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Conversion of Oxacillin-Resistant Staphylococci from Heterotypic to Homotypic Resistance Expression

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Staphylococci that acquire the *mecA* gene are usually resistant to β-lactam antibiotics (methicillin or oxacillin resistance). *mecA* encodes a penicillin-binding protein (PBP 2a) that has a reduced affinity for β-lactams. In some isolates with methicillin or oxacillin resistance, only a small proportion (≤0.1%) of the population expresses resistance to ≥10 μg of oxacillin per ml (heterotypic resistance [HeR]), while in other isolates most of the population expresses resistance (homotypic resistance [HoR]). In the present study, growth of *Staphylococcus aureus* or *Staphylococcus epidermidis* strains with HeR in concentrations of oxacillin (0.3 to 0.7 μg/ml) that produced a fall or a lag in optical density converted the strains from the HeR to the HoR phenotype. The conversion from the HeR to the HoR phenotype appeared to be due to the selection of a highly resistant mutant population, as determined by fluctuation analysis and the failure of populations with HoR to revert to HeR after 60 generations of growth in antibiotic-free media. The frequencies of conversion were as high as 10^{-3} to 10^{-2}. Conversion to HoR required an intact *mecA* gene and an increase in the level of *mecA* transcription since no highly resistant subpopulation could be selected after growth in oxacillin when *mecA* transcription was constitutively repressed or when *mecA* had been inactivated. In addition, in both *S. epidermidis* and *S. aureus* the level of resistance to vancomycin, which also acts directly on the staphylococcal cell wall, was greater among convertants with HoR than their isogenic parents. The conversion of a population from HeR to HoR involves the selection of a mutation(s) that occurs at a high frequency and most likely requires abundant PBP 2a.

Oxacillin (methicillin) resistance in staphylococci is usually mediated by an acquired gene (*mecA*) that encodes a penicillin-binding protein (PBP 2a) not inhibited by β-lactam antibiotics (7, 15, 26). While *mecA* is essential for oxacillin resistance, the phenotypic expression of resistance in cells containing *mecA* varies. The predominant phenotype is called heterotypic or heterogeneous and is characterized by the presence of a small population of cells (usually 0.1 to 0.01%) that are resistant to >10 μg of oxacillin per ml, while the majority of the population is killed by low concentrations of the antibiotic. The colony sizes of isolates that survive on agar containing oxacillin are also heterogeneous. In contrast, the homotypic or homogeneous resistance phenotype is characterized by a uniform, highly oxacillin resistant population that exhibits little variation in colony size on agar (25). When examined by enumeration of colonies on agar containing increasing concentrations of methicillin or oxacillin (efficiency of plating [EOP]) the heterotypic phenotype has been shown to be stable and reproducible. However, alteration of environmental conditions such as the temperature and the salt concentration markedly affects resistance expression (6, 11, 12, 25).

One of the most important observations that has been made about the phenotype is that an oxacillin-resistant population with homotypic resistance will arise from one that has heterotypic resistance following exposure of a staphylococcal isolate to a β-lactam antibiotic (12). It is not clear from studies that have examined this conversion if the population with homotypic resistance emerges due to the selection of mutational events or to induction of a resistance pathway. Single, highly resistant colonies that seem to be the result of a chromosomal mutation(s) have been characterized (20), and it has been proposed that highly resistant subpopulations may arise from a more susceptible background by some sort of genetic rearrangement similar to phase variation (8). However, the size of the resistant subpopulation (10^{-3} to 10^{-5}) in strains from which resistant mutants have been selected is larger than that usually seen with spontaneous mutations of phase variations. In addition, colonies with homotypic resistance that are selected by antibiotic exposure have been variously described as phenotypically stable, not reverting to heterotypy following growth in antibiotic-free medium (20), or unstable, exhibiting rapid phenotypic reversion (8). Phenotypic stability would suggest the selection of mutations, while instability would be more characteristic of induction.

Attempts to understand the molecular basis of heterotypy and homotypy have largely assessed mutations that converted a stable, homotypically resistant *S. aureus* strain, strain COL, to heterotypic resistance or a less resistant phenotype (3, 9). The resulting studies have revealed a number of important genes that are essential for normal cell wall construction. However, it is not clear if these same genes are necessary for or are even involved in conversion from the heterotypic to the homotypic resistance phenotype. Since it seems likely that an understand-

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ing of the conversion from the heterotypic to the homotypic resistance phenotype will be essential to obtaining an understanding of these phenotypes, we have undertaken a detailed characterization of the two populations and their selection. While at this stage the studies are still largely descriptive, we believe that careful definitions will assist with a search for molecular mechanisms.

MATERIALS AND METHODS

Bacterial strains. *Staphylococcus aureus* strains RN450M, N315, 67-0, and BMS1 along with *S. epidermidis* isolates SE42, SE60, SE53, and SE20 have been described previously (10, 18). All strains are oxacillin resistant and contain *mecA*. All strains except RN450M contain functional *mecR1* and *mecI* genes. Strain RN450M is strain RN450 transformed with the *mec* region from strain COL, and both *mecI* and the 3′ end of *mecR1* are deleted from RN450. The *mecI* gene of 67-0 contains a missense mutation that decreases but that does not abolish the repression of *mecA* transcription (17). All strains exhibited a heterotypic resistance pattern.

Materials and media. Mueller-Hinton broth and Mueller-Hinton agar (MHA) (both from BBL Microbiology Systems, Cockeysville, Md.) and brain heart infusion (BHI) broth and agar (Difco Laboratories, Detroit, Mich.), with and without selective additives (Sigma, St. Louis, Mo.; United States Biochemicals, Cleveland, Ohio), were used for subculture and maintenance of *Escherichia coli*, *S. epidermidis*, and *S. aureus* strains.

Conversion to homotypic resistance phenotype in broth. Conversion of the strains from the heterotypic to the homotypic resistance phenotype was performed as follows. The bacteria were grown in 5 ml of BHI broth without antibiotic overnight. Cultures were diluted to an optical density at 600 nm (OD_{600}) of ≈0.05 in 100 ml of BHI broth with 0.3 to 0.7 μg of oxacillin (Sigma) per ml and were grown at 37°C with shaking (180 rpm). The ODs were monitored every hour for 15 h. Starting at 5 h, a sample was streaked onto an oxacillin gradient plate with a concentration gradient from 0 to 100 μg/ml. In addition, the total number of viable bacteria was estimated by plating dilutions of the culture on MHA without antibiotic and counting the numbers of CFU after 24 h at 37°C. All plates were incubated at 30°C for 48 h. Conversion was verified by EOP assays as described by Chambers et al. (5), except that oxacillin was substituted for methicillin.

Reversion from homotypic to heterotypic resistance phenotype. A 1-μl culture of bacteria that had been converted from the heterotypic to the homotypic resistance phenotype as described above was inoculated into 100 ml of fresh BHI broth (an approximately 10^{-5} dilution) without antibiotic and incubated at 37°C overnight with shaking. This process was repeated for 5 days, giving approximately 60 generations of growth. At day 5, EOP assays were performed to determine the resistance phenotype. Strains that had converted from the heterotypic to the homotypic resistance phenotype were also passed on antibiotic-free agar to assess phenotypic reversion. Several colonies were randomly selected from an antibiotic-free MHA plate, and each colony was then streaked onto another MHA plate. This process was repeated for 10 days. At day 10, an EOP assay was performed on plates containing oxacillin to determine the resistance phenotype.

Conversion to homotypic growth on agar. EOP was determined for strain RN450M on oxacillin by using concentrations of antibiotic lower than those routinely used for this analysis (0.5, 1, 2, 5, 10, 20, 50, and 100 μg/ml). Following incubation at 30°C for 48 h, colonies were picked from the plates containing 1, 5, 10, 20, 50, and 100 μg of oxacillin per ml. Each colony was resuspended in 1 ml of BHI broth, and another EOP assay was performed with each colony.

MIC determinations for other cell wall-active agents. The MICs of vancomycin and oxacillin were determined by the agar dilution method with an inoculum of 10^4 CFU per spot, according to the guidelines of NCCLS (17). The plates used for determination of the oxacillin MICs were incubated at 35°C, while those used for determination of vancomycin MICs were incubated at 37°C. Rectangular plates containing a gradient of 100 μg of oxacillin per ml were swabbed along the gradient with a swab that contained a suspension that matched a 0.5 McFarland standard suspension.

Fluctuation analysis. The classic method of Luria and Delbruck (14) was used to investigate the likelihood that generation of a homotypically oxacillin resistant population from one that was heterotypically resistant was due to the selection of preexisting mutations rather than induction of phenotypic changes in response to the inducing agent. Overnight cultures were diluted so that the final colony count was 10^3 CFU/ml, and 2 ml of culture was pipetted into each well of a 24-well plate. The plates were incubated at 37°C for 24 h with shaking (180 rpm). Various dilutions (10^{-1} to 10^{-9}) were plated onto MHA with no antibiotic and MHA containing rifampin (1 μg/ml), oxacillin (20 μg/ml), or minocycline (1 μg/ml). Colonies were counted after 48 h of incubation at 30°C. The resistance frequency was obtained by dividing the number of colonies on agar containing antibiotic by the number of colonies on antibiotic-free medium.

Statistical methods. Our hypothesis (the null hypothesis) was that if conversion from heterotypic to homotypic resistance was due to the induction of resistance, there would be a small fixed chance (parameter P) that resistance would be induced in each bacterium upon exposure to an antibiotic (oxacillin, rifampin, or minocycline). A binomial distribution would be expected if the number (X) of resistant bacteria out of the total number (N) of bacteria had a probability (P) of resistance. In cases in which the rate of resistance was small, there would be a Poisson distribution. Because the number of bacteria (N) in each microtiter well varied, we normalized the number of bacteria in each well for each set of antibiotic trials to the highest number of CFU per milliliter obtained for that trial. When N is large, the binomial distribution is the same as the normal distribution with mean $N \cdot P$ and variance $N \cdot P \cdot (1 - P)$. We tested the goodness of the binomial (normal) distribution using the Kolmogorov-Smirnov goodness of fit (23). The failure of the data to conform to a normal distribution would result in a rejection of the null hypothesis. The calculations were carried out with the statistical software package S-Plus 2000 (S-Plus 2000 Guide to Statistics, Data Analysis Products Division, Math Soft, Seattle, Wash., 2000).

Monitoring of *mecA* expression during conversion to homotypy. *mecA* expression was monitored by use of *mecA-lacZ* transcriptional fusions pGO630 and pGO630(ΔR1) that had been integrated into the chromosomal lipase gene with the integration vector pSK950, as described previously (22). The pGO630 construct contained intact *mecA* regulatory genes *mecR1* and *mecI*, with the first 50 bp of *mecA* fused to the *lacZ* reporter (22). The construct with the *mecR1* mutation was made by cleaving *mecR1* at its unique *Cla*I restriction site, filling in the single-stranded overhang with the Klenow fragment (New England Biolabs, Beverly, Mass.), and ligating the blunt ends, creating a frameshift mutation. The presence of the mutation was confirmed by nucleotide sequence analysis. The assay itself was performed by the manufacturer's protocol (Galacto Light Plus; chemiluminescent reporter assay; Tropix, Bedford, Mass.), with minor changes. Briefly, at each time point 1 ml of culture was centrifuged at 10,000 × *g* for 1 min. The pellet was then washed in phosphate-buffered saline and resuspended in 100 μl of lysis buffer (Tropix) containing 50 μg of lysostaphin per ml. After a 15-min incubation at 37°C, the culture was centrifuged as described above and 20 μl of the supernatant was incubated in microtiter wells with 70 μl of Galactron-Plus (1,2-dioxetane; Tropix) for 1 h according to the manufacturer's instructions. The β-galactosidase activity was measured with a Tropix TR717 microplate luminometer with a 100-μl injector and a 1.6-s delay and by reading for 10 s. Activity was expressed as the number of relative light units per the specific OD_{600} at which the sample was taken.

Inactivation of *mecA*. The *mecA* gene was inactivated by allelic replacement. An *E. coli-S. aureus* shuttle vector was constructed by using temperature-sensitive pE194 (erythromycin resistant) as a staphylococcal replicon. pE194 contained *mecA* interrupted after 910 bp by *tet*(M) (5′ to the penicillin binding domain). Following growth at the nonpermissive temperature for plasmid replication (42°C), colonies that would grow on minocycline (5 μg/ml) but not erythromycin (10 μg/ml) were sought, indicating integration of *tetM* into *mecA* and loss of plasmid sequences by secondary recombination. Integration was confirmed by PCR and Southern hybridization. The construct and method of allelic replacement were similar to those described previously for inactivation of *mecI* (18).

RESULTS

Conversion from heterotypic to homotypic resistance phenotype in broth. Methicillin-resistant *S. aureus* MRSA strains RN450M, N315, 67-O, and BMS1 and methicillin-resistant *S. epidermidis* strains SE42, SE53, SE60, and SE20 were tested for their abilities to convert from the heterotypic to the homotypic resistance phenotype by growth in broth containing oxacillin. The results were comparable for all strains; the data for the methicillin-resistant *S. epidermidis* strains are presented elsewhere (10). After conversion was confirmed by EOP assays, all strains with the homotypic resistance phenotype were designated Ho. For example, RN450MHo represents strain RN450M converted to homotypy. A typical growth curve,

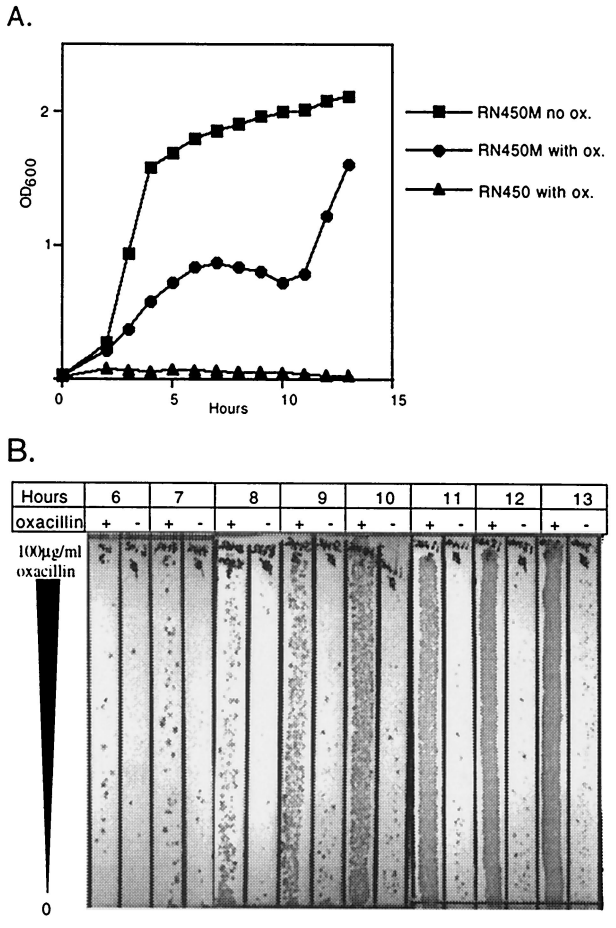

FIG. 1. Time course and phenotypic expression of conversion to homotypic oxacillin resistance of *S. aureus* strain RN450M. The linear OD_{600} is on the *y* axis, and time is on the *x* axis. (A) Growth curve of oxacillin-resistant strain RN450M and its isogenic oxacillin-susceptible parent, RN450, in the presence of oxacillin (ox.; 0.5 μg/ml). A growth curve for RN450M in the absence of oxacillin is included for comparison. (B) Oxacillin gradient plates streaked with cells of RN450M grown in the presence and absence of oxacillin. The concentrations in the gradient are indicated to the left. The time points correspond to those in the graph in panel A.

shown for strain RN450M, is presented in Fig. 1. Conversion to homotypy occurred following growth in oxacillin, but only if the concentration of antibiotic used was sufficient to cause either a growth lag or a drop in the OD. The concentrations of oxacillin that did not cause a growth lag or a drop in the OD compared to the growth curve for strains grown without oxacillin resulted in no change in phenotype. The minimum oxacillin concentration required for a growth lag was from 0.3 to 0.7 μg/ml for all strains, and conversion occurred at these concentrations after 6 to 8 h of growth. Higher concentrations also resulted in a phenotypic conversion, but the time of the growth lag was longer, varying directly with the antibiotic concentration.

Reversion from homotypic to heterotypic resistance phenotype. An assessment of reversion of *S. aureus* RN450MHo, 67-0Ho, and N315Ho and *S. epidermidis* SE42Ho, SE60Ho, and SE20Ho to heterotypy following passage in antibiotic-free medium is shown in Fig. 2. There was only a 1- to 2-log decrease in the numbers of CFU per milliliter for any strain on oxacillin-containing agar after growth in antibiotic-free broth for 60 generations. In no strain was there a reversion to the expression of the heterotypic resistance of the parent. In addition, no reversion was seen after 10 passages of five colonies each of *S. aureus* RN450MHo and *S. epidermidis* SE60Ho and SE20Ho on antibiotic-free agar.

Fluctuation analysis of conversion from heterotypic to homotypic resistance phenotype. *S. aureus* RN450M and *S. epidermidis* SE42 were used for fluctuation analysis of conversion from the heterotypic to the homotypic resistance phenotype. Since mutations could arise randomly at any point in time during any generation, one would not expect the number of bacteria growing on oxacillin-containing agar to be normally distributed among the wells if conversion to homotypy was due to the selection of mutants. In contrast, if resistance was the result of induction of a pathway(s) upon exposure to the antibiotic, the number of colonies on agar should be relatively uniform among wells and should conform to a normal distribution. As controls, we also inoculated each culture onto agar containing rifampin, resistance to which is known to be due to mutation of the target (2), and to minocycline, resistance to which is known to be due to induction of transcription of the resistance gene (24), which we inserted into the chromosome of one of the test strains (*S. aureus* RN450M).

Following exposure to minocycline, a mean of 0.28% of the

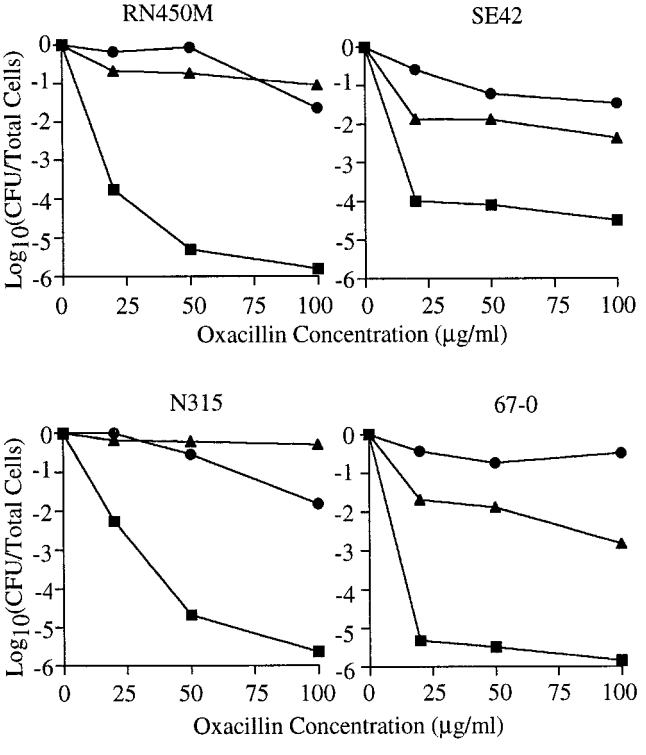

FIG. 2. EOP curves before conversion to a homotypic resistance phenotype (■), immediately after conversion (●), and following conversion to homotypy and after passage for 60 generations in antibiotic-free BHI broth (▲). Shown on the *y* axis are the numbers of *S. aureus* or *S. epidermidis* cells (in $\log_{10}$ CFU per milliliter on oxacillin/CFU per milliliter on MHA) remaining on the plates containing increasing concentrations of oxacillin (shown on the *x* axis). Strains RN450M, N315, and 67-0 are *S. aureus*; strain SE42 is *S. epidermidis*.

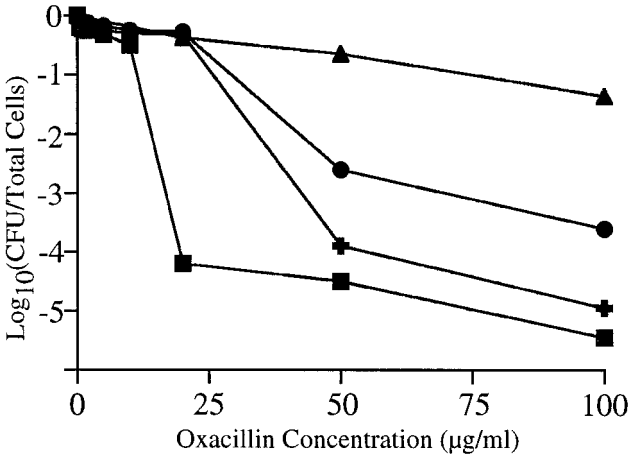

FIG. 3. EOP curves generated by testing cells obtained directly from plates containing RN450M colonies growing on various concentrations of oxacillin. Colonies to be tested were resuspended in 1 ml of BHI broth. Shown on the *y* axis is the number of *S. aureus* cells (in $\log_{10}$ CFU per milliliter on oxacillin/CFU per milliliter on MHA) remaining on the plates containing various concentrations of oxacillin (shown on the *x* axis). Not shown on the *x* axis are numbers for 0.5, 1.0, 2.0, and 5.0 μg. Cells were obtained from agar containing 1 μg (solid squares), 5 μg (solid crosses), 10 μg (solid circles), and 50 μg (solid triangles) of oxacillin per ml.

S. aureus RN450M population became resistant to the antibiotic (range, 0.019 to 1.4%; $n = 36$) and the resistant colonies were normally distributed ($P = 0.4994$). In contrast, the colonies of both *S. aureus* RN450M and *S. epidermidis* SE42 that arose after exposure to rifampin were not normally distributed ($P = 0.006$ for *S. aureus* and $P = 0.0167$ for *S. epidermidis*). Analysis of the distribution of resistant colonies that arose after exposure of the staphylococci to oxacillin revealed that the frequency of *S. aureus* RN450M resistance to oxacillin varied from 1.4×10^{-3} to 7.9×10^{-6} CFU/ml ($n = 44$) and that the frequency of *S. epidermidis* SE42 resistance to oxacillin varied from 6.1×10^{-2} to 2.1×10^{-7} CFU/ml ($n = 44$). This distribution did not conform to a normal distribution ($P = 0.0248$ for *S. aureus* and $P < 0.0001$ for *S. epidermidis*). The distribution of oxacillin-resistant colonies therefore resembled the distribution found following exposure to rifampin rather than the distribution found following exposure to minocycline.

Additional evidence that conversion of a population from the heterotypic to the homotypic resistance phenotype involved the selection of mutants was generated by growing inocula of *S. aureus* RN450M of different sizes in 0.3 μg of oxacillin per ml. The hypothesis was that at a constant concentration of antibiotic, induction should occur at all inoculum sizes; inoculum-dependent growth in the presence of antibiotic would suggest that mutations were being selected. At cell densities of 10^4 CFU/ml, all 24 cultures grew to turbidity, while at cell densities of 10^3 CFU/ml, only 2 of 24 cultures had visible growth and none of the clear wells had any colonies on subculture after 24 h of incubation. All 24 control cultures containing no antibiotics grew to turbidity after 24 h.

Conversion to homotypic growth on agar. The data generated in broth established that there were critical inoculum sizes and drug concentrations that determined conversion of a population from a heterotypic to a homotypic resistance phenotype. Furthermore, following growth in broth above a critical selection concentration, the entire population became homotypically resistant to oxacillin. We sought to replicate the results obtained in broth by growing *S. aureus* RN450M on agar plates containing incremental concentrations of oxacillin, starting at concentrations near those used for conversion to homotypy in broth. There was no reduction in the colony count from an inoculum of 10^9 CFU/ml with 0.5 and 1.0 μg of oxacillin per ml and less than a 1-log-unit reduction with 2.0 and 5.0 μg of oxacillin per ml. However, with oxacillin at between 5 and 20 μg/ml there was a progressive decrease in the colony count of more than 4 log units. Similar results were seen for the other *S. aureus* and *S. epidermidis* strains studied, although the rapid reduction in colony counts occurred for *S. epidermidis* at lower drug concentrations. These data suggest that no single critical concentration of oxacillin in agar is responsible for a reduction in the colony count of the susceptible subpopulation but, rather, that a range of concentrations is responsible for a reduction in the colony count. In order to further assess whether the presence of a single critical concentration is required for conversion from heterotypy to homotypy on agar, several colonies were picked from each plate containing 1, 5, 10, and 50 μg of oxacillin per ml and examined by EOP assay. The data are shown in Fig. 3. Each concentration produced colonies that expressed the homotypic resistance phenotype in the presence of that concentration and several higher concentrations, but no single concentration produced colonies that expressed the homotypic resistance phenotype in the presence of all higher concentrations of oxacillin.

Effect of homotypic conversion on susceptibility to vancomycin. The MICs of oxacillin and vancomycin for *S. aureus* and *S. epidermidis* were determined before and after conversion from heterotypy to homotypy. As shown in Table 1, the MICs of vancomycin were increased for all staphylococcal strains after conversion to homotypy. Differences were confirmed by passing each strain on antibiotic-free media for 5 days and performing another MIC determination.

Relationship of *mecA* transcription to conversion from heterotypy to homotypy. The chromosomal *mecA* gene was inactivated by allelic replacement mutagenesis in both *S. aureus* RN450M (heterotypic resistance expression) and COL (homotypic resistance expression). Both strains, RN450MΔ*mecA* and COLΔ*mecA*, respectively, were susceptible to oxacillin, could

TABLE 1. MIC determinations before and after conversion from heterotypic to homotypic resistance[a]

Strain	MIC (μg/ml)			
	Oxacillin		Vancomycin	
	Before	After	Before	After
RN450M	1	>512	1	4
N315	4	>512	1	4
67-0	8	256	1	8
BMS1	1	128	1	4
SE60	0.5	>512	2	4
SE53	1	256	0.5	2
SE42	0.25	>512	1	4
SE20	8	>512	1	4

[a] Cells of each strain were converted to the homotypic resistance phenotype by growth for 24 h in 0.7 to 1.0 μg of oxacillin per ml.

not survive growth in the presence of concentrations of the antibiotic above the MIC, and did not convert to higher levels of oxacillin resistance after growth in the presence of oxacillin concentrations below the MIC. In addition, *mecA* was inactivated in RN450MHo, designated RN450MHoΔ*mecA*, and the phenotype reverted to susceptible, with the MIC for RN450MHoΔ*mecA* being the same as that for the heterotypic knockout strain, RN450MΔ*mecA*.

In order to assess the need for an increase in *mecA* transcription to convert from heterotypic to homotypic resistance expression, two *S. aureus* RN450M strains were constructed that contained copies of *mecA* regulators *mecR1* and *mecI* integrated into the chromosomal lipase gene. The RN450M *mecA* region contains a natural deletion of these regulators (1, 18). The integrated chromosomal copy of *mecI* has been shown to repress *mecA* transcription in *trans* (18). One of the constructs, RN450M::pGO630, had an intact copy of *mecR1*, so that *mecI* repression of *mecA* transcription could be relieved by induction during growth in oxacillin. The other construct (RN450M::pGO630ΔR1) had a frameshift mutation in *mecR1*, producing constitutive repression of *mecA* and preventing an increase in the level of *mecA* transcription during growth in oxacillin. Repression and induction were monitored during growth by monitoring the activity of β-galactosidase, produced by a transcriptional fusion of *lacZ* to *mecA* divergently transcribed from the chromosomal regulators. As shown in Fig. 4, in RN450M::pGO630 there was conversion to homotypy and an increase in the level of β-galactosidase activity following growth in oxacillin. The increase in the level of β-galactosidase production, representing *mecA* transcription, paralleled the rapid outgrowth of the resistant subpopulation. In contrast, in RN450M::pGO630ΔR1, there was no conversion to homotypy and no increase in the level of β-galactosidase activity following growth in oxacillin. The level of β-galactosidase production fell to the baseline level when RN450M::pGO630 was grown in antibiotic-free medium, yet the homotypic resistance phenotype remained stable (data not shown). The data on *mecA* transcription, obtained by using β-galactosidase production from a *lacZ* fusion, illustrated in Fig. 4 were confirmed in strain RN450M by Northern blot analysis (data not shown). In addition, induction of *mecA* during conversion from heterotypic to homotypic oxacillin resistance was confirmed by Northern blot analysis in all of the other strains of *S. aureus* and *S. epidermidis* used in the study, each of which had an intact *mecA* regulator (data not shown).

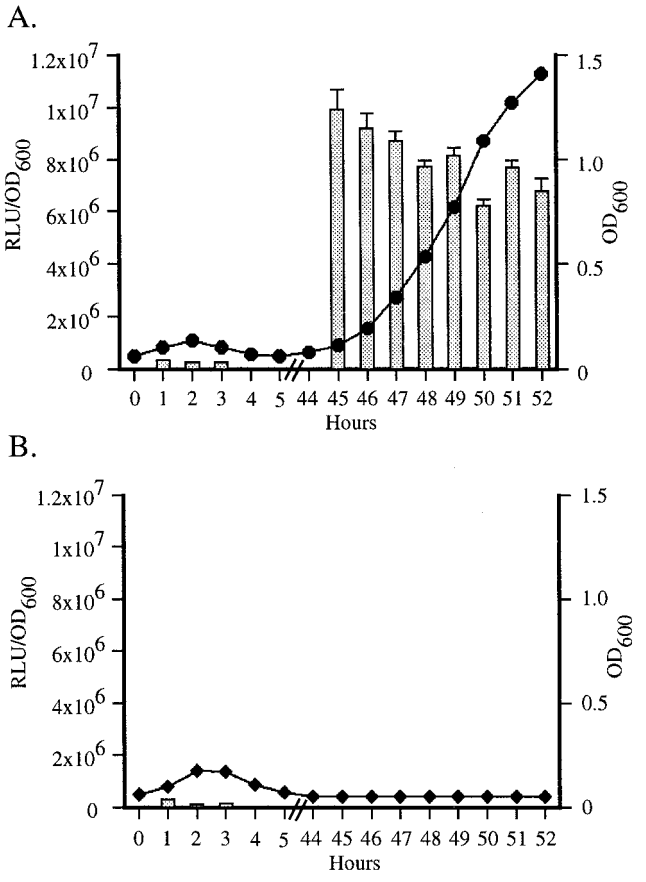

FIG. 4. Kinetics of transcription of a chromosomal *mecA*::*lacZ* fusion during conversion to homotypic oxacillin resistance. β-Galactosidase activity, indicated by the bars, is expressed as relative light units (RLU)/OD_{600} of the sample. The mean and standard error are provided for each sample, and a growth curve (circles or diamonds) is also shown. No measurements were performed between 4 and 44 h due to small numbers of cells. (A) Transcription of a *mecA*::*lacZ* fusion (RN450M::pGO630) over time in the presence of 0.3 μg of oxacillin per ml with both the *mecA* repressor (*mecI*) and the *mecA* inducer (*mecR1*) intact. (B) Transcription of a *mecA*::*lacZ* fusion (RN450M::pGO630ΔR1) over time in the presence of 0.3 μg of oxacillin per ml with *mecI* intact but *mecR1* inactivated.

DISCUSSION

In the present study, we have confirmed previous suggestions that conversion of oxacillin-resistant *S. epidermidis* and oxacillin-resistant *S. aureus* from heterotypic to homotypic resistance expression was due to the selection of chromosomal mutations or genetic rearrangements. First, we used the fluctuation analysis of Luria and Delbruck (14) to provide more rigorous support for this contention than has previously been offered. Luria and Delbruck, unaware of the molecular basis for mutation or heredity, used microbiological and mathematical analyses to prove that bacterial resistance to phage-mediated lysis was due to bacterial mutations rather than to "adaptation" of the bacteria to the phage, a process that we would now ascribe to the activation of genes by induction and signal transduction. Since we knew the mechanisms of induction and mutation, we were able to provide appropriate controls that statistically supported the mutational nature of conversion of oxacillin resistance from heterotypic to homotypic resistance expression.

A second means of support for the mutational nature of the conversion to homotypic resistance expression was provided by the stability of the converted phenotype. We found no reversion to heterotypic resistance expression of the parent after extensive passage either in broth or on agar. In the absence of selection for reversion, mutations would be expected to be stable, while induced changes would rapidly revert. Finally, conversion to homotypic expression was found to be inoculum rather than drug concentration dependent, suggesting that a sufficient quantity of bacteria was required in order to include the rare mutant population. Induction would be expected to

need a threshold drug concentration at which all isolates in the inoculum would develop the converted phenotype.

Many investigators have observed that the prevalence of the highly oxacillin resistant subpopulation can be high ($\approx 10^{-3}$), log units greater than that seen for the spontaneous mutation of a single gene (10^{-6} to 10^{-9}). The frequency of mutation of a single gene that leads to resistance was shown in the present study for rifampin resistance, usually due to selection of a single point mutation in the *rpoB* gene, which encodes the small subunit of RNA polymerase (2). The nature of the mutational event(s) that leads to high-level oxacillin resistance can be only a matter of speculation at this point. Possibilities include the following. First, there may be many independent mutations that can be selected, each one of which would give the observed phenotype. We noted, by doing EOP analysis on agar, that there was no single critical concentration at which the entire population would convert to high-level resistance. Rather, colonies growing on one concentration of oxacillin would be resistant only to concentrations 1 or 2 doubling dilutions higher. This suggests the progressive selection of either different mutations in a single gene or mutations in different genes at increasing drug concentrations. It is even possible that different mutations are responsible for homotypic resistance expression in each experiment. However, the reproducibility of conversion makes common mutations more likely. The observations made by others that many individual transposon insertions into the COL genome converted oxacillin resistance expression from homotypic to heterotypic suggest that many unrelated genes affect this phenotype (9).

Second, oxacillin-resistant *S. epidermidis* and oxacillin-resistant *S. aureus* may be examples of strains with hypermutable phenotypes that have defects in genes responsible for repair of DNA damage. Hypermutable strains of *Pseudomonas aeruginosa*, *E. coli*, and *Salmonella enterica* have been found that are more likely to be pathogenic or antibiotic resistant than nonmutators due to rapid environmental adaptation through increased genetic diversity (19). However, we have found no difference in the selection of rifampin-resistant mutants between isogenic pairs of *S. aureus* isolates with heterotypic and homotypic resistance (J. Finan and G. Archer, unpublished observations).

Third, one or more genes that mutate at high frequencies may be affecting cell wall construction. The presence of single-nucleotide or oligonucleotide repeat sequences has been shown in both *Neisseria* and *Haemophilus* to create selectable mutations at high frequencies due to slipping of DNA strands and base mispairing during replication (13, 21, 27). This produces both phase and antigenic variations at frequencies as high as 10^{-2} per cell per generation (21, 27).

Finally, there may be an invertible segment within the chromosome that switches expression between a gene important for homotypic resistance and an alternate gene important for heterotypic resistance. Under antibiotic pressure the homotypic state would be selected. This would be similar to the phase variation of the flagellar genes of *Salmonella* (28). Such a regulatory mechanism could result in variations at frequencies that are 2 to 3 orders of magnitude higher than those of the spontaneous mutation alone.

Several observations made in the present study suggest that the conversion of oxacillin resistance phenotypes from heterotypic to homotypic involves genes responsible for cell wall construction. First, the level of resistance to vancomycin, another agent that targets the cell wall, was increased in populations with homotypic resistance compared to that in populations with isogenic heterotypic resistance. While clinical *S. aureus* isolates that are homotypically resistant to oxacillin are not usually less susceptible to vancomycin, it is possible that decreased susceptibility to vancomycin is a less stable phenotype in vivo than high-level resistance to oxacillin.

Second, in the RN450M construct in which *mecA* was constitutively repressed, *mecA* was still transcribed at a detectable level. However, the inability of the level of *mecA* transcription to increase in this strain prevented its conversion from heterotypic to homotypic resistance expression when it was grown in the presence of oxacillin. This established a requirement for an increase in the level of *mecA* transcription—and, presumably, in the quantity of PBP 2a—for conversion to occur. However, other studies have noted a lack of correlation between the amount of the *mecA* transcript or PBP 2a and the resistance phenotype (heterotypy or homotypy) (6, 10, 16, 18). Our observations would suggest that some increase in the level of *mecA* transcription and the quantity of PBP 2a is necessary but not sufficient for conversion to homotypic resistance expression. Since the cell wall made by PBP 2a during growth in the presence of β-lactam antibiotics has been shown to be abnormal compared to that of the wild type (4), it is reasonable to assume that the cell wall construction catalyzed by this enzyme is inefficient and that an increased quantity would be required for high-level β-lactam resistance.

The observations made in the present study may promote the use of the powerful tools of bioinformatics, proteomics, and microarray or microchip analysis of gene expression to provide molecular definitions for a complex phenotype.

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