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# Spontaneous Deletion of the Methicillin Resistance Determinant, *mecA*, Partially Compensates for the Fitness Cost Associated with High-Level Vancomycin Resistance in *Staphylococcus aureus*▽

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**Treatment of infections caused by *Staphylococcus aureus* is often confounded by the bacterium's ability to develop resistance to chemotherapeutic agents. Methicillin-resistant *S. aureus* (MRSA) arises through the acquisition of staphylococcal chromosomal cassette *mec* (SCC*mec*), a genomic island containing the methicillin resistance determinant, *mecA*. In contrast, resistance to vancomycin can result from exposure to the drug, a mechanism that is not dependent upon a gene acquisition event. Here we describe three MRSA strains that became resistant to vancomycin during passage in the presence of increasing concentrations of the drug. In each case two derivative strains were isolated, one that had lost *mecA* and one that retained *mecA* during passage. Strain 5836VR lost *mecA* by the site-specific chromosomal excision of SCC*mec*, while the other two strains (strains 3130VR and VP32) deleted portions of their SCC*mec* elements in a manner that appeared to involve IS*431*. Conversion to vancomycin resistance caused a decrease in the growth rate that was partially compensated for by the deletion of *mecA*. In mixed-culture competition experiments, vancomycin-resistant strains that lacked *mecA* readily outcompeted their *mecA*-containing counterparts, suggesting that the loss of *mecA* during conversion to vancomycin resistance was advantageous to the organism.**

*Staphylococcus aureus* is an aggressive human pathogen and a leading cause of disease in the community and health care settings (14, 32). *S. aureus* infections range in severity from uncomplicated skin and soft tissue infections to the more severe infections necrotizing pneumonia, endocarditis, and sepsis. Treatment of staphylococcal disease is complicated by the organism's innate ability to become resistant to chemotherapy. Staphylococci become resistant to methicillin and other β-lactam antibiotics by acquiring a genomic island known as staphylococcal chromosome cassette *mec* (SCC*mec*) (5). SCC*mec* contains the *mecA* gene, which mediates β-lactam resistance, as well as site-specific recombinase genes (*ccrAB* or *ccrC*) that are involved in the element's mobility (25–27, 29, 34). *mecA* encodes an alternate penicillin binding protein (PBP 2a) that has a low affinity for β-lactam antibiotics. PBP 2a is responsible for the continued cross-linking of peptidoglycan substituents in the cell wall when β-lactam antibiotics inhibit the penicillin binding proteins normally involved in cell wall synthesis (4, 9). The phenotypic expression of methicillin resistance is variable among methicillin-resistant *S. aureus* (MRSA) strains. In strains exhibiting heterotypic methicillin resistance, only a subpopulation of bacteria expresses high-level resistance, whereas homotypic resistance is characterized by the expression of high-level resistance by the entire population (3, 18).

MRSA infections now have a higher incidence than methicillin-susceptible *S. aureus* infections in some settings. MRSA strains were responsible for 59% of skin and soft tissue infections in 11 U.S. emergency departments, and MRSA made up 59.5% of all *S. aureus* infections in intensive care unit patients in 2004 (32, 37). In addition, the incidence of MRSA disease in the community more than doubled from 2002 to 2004 (14). The increasing rate of MRSA infections has shifted chemotherapy away from β-lactam antibiotics and toward drugs effective against MRSA, such as vancomycin. As a result, *S. aureus* strains with reduced susceptibility to vancomycin are emerging (6, 10, 22, 24). *S. aureus* becomes resistant to vancomycin in two ways. One mechanism is rare and involves the acquisition of the *van* operon contained on Tn*1546* (or other similar elements), which is typically present on a conjugative plasmid (6–8). Another mechanism of vancomycin resistance involves alterations in the cell wall structure, presumably as a result of mutations. This type of resistance is thought to be mediated by a thickened, poorly cross-linked cell wall that contains sufficient D-Ala–D-Ala targets in the periphery that bind to vancomycin, preventing the drug from accessing more lethal targets at the interior of the cell wall, where cell wall synthesis occurs (15–17).

Several lines of evidence suggest that there is an incompatibility between the simultaneous expression of high-level vancomycin resistance and high-level methicillin resistance in *S. aureus*. PBP 2a is not capable of cross-linking peptidoglycan containing stem peptide modified by the *van* genes (21, 39). Although it is not universally seen, there are several observations that MRSA passaged in the presence of increasing concentrations of vancomycin has a decrease in oxacillin resistance or has a deleted *mecA* (1, 36, 41). In the study described here we investigated the genetic and fitness alterations that occurred in three MRSA strains that became oxacillin susceptible during passage to high-level vancomycin resistance in compar-

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TABLE 1. *Staphylococcus aureus* strains[a]

| Strain | Characteristic(s) | Description | MIC(μg/ml) | | Reference |
|---|---|---|---|---|---|
| | | | Vm | Ox | |
| 3130 | $Ox^r$ (ho) $Vm^S$ | Clinical isolate, SCOPE surveillance study | 0.5 | >8 | 43 |
| 3130VR | $Ox^s$ $Vm^r$ | 3130 passaged to $Vm^r$ | 32 | 0.5 | This study |
| 3130V32 | $Ox^r$ (ho) $Vm^r$ | 3130 passaged to $Vm^r$ | 32 | >8 | This study |
| 5827 | $Ox^r$ (ho) $Vm^i$ | Clinical VISA isolate, Michigan, 1997 | 4 | >8 | 42 |
| VP32 | $Ox^s$ $Vm^r$ | 5827 passaged to $Vm^r$ | 32 | 0.5 | 31 |
| 5827V32 | $Ox^r$ (ho) $Vm^r$ | 5827 passaged to $Vm^r$ | 32 | >8 | This study |
| 5836 | $Ox^r$ (he) $Vm^i$ | Clinical VISA isolate, New Jersey, 1997 | 8 | >8 | 42 |
| 5836VR | $Ox^s$ $Vm^r$ | 5836 passaged to $Vm^r$ | 32 | 0.25 | This study |
| 5836V32 | $Ox^r$ (ho) $Vm^r$ | 5836 passaged to $Vm^r$ | 32 | >8 | This study |
| 450MHomo | $Ox^r$ (ho) | RN450M (8325-4) passaged to homotypic oxacillin resistance | ND | >8 | This study |
| 450MHomoex | $Ox^s$ | 450MHomo, SCC*mec* excised | ND | 0.5 | This study |
| 450MHomoΔ*mecA* | $Ox^s$ $Tc^r$ | 450MHomo, *mecA*::*tetM* | ND | 0.5 | This study |
| 3130V32Δ*mecA* | $Ox^s$ $Tc^r$ $Vm^r$ | 3130V32 *mecA*::*tetM* | 32 | 0.5 | This study |

[a] Abbreviations: Ox, oxacillin; Vm, vancomycin; Tc, tetracycline; r, resistant; s, sensitive; i, intermediate; (ho), homotypic; (he), heterotypic; ND, not determined.

ison with those of isogenic strains that retained oxacillin resistance during the same vancomycin passage experiments.

## MATERIALS AND METHODS

**Bacterial strains and media.** All bacterial strains used in this study are listed in Table 1. The bacteria were grown in brain heart infusion (BHI) broth or agar (Becton Dickinson, Sparks, MD) at 37°C with shaking at 220 rpm, unless otherwise noted. Strain 450MHomo is a derivative of strain 450M that was passaged on 6 μg/ml of oxacillin until the strain was homotypically resistant, as determined by population analysis profiling (23, 44). 450MHomoΔ*mecA* is strain 450MHomo with *mecA* insertionally inactivated by *tetM*. This was accomplished by transducing plasmid pΔ*mecA* into 450MHomo. This plasmid contains *mecA*::*tetM* and a temperature-sensitive origin of replication (20). Transductant colonies were picked and grown on tryptic soy agar with tetracycline at 42°C, the nonpermissive temperature for plasmid replication. Any colonies that grew were then screened for tetracycline resistance, oxacillin sensitivity, and erythromycin sensitivity (resistance is encoded by the pΔ*mecA* backbone). Bacteria with the correct resistance profile were confirmed to contain *mecA*::*tetM* by PCR. Strain 3130V32Δ*mecA* was constructed from strain 3130V32 in the same manner. Strain 450Mex is strain 450M with SCC*mec* site specifically excised from the chromosome. To accomplish this, plasmid-borne *ccrAB* was transduced into 450M (34). The transductants were passaged overnight, and an oxacillin-susceptible colony was isolated. PCR amplification across the SCC*mec*-chromosomal junction was used to confirm the CcrAB-mediated excision of SCC*mec*.

**Vancomycin passage technique.** A single colony of each strain was grown in broth with increasing levels of vancomycin (0.5, 1, 2, 4, 8, 12, 16, 20, 24, 28, and 32 μg/ml; Sigma Aldrich, St. Louis, MO) until a thick culture (optical density at 600 nm [$OD_{600}$] > 1.0) grew (up to 48 h). The strains were then streaked onto BHI agar with the same concentration of vancomycin as that in their most recent growth broth to confirm their stability and resistance level. A single colony was then selected from the plate and used for continued passage. The strains were passaged once in broth at each concentration of vancomycin up to 32 μg/ml, passaged three times at 32 μg/ml, and then maintained on BHI agar containing 32 μg/ml of vancomycin. Colonies were selected from the 32-μg/ml vancomycin plate and streaked onto plates containing 6 μg/ml of oxacillin. Isolate pairs that could (strains 3130V32, 5827V32, and 5836V32; oxacillin resistant) and could not (strains 3130VR, VP32, and 5836VR; oxacillin susceptible) grow on oxacillin were saved (Table 1).

**Antimicrobial susceptibility testing.** MICs were determined by broth microdilution in cation-adjusted Mueller-Hinton broth (Becton Dickinson, Cockeysville, MD), according to the Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines (33).

**PFGE.** The preparation of genomic DNA and separation by pulsed-field gel electrophoresis (PFGE) were adapted from the method of Bannerman et al. (2). Agarose plugs containing genomic DNA were prepared from overnight cultures and digested with SmaI (Promega, Madison, WI). Each plug was placed into a well of a 1% agarose gel and run under the following parameters: 6 V/cm; initial switching time, 1 s; final switching time, 30 s; run time, 22 h; run temperature, 14°C. The bands were visualized with UV light after ethidium bromide staining.

**PCR and DNA sequencing.** PCR was performed with the *Taq* PCR master mix kit (Qiagen, Valencia, CA); the annealing temperature was generally 52°C, and extensions were at 72°C for 1.5 min/kb of amplification products. All PCR primers are shown in Table 2. For amplification products greater than 3 kb in length, Platinum PCR SuperMix high fidelity (Invitrogen, Carlsbad, CA) was used according to the manufacturer's suggested parameters. Sequencing of all PCR amplification products was performed by the Nucleic Acid Research Facility at Virginia Commonwealth University (Richmond).

**Growth analysis.** Overnight cultures were diluted to an $OD_{600}$ of <0.1, and 150-μl aliquots were placed in each well of a 96-well plate. The plates were incubated at 37°C with shaking for 18 h in a Multiskan Ascent apparatus (Thermo Lab Systems, Franklin, MA), and the $OD_{595}$ was measured every 15 min. The doubling times were determined by averaging the time required for the $OD_{595}$ to double over two or more time intervals within log-phase growth.

**Competition experiments.** When a chromosomal selectable marker was not available, a plasmid-borne selectable marker was introduced into each of the two strains being competed to provide a means of distinguishing the two strains contained in a mixed culture. pRN5543 encodes chloramphenicol resistance, and pCN36 encodes tetracycline resistance (Table 2). There is a possibility that pRN5543 and pCN36 may impose differential fitness costs on strains. To control for this, each competition was repeated after the resistance plasmids contained by each of the two strains were swapped. The strain pairs competed in this work included 3130VR/pRN5543 versus 3130V32/pCN36 and 3130VR/pCN36 versus 3130V32/pRN5543 (see Fig. 5, top left panel), VP32/pRN5543 versus 5827V32/pCN36 and VP32/pCN36 versus 5827V32/pRN5543 (see Fig. 5, top right panel), 5836VR/pRN5543 versus 5836V32/pCN36 and 5836VR/pCN36 versus 5836V32/pRN5543 (see Fig. 5, bottom panel), 3130V32/pRN5543 versus 3130V32Δ*mecA* (see Fig. 6), 450MHomo versus 450MHomoΔ*mecA* (see Fig. 7, top left panel), 450MHomo versus 450MHomoex (see Fig. 7, top right panel), and 450MHomoex versus 450MHomoΔ*mecA*. Overnight cultures of the strain pairs to be competed were diluted, and $10^5$ cells of each strain were inoculated into one 5-ml BHI broth culture and grown at 37°C for 20 to 26 h until thick cultures ($OD_{600} \geq 1.0$) were achieved. At this point, the cultures were serially diluted and plated onto selective medium containing 30 μg/ml of chloramphenicol, 10 μg/ml of tetracycline, or 6 μg/ml of oxacillin (Sigma Aldrich) for enumeration of the selected strain. At the same time, each mixed culture was diluted 1:50,000 in fresh medium. The process of enumerating each strain in the mixed culture, diluting 1:50,000 in fresh medium, and regrowth was repeated daily for up to 9 days.

**SCC*mec* excision.** Plasmid-borne *ccrAB* was introduced into strains 3130 and 5827 by phage 80α transduction, and the strains were grown for 24 h in BHI broth prior to the isolation of total cellular DNA, as described previously (34). SCC*mec* excision was detected by a PCR-based approach. Primers were designed to amplify across the chromosomal junction from which SCC*mec* excised (primers I1F and MRSAexrev for strain 3130, primers I1F and I1R for strain 5827) and the excised, circular SCC*mec* element (primers WK1R and N315attRfor). The nucleotide sequences of the amplification products were determined to confirm that CcrAB-mediated SCC*mec* excision had occurred.

**Real-time RT-PCR.** Overnight cultures of the strains were diluted 1:1,000 in fresh BHI broth (Becton Dickinson, Cockeysville, MD), BHI broth containing 1

TABLE 2. Plasmids and primers

| Plasmid or primer | Sequence | Description[a] | Reference or source |
|---|---|---|---|
| Plasmids | | | |
| p$\Delta$*mecA* | | Em$^r$ Tc$^r$ | 20 |
| pRN5543 | | Cm$^r$ | 13 |
| pCN36 | | Tc$^r$ | 11 |
| Primers | | | |
| I1F | GTTCCAGACGAAAAAGCACCAG | Amplifies across the junction when SCC*mec* is excised | 34 |
| I1R | CATTTTATGAGTCTCGCAAATTGTCAG | Amplifies across the junction when SCC*mec* is excised | 34 |
| MRSAexrev | CCTTTTGTATAATATATTCACATCACC | Amplifies across the junction when SCC*mec* is excised | This study |
| WK1R | CCGTAATTTACTATATTTAGTTGC | Amplifies the excised, circular SCC*mec* element | This study |
| N315attRfor | GATCCTCGAGGAAATTCATTCGCATCAA ACCTTTGATAC | Amplifies the excised, circular SCC*mec* element | This study |
| mecAF | CTCATATAGCTCATCATACACTTTACC | Detects *mecA* | This study |
| mecAR | CACTTATTTTAATAGTTGTAGTTGTCGG | Detects *mecA* | This study |
| unirev | GCACAGTGGGAATTAATCGAAGC | Long-range PCR primer | This study |
| 252rev | CCACTATTTAACTGACTTGATATACC | Long-range PCR primer | This study |
| Map1F | GGAAGATCTGATTGCTTAACTGC | Maps SCC*mec* deletion | This study |
| Map1R | CTCTCTTGGTCGTCAGACTGATGG | Maps SCC*mec* deletion | This study |
| Map2F | GCATGCTGCTTGCCTTAGG | Maps SCC*mec* deletion | This study |
| Map2R | CACACAGCCAAAGCAATCAGC | Maps SCC*mec* deletion | This study |
| Map3F | CCATTTGGCAGTTCTAAAAATCCG | Maps SCC*mec* deletion | This study |
| Map3R | CGTAATACATTCGGTCATTGGGAAGC | Maps SCC*mec* deletion | This study |
| Map4F | GGTTTCATGTTTGTGCTTCAGG | Maps SCC*mec* deletion | This study |
| Map4R | CACGATACAAATCAAAAAAAGGTTGG | Maps SCC*mec* deletion | This study |
| Map6F | CGTATCCTTTACAGGATATTTTGC | Maps SCC*mec* deletion | This study |
| Map6R | CTGCATATTCTTGAATTTAAAAAGG | Maps SCC*mec* deletion | This study |
| Map7F | GTTTCAGACTTTAGCGAGGAATGG | Maps SCC*mec* deletion | This study |
| Map7R | CTATGTTGTATTTATCTTCGATAATGG | Maps SCC*mec* deletion | This study |
| Map8F | GTGTTGCATTTGGTAGCC | Maps SCC*mec* deletion | This study |
| Map8R | CGATGAGTTAAGAGCACGTATC | Maps SCC*mec* deletion | This study |
| Map9F | CCGTTCGTTATAAATACTGCC | Maps SCC*mec* deletion | This study |
| Map9R | CATGGAAAGTACATATAAAAAAAGAGG | Maps SCC*mec* deletion | This study |

[a] Cm, chloramphenicol; Em, erythromycin; Tc, tetracycline; r, resistant.

$\mu$g/ml of vancomycin, or BHI broth containing 1 $\mu$g/ml of oxacillin and were grown to an $OD_{600}$ of 0.5 to 0.7. RNA was stabilized with the RNAprotect reagent (Qiagen) and was isolated by using the RNeasy kit (Qiagen), according to the manufacturer's suggested protocol. DNA was removed from the RNA samples by treatment with the DNA-free reagent (Ambion, Austin, TX), according to the manufacturer's parameters. Reverse transcription-PCR (RT-PCR) was performed by the Nucleic Acid Research Facility at Virginia Commonwealth University by using the ABI Prism 7900 sequence detection system from Applied Biosystems in conjunction with a TaqMan probe.

**Statistical analysis.** Statistical analysis was performed by using the two-tailed, paired *t* test on the data sets with Microsoft Excel software. Significance was reached with a *P* value of $\leq 0.05$.

## RESULTS

**SCC*mec* deletion mapping.** The loss of *mecA* during the passage of *S. aureus* to high-level vancomycin resistance has been observed previously (1, 31, 36, 41). In order to further characterize the loss of *mecA* during vancomycin passage, we examined three sets of strains. Each set of strains contained the parent (strains 3130, 5827, and 5836) and two passage-derived high-level vancomycin-resistant isolates, one methicillin resistant (strains 3130V32, 5827V32, and 5836V32) and one methicillin susceptible (strains 3130VR, VP32, and 5836VR). *mecA* was not detected by PCR in strain 3130VR, VP32, or 5836VR, while it was present in their parental strains and their methicillin-resistant, vancomycin-resistant derivatives (Fig. 1A). SmaI restriction digestion of total cellular DNA and PFGE were used to confirm the identity of each strain and exclude the possibility that a contaminant was isolated as well as to visualize a chromosomal deletion encompassing *mecA* (Fig. 1B). Strains 3130VR, VP32, and 5836VR differed from their parental strains by a single band shift, suggesting that in each case the loss of *mecA* is coincident with the loss of a rather large fragment of the chromosome.

CcrAB have been shown to catalyze the precise chromosomal excision of SCC*mec*, and so a likely explanation for this loss of DNA is that the SCC*mec* element was site-specifically excised from the chromosomes of strains 3130VR, VP32, and 5836VR (25, 27, 34). The excision of SCC*mec* from the chromosome can be detected by PCR amplification across the chromosomal junction from which SCC*mec* was excised by using primers I1F and I1R, as described previously (34). By this approach, strain 5836VR yielded a positive amplification product, and DNA sequencing of this product revealed that the loss of *mecA* in this strain was due to site-specific SCC*mec* excision (data not shown). However, site-specific SCC*mec* excision from strains 3130VR and VP32 was not detected.

*orfX*, the open reading frame into which SCC*mec* inserts, was detected by PCR in both strain 3130VR and strain VP32, as was a region of the staphylococcal chromosome present outside of SCC*mec*. Primers were designed in these areas for long-range PCR amplification across the SCC*mec* insertion site in each of these strains (primers I1F and unirev for strain VP32 and primers I1F and 252rev for strain 3130VR). A 6-kb

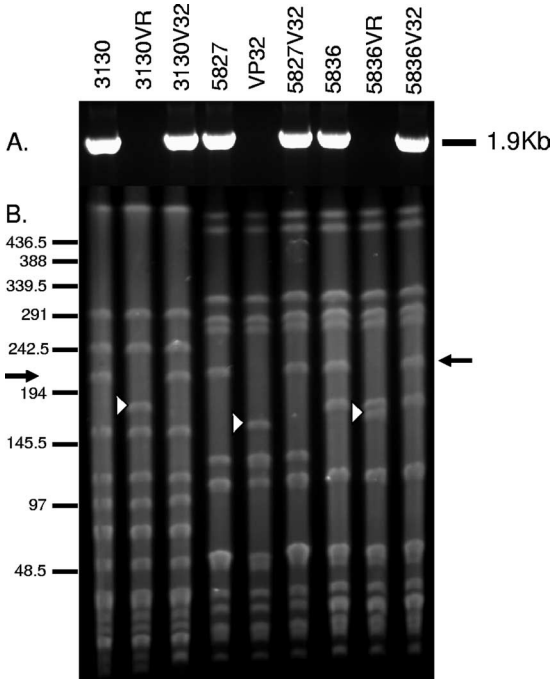


FIG. 1. Detection of *mecA*. (A) PCR for the detection of *mecA* from the strains indicated along the top. (B) SmaI-digested total cellular DNA separated by PFGE. Black arrows show bands present in the parental strains and the vancomycin-resistant, *mecA*-positive strains that shift (white arrowheads) in the vancomycin-resistant, *mecA*-negative strains, indicating a loss of DNA. Molecular weights (in kilobase pairs) are indicated on the left.

long-range PCR amplification product was generated from VP32, but there was no amplification product generated from 3130VR (data not shown). The long-range PCR fragment from VP32 was sequenced to determine the precise deletion. Strains 5836, 5827, and 3130 were found to contain SCC*mec* type II by using the method described by Ito et al. (25) (data not shown). Primers were designed to amplify various regions along the type II SCC*mec* element in an effort to map the *mecA* deletion in strain 3130VR. By this approach, an approximately 32-kb deletion was identified and 2 kb around the deletion site was amplified and sequenced to precisely map the deletion.

Figure 2 shows a schematic of the SCC*mec* elements present in the parental strains as well as the deletions from the vancomycin-resistant derivatives. Strain 3130VR had a 32-kb deletion of SCC*mec* extending from the IS*431* insertion element that flanks the integrated pUB110 to a region just beyond *ccrA*. VP32 had a larger deletion of portion of SCC*mec* that extended from the IS*431* element flanking pUB110 to a region nearly 7 kb outside the left end of SCC*mec*. As indicated above, strain 5836VR site-specifically excised the entire SCC*mec* element.

SCC*mec* elements contain recombinase genes (*ccrAB*) responsible for their mobility, so it was surprising that CcrAB-mediated recombination appeared to be responsible for the loss of *mecA* in only one of the three strains. To determine if CcrAB-mediated SCC*mec* excision is possible in strains 3130 and 5827, *ccrAB* was introduced into each strain on a multicopy plasmid, and the excision of SCC*mec* was monitored by PCR, as described previously (34). This method amplifies across the chromosomal junction from which SCC*mec* has excised as well as amplifies a fragment of the excised, circular SCC*mec* element. As shown in Fig. 3, both the chromosomal junction and the circular SCC*mec* element were amplified from strains 3130 and 5827, which contain *ccrAB* in *trans*, and sequencing of these fragments confirmed that CcrAB-mediated SCC*mec* excision was possible in these strains.

**Fitness assessment.** The fact that three strains deleted portions of SCC*mec* during passage to vancomycin resistance suggested that the loss of this DNA was somehow advantageous to the bacteria. The growth rate of each strain was monitored, and the doubling times during the exponential growth phase are shown in Fig. 4. In each case, passage to vancomycin resistance caused an increase in the doubling time compared to that of the parent strain. This decrease in growth rate was partially compensated for by the deletion of SCC*mec*, as strains 3130VR, VP32, and 5836VR each grew faster than their SCC*mec*-containing counterparts, strains 3130V32, 5827V32, and 5836V32, respectively (the differences were not statistically significant by a paired *t* test).

To further assess the fitness advantage of deleting portions of SCC*mec*, mixed-culture competition experiments were employed. Strains 3130VR and 3130V32, VP32 and 5827V32, and 5836VR and 5836V32 were competed in mixed cultures. Representative results of three independent competition experiments are shown in Fig. 5. After 3 days of competition, 3130V32 was not recovered from the mixed cultures, indicating that 3130VR had outcompeted 3130V32 for the limited resources available for growth. Similar results were seen with 5827V32 after 5 days of competition and with 5836V32 after 3 days of competition. These data reveal that, in each case, the deletion of a portion of SCC*mec* provided a significant fitness advantage that allowed the strains (3130VR, VP32, and 5836VR) to completely outcompete their SCC*mec*-containing counterparts (3130V32, 5827V32, and 5836V32) in less than 5 days.

Strains 3130VR, VP32, and 5836VR each lost a significant amount of DNA containing the *mec* operon (*mecI*, *mecR1*, and *mecA*), including the identified regions *ccrAB*, Tn*554*, and pUB110. It was not clear exactly which portion of this deleted DNA was responsible for the change in fitness. To further assess the role of *mecA* in fitness, *mecA* was insertionally inactivated in strain 3130V32 by insertion of the tetracycline resistance gene, *tetM* (Table 1), to yield strain 3130V32Δ*mecA*. To determine if the inactivation of *mecA* caused a change in fitness, strains 3130V32/pRN5543 (chloramphenicol resistant) and 3130V32Δ*mecA* (tetracycline resistant) were competed in mixed culture (representative results are shown in Fig. 6). After 8 days of passage, 3130V32/pRN5543 was not recovered from the mixed culture, indicating that the inactivation of *mecA* in strain 3130V32Δ*mecA* provided a gain in fitness that enabled it to readily outcompete an isogenic counterpart containing *mecA* (3130V32/pRN5543). To determine if the *mecA*-imposed fitness cost is limited to vancomycin-resistant *S. aureus* strains, vancomycin-susceptible strains 450MHomo, 450MHomoex, and 450MHomoΔ*mecA* were examined by competition. Strain 450MHomo contains the intact type I SCC*mec* and is homotypically oxacillin resistant, 450MHomoex is strain

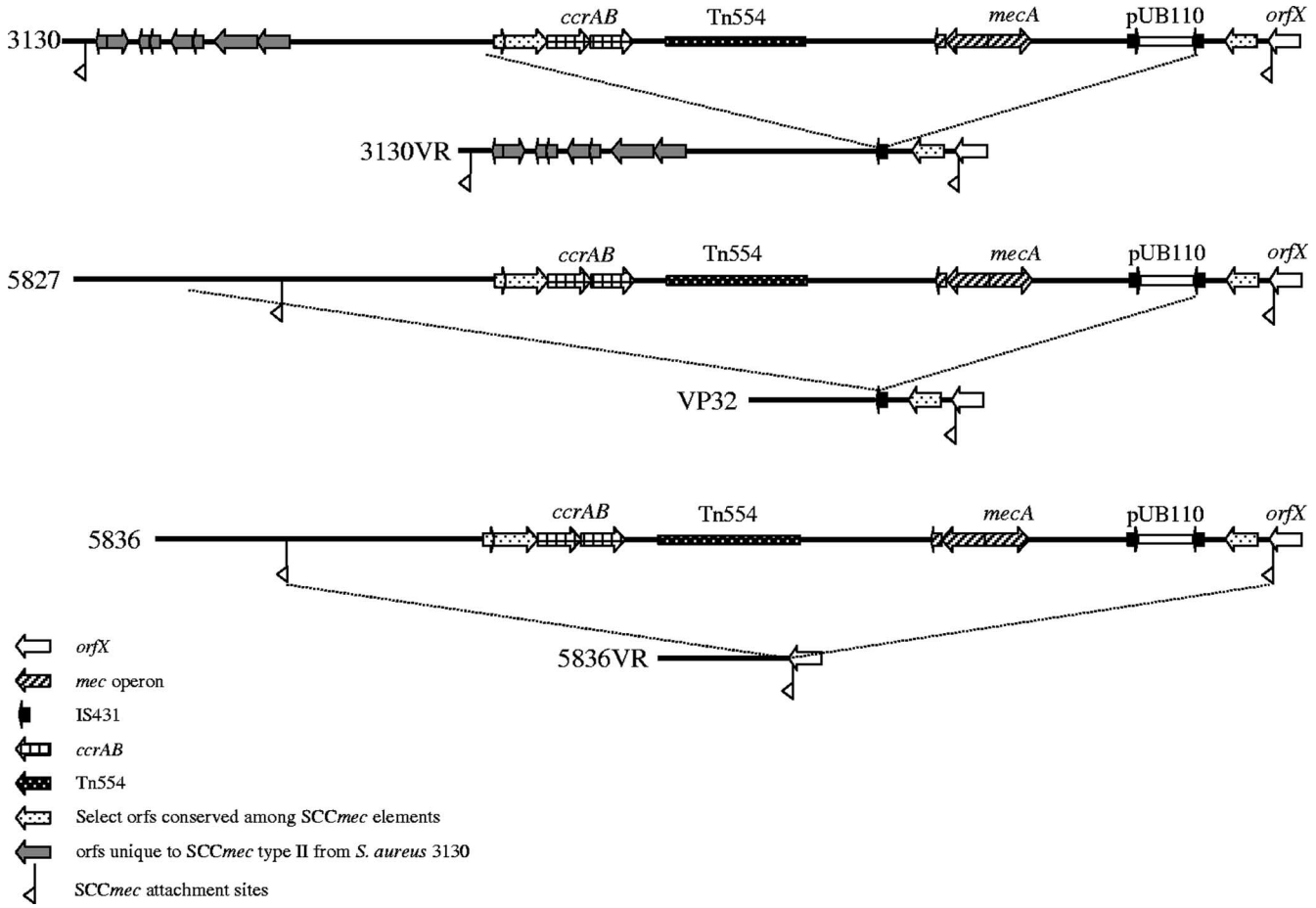


FIG. 2. Schematic of SCC*mec* elements and the deletions in vancomycin-resistant derivatives. Depicted are the SCC*mec* elements of the parental strains as well as the remnants of SCC*mec* present in the vancomycin-resistant, methicillin-susceptible derivatives. The region deleted from the parent to the derivative is indicated by dotted lines. Selected open reading frames (orfs) are shown as block arrows, identified in the key at the bottom. All parental strains contained SCC*mec* type II. The deletions in strains 3130VR and VP32 mapped precisely to the copy of IS*431* flanking PUB110, while 5836VR deleted SCC*mec* in a manner consistent with CcrAB-mediated recombination. All deletions included the *mec* operon (*mecA*, *mecRI*, and *mecI*) as well as *ccrAB*.

450MHomo with the SCC*mec* element site-specifically excised, and 450MHomoΔ*mecA* is 450MHomo with *mecA* insertionally inactivated by *tetM*. For these competition experiments, 450MHomo was selected by using oxacillin and 450MHomo Δ*mecA* was selected by using tetracycline. Strain 450MHomoex lacks a selectable marker. Therefore, when 450MHomoex was present in mixed cultures, the total number of cells on nonselective medium is shown. Representative results of these competition experiments are shown in Fig. 7. Strain 450MHomo, which contains an intact *mecA*, was outcompeted by 450MHomoΔ*mecA* and 450MHomoex, neither of which contained *mecA* (Fig. 7, top panels). However, there was no detectable decline in 450MHomoΔ*mecA* when it was competed with 450MHomoex (Fig. 7, bottom panel), suggesting that these two strains have similar fitness levels. Taken together, these data show that there is not an appreciable difference in fitness between a strain that has lost the entire SCC*mec* element and a strain in which *mecA* is inactivated, although both show a fitness advantage over the fitness of a strain containing *mecA*. Therefore, the fitness cost associated with *mecA* is not limited to vancomycin-resistant strains, with vancomycin-susceptible, homotypic MRSA strains displaying similar fitness costs.

Strains 3130V32, 5827V32, and 5836V32 each carried the type II SCC*mec* element, which contains the intact *mec* operon, consisting of *mecA* and the regulatory genes, *mecR1* and *mecI*. They also produce β-lactamase and carry *blaZ* as well as the regulatory genes, *blaR1* and *blaI*. Both MecI and BlaI repress *mecA* transcription in the absence of β-lactam antibiotics. *mecI* and *blaI* were amplified by PCR from strains 3130V32, 5827V32, and 5836V32; and their nucleotide sequences were determined. Each strain was found to contain at least one intact repressor of *mecA* transcription (data not shown). Neither the parental strains nor the vancomycin-resistant derivatives were exposed to β-lactam antibiotics, and therefore, *mecA* should have been repressed and expressed at low levels. However, low-level *mecA* expression would not be expected to impose a fitness cost on the bacterium. To gain a better understanding of the *mecA* expression levels in the parental and vancomycin-resistant derivative strains, real-time RT-PCR was performed with uninduced strains, strains grown in 1 μg/ml of oxacillin, and strains grown in 1 μg/ml of vancomycin; and the results are shown in Table 3. Induction with oxacillin was not required for the levels of *mecA* transcription to be detectable, as *mecA* was expressed from all strains at submaximal levels when it was uninduced or exposed to vancomycin, suggesting that MecI/

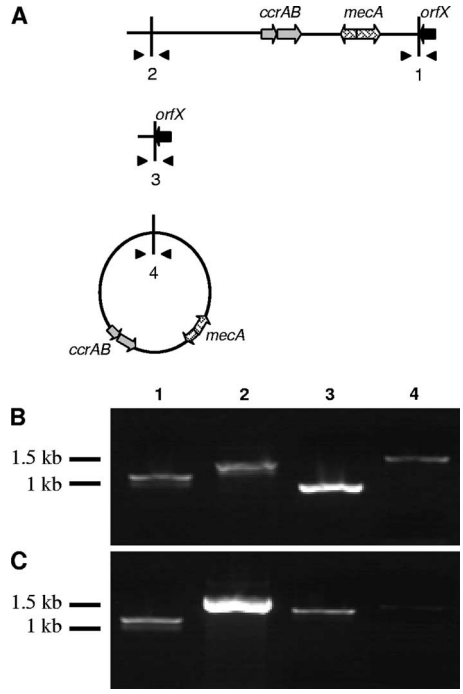


FIG. 3. Site-specific SCC*mec* excision in strains 3130 and 5827. CcrAB-mediated excision of SCC*mec* was monitored by use of a PCR-based technique. PCR was performed with total cellular DNA isolated from strains 3130 and 5827 containing *ccrAB* on a multicopy plasmid. (A) Schematic of the region of the staphylococcal chromosome where SCC*mec* is integrated (top), the staphylococcal chromosome where SCC*mec* has excised (middle), and the excised, circular SCC*mec* element (bottom). Selected open reading frames are shown as block arrows for orientation; vertical lines indicate SCC*mec* attachment sites; and small triangles represent the primers used to amplify the right SCC*mec* attachment site (triangles 1), the left SCC*mec* attachment site (triangles 2), the chromosomal junction from which SCC*mec* excised (triangles 3), and the excised, circular SCC*mec* element (triangles 4). (B and C) Agarose gel electrophoresis of PCR amplification products from strain 3130 and 5827, respectively. The numbers used to designate the lanes correspond to the primer sets depicted by triangles in panel A.

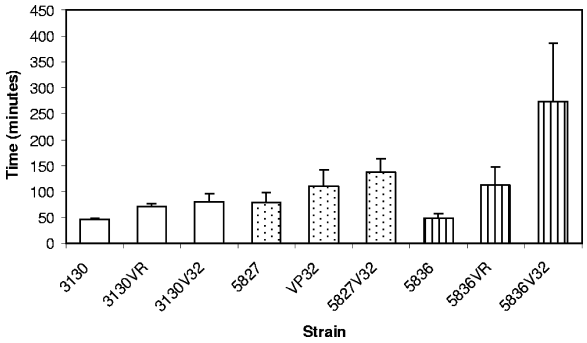


FIG. 4. Growth analysis. The doubling time is shown for each strain listed. Doubling times with error bars (standard deviations) were calculated from the exponential growth phases of seven independent growth curves prepared for each strain. Statistical analysis for comparison of the results for each derivative strain to those for its parent strain was performed by a paired *t* test.

BlaI-mediated repression is incomplete in these strains. Furthermore, when they were exposed to vancomycin, the vancomycin-resistant derivative strains expressed *mecA* at levels greater than those seen in their vancomycin-susceptible parents. While these levels were not maximal, they may have resulted in the production of sufficient PBP 2a to impose a fitness cost on the vancomycin-resistant strains.

## DISCUSSION

Passage of *Staphylococcus aureus* in the presence of increasing concentrations of vancomycin leads to vancomycin resistance mediated by changes in cell wall structure. Although this is a laboratory phenomenon, it is thought to mimic the mechanism of resistance seen in vancomycin-intermediate resistant *S. aureus* (VISA) clinical isolates (MICs = 8 μg/ml). However, high-level vancomycin resistance (MICs ≥ 32 μg/ml) due to this resistance mechanism is not seen among clinical isolates. Here we show that high-level, passage-induced vancomycin resistance is associated with a decrease in the growth rate and the loss of SCC*mec*, including *mecA*, observations that have also been made by others (1, 31, 36, 41). These SCC*mec* deletions resulted in strains with a faster growth rate and an ability to outcompete their *mecA*-containing vancomycin-resistant counterparts in mixed culture competition experiments (Fig. 4 and 5). These data indicate that deletion of this DNA is not just coincident with passage on vancomycin but also advantageous to the bacterium during the gradual conversion to high-level vancomycin resistance. All strains studied contained SCC*mec* type II. While it is unlikely, it is possible that different SCC*mec* types may behave differently. We also provide evidence indicating that it was the loss of *mecA* and not other regions of SCC*mec* that was responsible for the gain in fitness, since the insertional inactivation of *mecA* in otherwise isogenic vancomycin-resistant strains provided a fitness advantage in mixed-culture competition experiments (Fig. 6). By using a vancomycin-susceptible laboratory strain in mixed-culture competition experiments, the excision of SCC*mec* provided no fitness advantage over the inactivation of *mecA* (Fig. 7). It seems that *Staphylococcus aureus* has difficulty simultaneously expressing high-level resistance to methicillin and high-level, passage-induced resistance to vancomycin. When high-level resistance to both is achieved, it comes at a great fitness cost to the bacterium. This may explain the absence of strains with high-level, passage-induced vancomycin resistance among clinical isolates. The fitness disadvantage would make them poor pathogens, and the loss of *mecA* with increasing vancomycin resistance would allow them to become susceptible to β-lactam antibiotics. The loss of β-lactam resistance also provides a rationale for treating infections due to VISA isolates with both vancomycin and β-lactams, the benefit of which has been demonstrated in an animal model of endocarditis (12).

Several lines of evidence suggest that the expression of methicillin resistance or *mecA* is deleterious to the staphylococcal cell. Ender et al. found an inverse correlation between oxacillin resistance levels and growth rate by competing strains with heterotypic and homotypic expression of oxacillin resistance (19). Also, Katayama et al. have shown that a naïve methicillin-sensitive *S. aureus* strain did not tolerate the introduction of plasmid-borne, unregulated *mecA*, as *mecA* was often mutated or deleted to circumvent the expression of PBP 2a (28). When

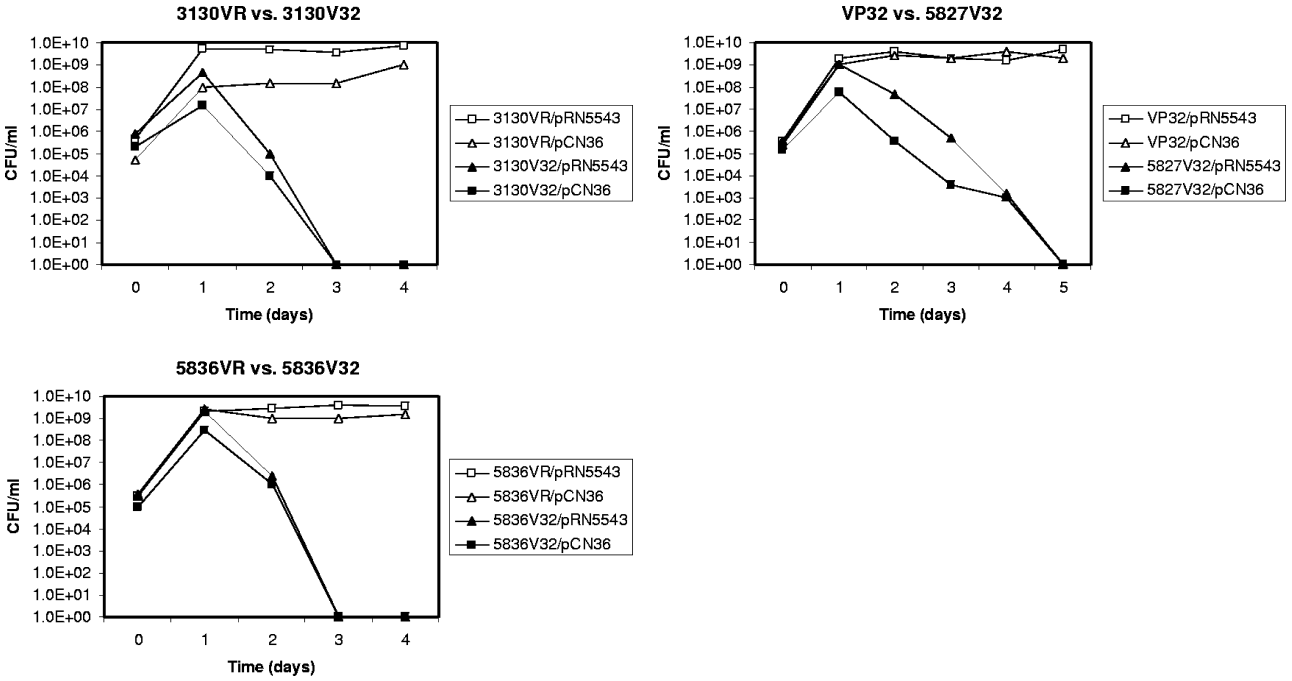


FIG. 5. Mixed-culture competition experiments. Equal numbers of two different strains were mixed in liquid culture and passaged for up to 5 days. The bacteria were enumerated by plating them on differential selective medium. Differential selectable markers were introduced on plasmids pRN5543 (chloramphenicol) and pCN36 (tetracycline). Each strain pair was competed twice, with the plasmids switched to correct for the possibility of differential fitness costs of each plasmid.

either the *mec* or *bla* regulatory gene was introduced along with *mecA*, the system was tolerated (28). The fitness cost associated with *mecA* in the current study is puzzling, because the strains in this study were not exposed to β-lactams, and therefore, the transcription of *mecA* should have been tightly regulated by the MecI or the BlaI repressor. As shown in Table 3, *mecA* was expressed at detectable levels in these strains without exposure to β-lactams, suggesting that, even though they carried intact repressors, the repression of *mecA* transcription was incomplete. McAleese et al. found that *mecA* transcription was down-regulated 2.9-fold in a VISA clinical isolate compared to the level of transcription in its vancomycin-sensitive parent (30). These strains contained *mecI*, yet the level of *mecA* transcription was reduced below the MecI-repressed levels as the level of vancomycin resistance increased. The data for our three passaged strains were similar, in that the level of *mecA* transcription in each vancomycin-resistant derivative was less than that seen in the parent. This suggests that the MecI-repressed levels of *mecA* transcription in the parent were in excess of what could be tolerated by the VISA or passage-derived vancomycin-resistant derivatives. Interestingly, we also saw an increase in *mecA* transcription in the vancomycin-resistant derivatives upon vancomycin exposure, possibly providing the trigger for *mecA* excision. Therefore, although PBP 2a was not quantified, it is likely that the level of *mecA* transcription observed, despite the MecI/BlaI repression, produced sufficient PBP 2a to place an additional burden on the bacterium during passage to vancomycin resistance, resulting in the fitness costs seen in this study.

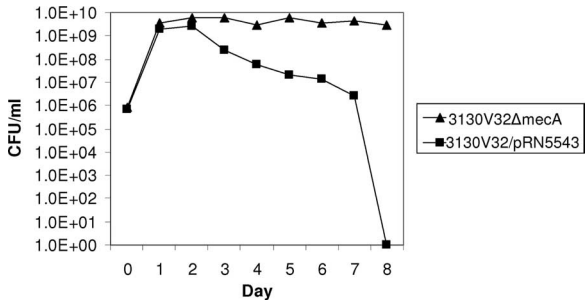


FIG. 6. Competition of 3130V32/pRN5543 and 3130V32Δ*mecA*. To determine if the fitness differential evident with the deletion of portions of SCC*mec* was due to the loss of *mecA*, 3130V32/pRN5543 and 3130V32Δ*mecA* were analyzed in mixed-culture competition experiments. Strain 3130V32Δ*mecA* is strain 3130V32 with *mecA* insertionally inactivated by *tetM*. Equal numbers of 3130V32/pRN5543 and 3130V32Δ*mecA* cells were mixed in liquid culture and passaged for 8 days. The bacteria were enumerated by plating them on differential selective medium (3130V32/pRN5543 on chloramphenicol and 3130V32Δ*mecA* on tetracycline).

The mechanism by which PBP 2a imposes a fitness cost on the bacterium is unclear. It may be that PBP 2a interferes directly with the altered cell wall biosynthesis of vancomycin-resistant strains. However, *mecA* was found to impose a fitness cost on a vancomycin-susceptible, homotypic methicillin-resistant strain (strain 450MHomo), indicating a cost associated with PBP 2a in the absence of the aberrant cell wall synthesis of vancomycin-resistant strains.

Parent strain 5836 displayed heterotypic resistance to methicillin, while strain 5836V32 was homotypically resistant to methicillin (as determined by population analysis profiling [data not shown]). Similarly, in our laboratory, all heterotypic

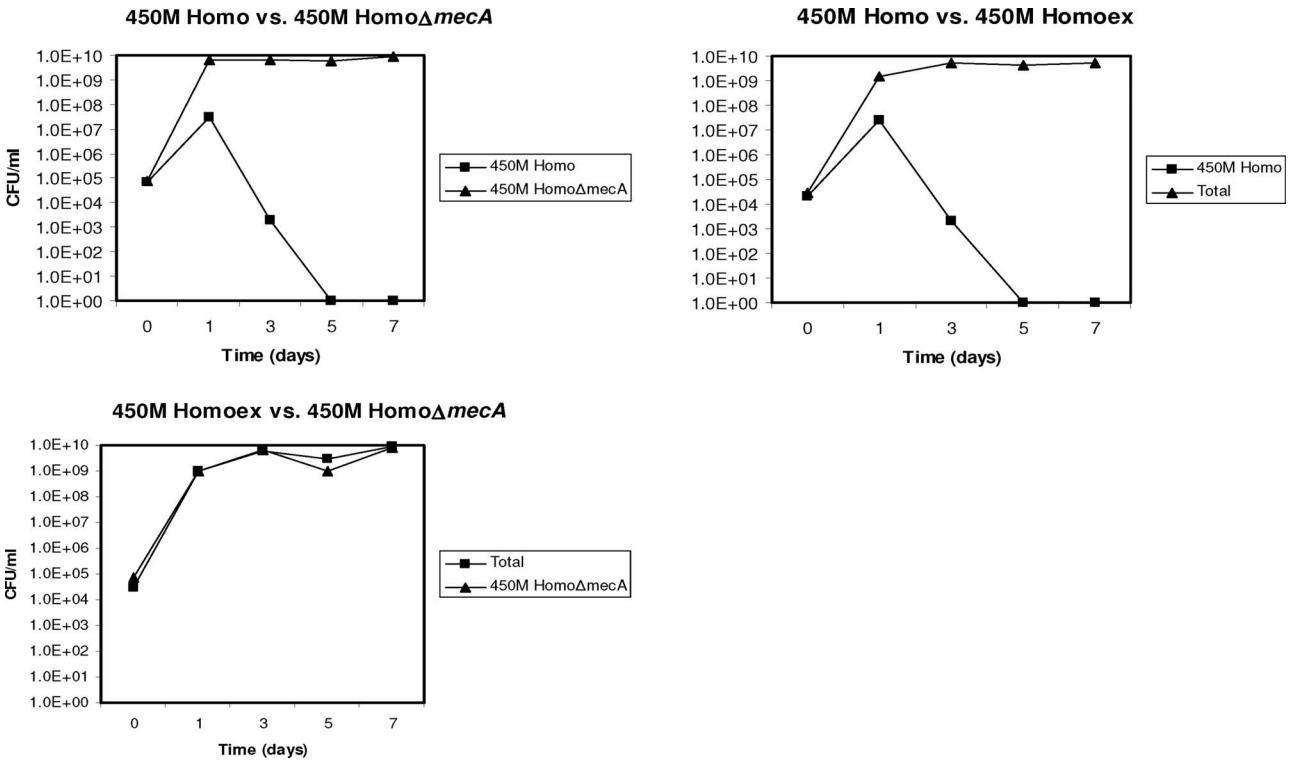


FIG. 7. Competition of 450MHomo, 450MHomoex, and 450MHomoΔ*mecA*. Equal numbers of two different strains were mixed in liquid culture and passaged for up to 5 days. The bacteria were enumerated by plating them on differential selective medium (450MHomo on oxacillin, and 450MHomoΔ*mecA* on tetracycline; 450MHomoex could not be directly selected for, and so the total colony count on antibiotic-free medium is shown).

MRSA strains that have been passaged to high-level vancomycin resistance and have retained methicillin resistance have converted to homotypic methicillin resistance ($n = 7$; data not shown). Therefore, it may be that the *mecA*-associated fitness cost is a result of the conversion to the homotypic expression of methicillin resistance and not a direct consequence of increasing resistance to vancomycin, as previously shown by Ender et al. (19). The conversion from heterotypic to homotypic methicillin resistance is not well understood. However, it is has been shown that the conversion is due to mutational events occurring outside of SCC*mec* and is not a result of increased *mecA* expression (20, 38). Further investigation into the mechanism of heterotypic-homotypic conversion may shed light on the cause of the *mecA*-associated fitness cost in these strains.

Strain 5836VR lost *mecA* by site-specific excision of SCC*mec*, while strains 3130VR and VP32 both deleted portions of SCC*mec* in a manner inconsistent with CcrAB-mediated SCC*mec* excision. The introduction of *ccrAB* in *trans* did lead to the excision of SCC*mec* from the chromosomes of strains 3130 and 5827 (Fig. 3), suggesting that these strains are capable of CcrAB-mediated SCC*mec* excision, yet this was not the mechanism of *mecA* deletion in 3130VR and VP32. This may indicate that, in some cases, SCC*mec* mobility is accomplished by mechanisms not involving CcrAB. Despite the deletion of fragments of different sizes, the DNA deleted in both 3130VR and VP32 maps precisely to an IS*431* element located adjacent to pUB110 in SCC*mec*. Reipert et al. also noted a chromosomal deletion encompassing *mecA* in a vancomycin-resistant *S. aureus* strain, and this deletion also mapped to the IS*431* element present downstream of *mecA* (36). IS*431* is present in all known SCC*mec* elements just downstream of the *mecA* operon, and in some cases, the *mec* operon is flanked by IS*431* on either side (35, 40). These findings suggest a role for IS*431* in the deletion of portions of SCC*mec*, and therefore, it is possible that IS*431* transposition/recombination may also play a role in the mobility and transfer of *mecA*.

TABLE 3. *mecA* expression

| Strain | Relative transcriptional units[a] | | |
|---|---|---|---|
| | UI | Vm | Ox |
| 3130 | 543.8 ± 126.3 | 337.5 ± 93.3 | 1,570.8 ± 591.9 |
| 3130V32 | 438.4 ± 168.5 | 639.3 ± 174.8 | 1,911.3 ± 1,587.8 |
| 5827 | 229.1 ± 232.9 | 125.2 ± 80.8 | 1,545.4 ± 489.4 |
| 5827V32 | 137.0 ± 68.3 | 270.9 ± 177.3 | 827.9 ± 747.1 |
| 5836 | 221.6 ± 179.6 | 43.4 ± 75.2 | 287.4 ± 277.1 |
| 5836V32 | 81.8 ± 16.3 | 200.7 ± 108.4 | 923.8 ± 224.4 |

[a] Relative to 16S rRNA. UI, uninduced; Vm, induced with vancomycin; Ox, induced with oxacillin.

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