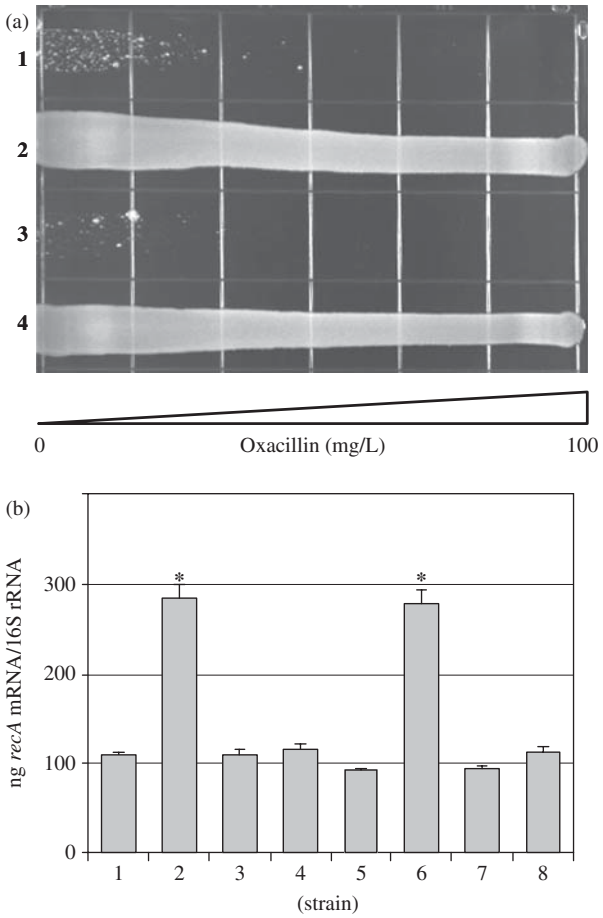


**Figure 4.** (a) Oxacillin gradient plate (0–100 mg/L) of SA13011-HeR (lane 1), SA13011-HoR (lane 2), LMR-HoR (lane 3) and LMR1-HoR-complemented (lane 4). A difference in the confluent growth is observed consistently with a decrease in the number of oxacillin-resistant cells in LMR1-HoR (mutant *lexA*+0.5 mg/L oxacillin). A representative picture of three independent cultures is shown. (b) Quantification of *recA* mRNA by real-time RT-PCR in SA13011 strains 1–8 (Table 1). Nanograms of *recA* mRNA/16SrRNA are shown on the vertical axis. *Significantly higher than sample 1; $P<0.001$. Three independent cultures were sampled in triplicate to minimize error caused by inter- and intra-sample variation.

clinical MRSA strain displaying a phenotype of extreme heterogeneity. Several groups including ourselves have investigated the emergence of new MRSA strains that are similar to the one we described in this study.[6–8]

Since exposure to subinhibitory concentrations of β-lactams allows the selection of high level resistance, the appearance of these strains makes them clinically relevant. Our results indicate that: (i) HeR–HoR selection is determined by the pre-existence of a hypermutable population that favours the HoR selection following exposure to oxacillin; and (ii) oxacillin-mediated SOS response is associated with this process and responsible for increased mutation generation. These results are in agreement with the concept that blockade of LexA cleavage results in reduction of oxacillin resistance in the selected homotypic population. Similar phenomena have been shown to occur in cells exposed to quinolones, well-known SOS-inducing agents.[29] In this case, the proportion of heteroresistant strains expressing methicillin resistance was determined to increase following exposure to fluoroquinolones and, conversely, resistance to fluoroquinolone appeared following exposure to oxacillin.[29] In a related study, Chambers *et al.*[30] demonstrated that exposure of community-associated MRSA to fluoroquinolones selected in a non-lethal manner for stable methicillin resistance variants at a frequency higher than that attributable to spontaneous mutation rates.[30] The authors associated this phenomenon with altered expression of stress-response genes and regulatory elements. The present results show that exposure of the clinical SA13011 strain to subinhibitory concentrations of either rifampicin or mitomycin led to HeR–HoR selection with a moderate increase in *mecA* expression, as previously described previously by using fluoroquinolone agents as SOS inducers.[30] These results support the concept that while β-lactam-mediated HeR–HoR selection involves induction of an SOS response, in order to reach maximal level of resistance, induction of *mecA* is absolutely required, and this effect is only achieved after exposure to this group of antibiotics. In fact, as we previously demonstrated previously in the laboratory-constructed SA450M,[9] inactivation of *mecA* prevented oxacillin-mediated HeR–HoR selection,[9] indicating that oxacillin-mediated selection of a highly resistant HoR population results from induced *mecA* expression accompanied by an SOS-mediated increased mutation rate.

β-Lactam-mediated SOS induction has previously been shown in *E. coli*.[31] In this model, inactivation of PBP3, either genetically or by exposing cells to ceftazidime, induced *dpiBA*, an operon encoding a response-effector two-component system.[32] This event, which involved cell wall stress and transiently halted bacterial cell division, required SOS-promoted cleavage of RecA and LexA.[31,32] Exposure to β-lactam antibiotics also induced the transcription of *dinB*, which codes for DNA polymerase IV, via an SOS-independent pathway.[33] Similarly, our findings indicate that in low-level resistance clinical MRSA strains, β-lactam antibiotics may represent extracellular stimuli of the SOS response due to cell wall biosynthesis perturbation rather than DNA damage.

Future studies should demonstrate the potential role of different cell wall components as mediators of SOS induction during HeR–HoR selection in strains like SA13011. Although anticipated, our results reinforce the need for great caution in the use of β-lactams in MRSA misinterpreted as MSSA in which these antibiotics are considered first option treatment.

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## Transparency declarations

None to declare.

## Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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