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Jumping the Barrier to β-Lactam Resistance in *Staphylococcus aureus*

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Although the staphylococcal methicillin resistance determinant, *mecA*, resides on a mobile genetic element, staphylococcus cassette chromosome *mec* (SCC*mec*), its distribution in nature is limited to as few as five clusters of related methicillin-resistant *Staphylococcus aureus* (MRSA) clones. To investigate the potential role of the host chromosome in clonal restriction of the methicillin resistance determinant, we constructed plasmid pYK20, carrying intact *mecA*, and introduced it into several methicillin-susceptible *Staphylococcus aureus* strains, five of which were naive hosts (i.e., *mecA* not previously resident on the host chromosome) and five of which were experienced hosts (i.e., methicillin-susceptible variants of MRSA strains from which SCC*mec* was excised). We next assessed the effect of the recipient background on the methicillin resistance phenotype by population analysis, by assaying the *mecA* expression of PBP2a by Western blot analysis, and by screening for mutations affecting *mecA*. Each experienced host transformed with pYK20 had a resistance phenotype and expressed PBP2a similar to that of the parent with chromosomal SCC*mec*, but naive hosts transformed with pYK20 selected against its expression, indicative of a host barrier. Either inducible β-lactamase regulatory genes *blaR1-blaI* or homologous regulatory genes *mecR1-mecI*, which control *mecA* expression, acted as compensatory elements, permitting the maintenance and expression of plasmid-carried *mecA*.

Staphylococcus aureus is an important human pathogen causing both community and hospital-associated infections. Penicillins and related β-lactams have dramatically reduced the morbidity and mortality of *S. aureus* infections, but steadily rising resistance threatens to erode their utility (5). Staphylococcal resistance to β-lactam antibiotics is mediated by either of two mechanisms: (i) production of β-lactamase and (ii) production of an altered target penicillin-binding protein (PBP), PBP2a.

β-Lactamase, encoded by *blaZ*, is an inducible, typically plasmid-carried, narrow-spectrum penicillinase that inactivates penicillin G and structurally related penicillins. It is regulated by two genes, *blaR1-blaI* (1), which are located immediately upstream and transcribed in the direction opposite that of *blaZ*.

PBP2a, which confers broad resistance to the entire β-lactam class (which is termed methicillin or oxacillin resistance) is a bacterial cell wall synthetic PBP that probably functions as a transpeptidase. The β-lactam antibiotics that are currently used clinically do not bind PBP2a at therapeutic concentrations and therefore lack efficacy in infections caused by methicillin-resistant staphylococci. PBP2a is encoded by *mecA* (8, 11, 33) and, like β-lactamase, also is inducible. Upstream from *mecA* are two genes, *mecR1-mecI*, which are homologs of *blaR1-blaI* (12, 18, 28, 29). MecI and BlaI are repressors of *mecA* as well as *blaZ* transcription (19, 20). MecR1 and BlaR1, which are specific for their cognate repressors and cannot substitute for each other (20), are the corresponding sensor-transducer molecules. A transmembrane signal is generated by binding of the inducer β-lactam to the extracellular sensor domain, which triggers the cleavage of the sensor transducer (19, 35) and the repressor, enabling the transcription of both *blaZ* and *blaR1-blaI* transcripts and *mecA* and *mecR1-mecI* transcripts, respectively (28).

mecA is located on a mobile element, staphylococcal cassette chromosome *mec* (SCC*mec*), which is horizontally transferable among staphylococcal species (14–16, 24). Four major SCC*mec* types, ranging in size from approximately 20 to over 50 kb, have been identified. Despite this potential mobility, *mecA* is nevertheless restricted to relatively few closely related methicillin-resistant *Staphylococcus aureus* (MRSA) clonal complexes, approximately five worldwide (10, 22), possibly because three of the four elements are too large to be transducible. Although type IV SCC*mec* is small enough to be transducible, it is found in the same clonal clusters that harbor the other types, suggesting that some genetic backgrounds are better adapted than others to whatever fitness burden might be imposed by SCC*mec* or *mecA*.

Chromosomal elements residing outside SCC*mec* are known to influence, sometimes dramatically, the methicillin resistance phenotype, which classically is heterogeneous, such that the vast majority of cells, although fully expressing PBP2a, can nevertheless be susceptible or express only low-level resistance. In the course of experiments to elucidate the contribution of the genome "hosting" *mecA* to the phenotype, we found that the host also can influence the genotype by restricting the horizontal acquisition of *mecA*. Differences among potential methicillin-susceptible recipient strains in the ability to tolerate *mecA* expression are another factor, in addition to SCC*mec* size and antibiotic selective pressure, that could account for the relatively limited clonal distribution of *mecA* in nature. Regulatory genes appear to have an important permissive role that allows an otherwise restrictive host genome to become parasitized by *mecA*.

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TABLE 1. Strains and plasmid vectors

Strain or plasmid	Description	Relevant phenotype or genotype[a]	Reference(s) or source
Strains			
MRSA			
COL	Homogeneous methicillin-resistant strain	Mc^r Tc^r	31
COLn	Tc^s isolated derivative of COL	Mc^r	This study
COL52	Antibiotic-selected mutant of COLn expressing higher level of resistance	Mc^r	6
COL8a	Methicillin-susceptible spontaneous mutant of COLn; stop codon at position of amino acid residue 115 in PBP2a	Mc^s	6
BB270	Homogeneous methicillin-resistant strain; SCC*mec*-positive transductant of NCTC 8325	Mc^r	2
N315	Heterogeneous methicillin-resistant strain carrying functional *mecI-mecR1* regulatory genes of *mecA*	Mc^r; β-lac	12
mecA-negative experienced hosts			
COLnex	SCC*mec* excision, strain obtained from COLn	Mc^s	This study
COL52ex	SCC*mec* excision strain obtained from COL52	Mc^s	This study
COL8aex	SCC*mec* excision strain obtained from COL8a	Mc^s	This study
BB270ex	SCC*mec* excision strain obtained from BB270	Mc^s	This study
N315ex	SCC*mec* excision strain obtained from N315	Mc^s; β-lac	15
mecA-negative naive hosts			
8325-4	Derivative of NCTC 8325 cured of all known prophages	Mc^s	23
RN4220	Restriction-deficient derivative of 8325-4	Mc^s	17
1-63	Wild-type clinical isolate	Mc^s	7, 8
83A	Wild-type strain ATCC 27706	Mc^s	
Newman	Wild-type strain ATCC 25904	Mc^s; β-lac	
NewmanP	Newman cured of β-lactamase plasmid	Mc^s	This study
Plasmids			
pAW8	*E. faecalis ori* in pAMα*1; E. coli ori* in ColEI	Tc^r; 5.1 kb	34
pYK20	2.8-kb PCR product; *mecA* gene cloned into pAW8 (*Bam*HI)	Tc^r; 7.9 kb	This study
pSR2	*ccr-2* complex cloned into temperature-sensitive pYT3	Tc^r; 12.2 kb	16
pYK644	*mecI-mecRl-mecA* fragment from pGO644 cloned into pAW8 (*Bam*HI·*Eco*RI)	Tc^r; 9.3 kb	This study
pRN5542	pSK256 with *Hin*dIII site in multiple cloning site deleted; derivative of pC194	Cm^r; 3.0 kb	19
pZRI	*blaI-blaR1-blaZ* complex gene cloned into pRN5542 and pBluescript-SK	Cm^r Amp^r; 9.2 kb	35
$pZR_{E/A}I$	Contains an amino acid substitution (Glu^{202} to Ala) on B1aR1 in pZRI	Cm^r Amp^r; 9.2 kb	35
pZRIc	Termination of *blaI* after amino acid 101 (*Bla*I cleavage site) in pZRI	Cm^r Amp^r; 9.0 kb	This study

[a] Abbreviations: Mc^r, methicillin resistant; Tc^r, tetracycline resistant; Mc^s, methicillin susceptible; Cm^r, chloramphenicol resistant; Amp^r, ampicillin resistant; β-lac, carrying wild-type β-lactamase plasmid.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains are shown in Table 1. Five strains carried *mecA*: COLn (nafcillin MIC, 400 μg/ml); COL52 (nafcillin MIC, 3,200 μg/ml); BB270 (nafcillin MIC, 400 μg/ml); COL8a, a spontaneous methicillin-susceptible variant of COLn; and N315 (nafcillin MIC, 50 μg/ml). All MRSA strains were tetracycline susceptible and β-lactamase negative, except for N315. We constructed *mecA*-negative, experienced *S. aureus* variants (i.e., a genetic background that has harbored *mecA*) from the *mecA*-carrying strains by introducing into them plasmid pSR (provided by K. Hiramatsu [16]). pSR provides two chromosomal cassette recombinase genes, *ccrA* and *ccrB*, in *trans* to precisely excise SCC*mec*, and mutants are identified by selecting for the methicillin susceptibility phenotype as described previously (16). The suffix "ex" designates an experienced *S. aureus* variant from which *mecA* has been excised (e.g., COLnex is the SCC*mec* excision mutant of COLn). All *S. aureus* strains and transformants were grown in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) or on Trypticase soy agar with aeration for 37°C unless indicated otherwise. Tetracycline and chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) were used at a concentration of 10 μg/ml unless indicated otherwise.

Plasmids and DNA manipulations. All plasmids and primers used in this study are shown in Tables 1 and 2, respectively. For transformation experiments, *mecA* was cloned into low-copy-number plasmid pAW8, an *Enterococcus faecalis-Escherichia coli* shuttle vector with a tetracycline-selectable marker (34). The *mecA* product, including the promoter and the first 223 nucleotides (nt) of *mecR1*, was obtained by PCR (35) amplification of COLn *mecA* with primers K34 and K38 and 1 U of Clontaq polymerase (Clontech, PaloAlto, Calif.). Plasmid pYK20 was isolated from *E. coli* DH5α by standard procedures. Plasmid pYK644, derived from pGO644 (provided by G. L. Archer), carries *mecA* complex genes (*mecI-mecR1-mecA*) from strain N315 (12). The *Bam*HI-*Eco*RI-digested fragment from pGO644 was inserted into those sites in pAW8. Plasmid pZRI contains *blaZ* complex genes (*blaI-blaR1-blaZ*), and plasmid $pZR_{E/A}I$ contains an amino acid substitution ($Glu^{202} \rightarrow$ Ala) in BlaR1, as previously described (35). Plasmid pZRIc was constructed by PCR amplification of the 2.1-kb fragment of pCH2278 carrying *blaR1* minus the first 13 nt through *blaI* minus the last 78 nt with primers P1-101 (Table 2) and P4 (35). The product was digested with *Hin*dIII and ligated to the *Hin*dIII site of pSK1.0 (35), restoring the *bla* region, with termination of *blaI* after amino acid residue 101, which is the *Bla*I cleavage site. All *blaZ* complex plasmids were cloned into *S. aureus* chloramphenicol-selectable plasmid vector pRN5542 (2). We confirmed the absence of mutations by nucleotide sequencing (performed at the UCSF Biomolecular Resources Center DNA Sequencing Facility). β-Lactamase activity was assessed with nitrocefin disks (Becton-Dickinson and Company, Sparks, Md.) (35). Naive and experienced host strains were transformed with plasmids by electroporation (16). After 48 h of incubation, transformants were tested for *mecA* by PCR amplification with vector-specific primers for the sequence immediately adjacent to the insert (K3 and K4).

TABLE 2. Synthetic oligonucleotide primers

Genetic element[a] and primer designation	Sequence[b]	Nucleotide positions
mecA		
K15	5′-AGCACACCTTCATATGACGTCT-3′	32473–32494
K16	5′-TGGATCAAAATTGGGTACAAGA-3′	31991–32012
K17	5′-AGTTGTAGTTGTCGGGTTTGGT-3′	31425–31446
K18	5′-TGGCAATATTAACGCACCTCACT-3′	33012–33034
K34	5′-TATGCGGATCCTCGTGTCAGATACATTTCGATTCA-3′	31068–31091
K38	5′-ATTTCGGATCCGTTGTAGCAGGAACACAAATGAATAAC-3′	33645–33619
lacZ		
K3	5′-TATGTTGTGTGGAATTGTGAGCGGA-3′	167–191
K4	5′-AGGCGATTAAGTTGGGTAACGCCAG-3′	329–353
blaR1-blaI, P1-101	5′-GTCGATAAGCTTTTAATTCAGCACTAAACTTTTCATG-3′	2205–2184

[a] The primers listed are specific for *mecA* genes of strain NCTC 10442 (GenBank accession no. AB033763), for *lacZ* in pUC119 (GenBank accession no. U07649), and for *blaR1-blaI* in pI258 (GenBank accession no. M62650).
[b] Underlining indicates *Bam*HI sites introduced in K34 and K38 and *Hin*dIII site introduced in P1-101.

Construction of pYK20 or pYK644 transformants. Plasmid pYK20 carrying intact *mecA* or pYK644 carrying the *mecI-mecR1-mecA* complex but lacking other SCC*mec* DNA (12, 14, 18) was introduced by electroporation into several methicillin-susceptible *S. aureus* strains, which were either naive (i.e., naturally free of *mecA*) or experienced (i.e., methicillin-susceptible variants of MRSA strains from which SCC*mec* had been excised, denoted by the "ex" suffix). β-Lactam antibiotic selection, which could affect phenotype, was avoided by selecting for the tetracycline resistance marker of the plasmid vector. We then assessed the effect of recipient background on the methicillin resistance phenotype by population analysis to determine the number of highly resistant CFU and assayed the *mecA* expression of PBP2a by Western blot analysis.

Population analysis. Population analysis was done by the agar plate method, in which approximately 10^8 CFU are quantitatively inoculated onto a series of agar plates containing increasing concentrations of nafcillin (Sigma) (32).

Detection of PBP2a. We assayed for PBP2a production by Western blotting. *S. aureus* membrane proteins were prepared from late-exponential-stage cultures, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred as described previously (35). PBP2a was detected by using a mouse monoclonal anti-PBP2a antibody (a gift from Denka Seiken Co., Ltd., Niigata, Japan) as the primary antibody, diluted 1:100,000, and alkaline phosphatase-conjugated anti-mouse immunoglobulin (Promega, Madison, Wis.). We detected bound antibodies by color development as directed by the manufacturer. Relative amounts of PBP2a proteins were measured by scanning densitometry.

COLnex reporter assay. We screened for *mecA* mutations in pYK20 transformants by using COLnex which, when transformed with wild-type *mecA* in pYK20, expresses homogeneous resistance. A representative colony of the transformant yielding the predicted 2.8-kb PCR *mecA* amplification product was diluted $1{:}10^4$ and regrown to a cell density of approximately 5×10^8 CFU/ml. Plasmid pYK20 was extracted, purified, and introduced into host strain COLnex by electroporation. After 48 h of incubation, COLnex(pYK20) transformants were selected on tetracycline-containing agar and replicated on Trypticase soy agar containing both tetracycline and nafcillin at concentrations of 0, 2.5, 10, and 100 μg/ml. After 24 h of incubation, CFU growing at each nafcillin concentration were counted, and the proportions were calculated relative to CFU growing on nafcillin-free agar. The COLn homogeneous phenotype is 100% growth of CFU at 100 μg of nafcillin/ml.

RESULTS

Phenotypes of naive and experienced host transformants. The resistance phenotype in a population analysis of experienced host transformants with pYK20 carrying unregulated *mecA* was in every instance identical to that of the parent (Fig. 1): COLnex and COL52ex transformants exhibited their characteristic homogeneous phenotype (i.e., >1% of plated CFU grew on agar containing 100 μg of nafcillin/ml). BB270ex, N315ex, COL8a, and COL8aex transformants had class 3 heterogeneous resistance (i.e., 1 CFU in 10^2 to 10^3 CFU at 100 μg of nafcillin/ml), as defined by Tomasz et al. (32). Resistance was extremely heterogeneous for naive host transformants carrying pYK20 (Fig. 2), with a class 2 pattern (only 1 CFU in 10^5 to 10^6 CFU at 100 μg of nafcillin/ml) for 1-63 and 8325-4 and a class 1 pattern (only 10^3 CFU in 10^8 CFU at 6.25 μg of nafcillin/ml) for RN4220.

The phenotype of each of the experienced hosts transformed with pYK644 carrying *mecR1-mecI*-regulated *mecA* was heterogeneous, except for strain COL52 (Fig. 1). The curve for N315ex(pYK644) was identical to that for parent strain N315, both of which expressed a more heterogeneous pattern than N315ex(pYK20), in which *mecA* regulatory genes are absent. Likewise, COL8a(pYK644) and BB270ex(pYK644) transformants, with intact *mecR1-mecI*, had a more heterogeneous pattern of resistance than their respective pYK20 transformants. pYK644 transformants of naive host 8325-4 and its derivative strain, RN4220, also had a more heterogeneous pattern of resistance than their pYK20 counterparts. In contrast, the phenotypes of naive host NewmanP(pYK644) and 1-63(pYK644) transformants and pYK20 transformants were the same (Fig. 2).

Stability of plasmid pYK20 in naive and experienced hosts. The expression of PBP2a detected in Western blots for each of the experienced host transformants carrying pYK20 was similar to that detected for the COLn(pAW8) control strain, in which an unregulated, single copy of *mecA* naturally resides on SCC*mec* (Fig. 3a). PBP2a expression varied greatly among the naive host transformants carrying pYK20: 8325-4 was similar to the COLn(pAW8) control strain, RN4220 was weakly positive, and 1-63 had no PBP2a detected.

As regulatory elements that could repress *mecA* expression are absent in these constructs, the diminished amounts of PBP2a in naive host transformants suggested the presence of mutations affecting *mecA* expression. We screened transformants for *mecA* deletions by PCR. Of 36 COLnex(pYK20) transformants tested, all had the expected 2.8-kb fragment. In contrast, 24.4, 4.7, and 70%, respectively, of transformants of naive hosts RN4220, 8325-4,and 1-63 carrying pYK20 yielded

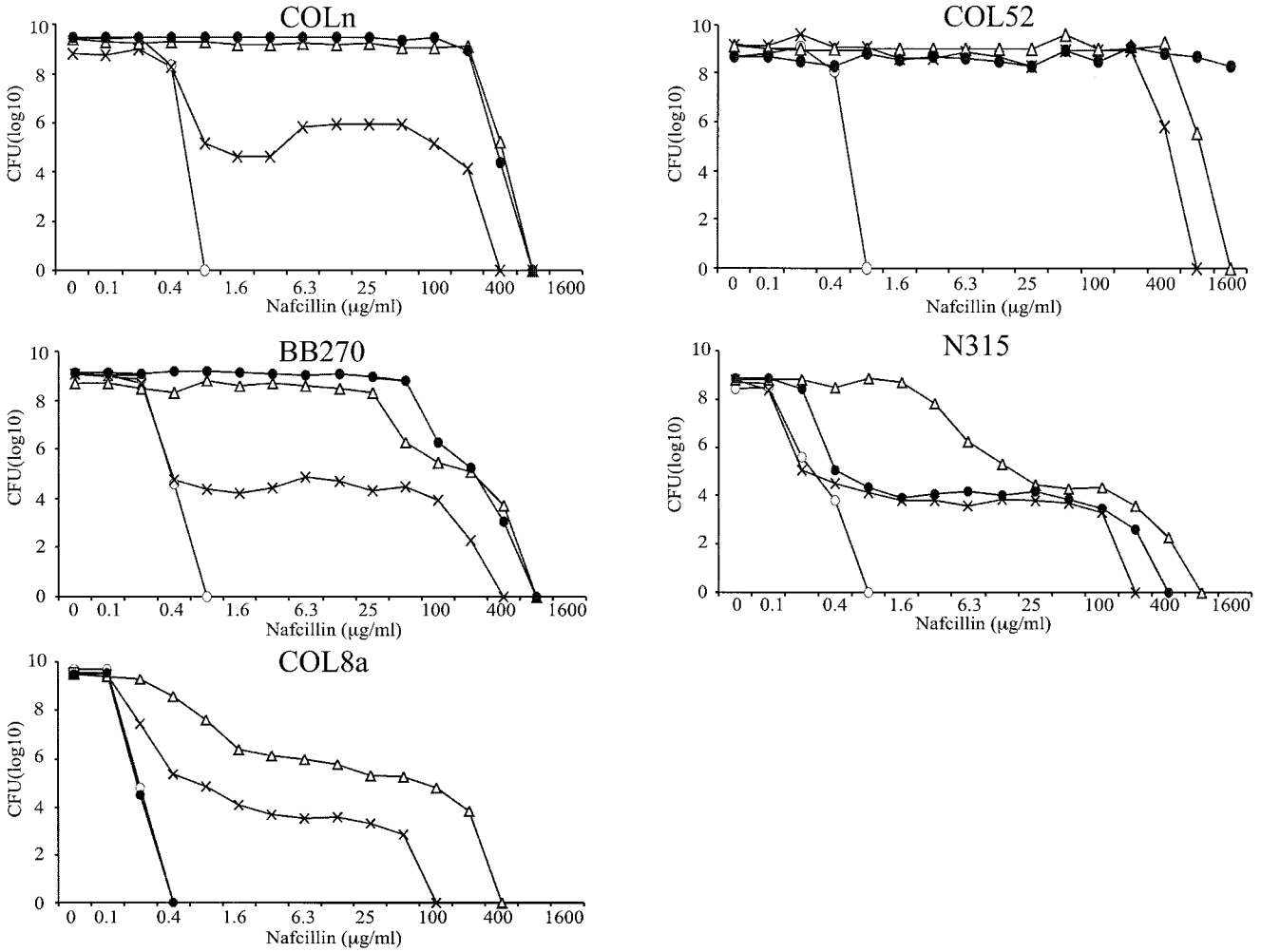

FIG. 1. Population analysis with nafcillin for five MRSA strains and experienced host MRSA (MRSAex) transformants. Symbols: ●, wild-type MRSA with SCC*mec* present plus cloning vector pAW8 only; ○, MRSAex with SCC*mec* excised plus cloning vector only; △, MRSAex transformed with pYK20, carrying unregulated *mecA*; ×, MRSAex transformed with pYK644, carrying the intact *mecI-mecR1-mecA* complex. Shown on the *y* axis is the number of cells (per milliliter) growing on nafcillin-containing agar.

PCR products of <2.8 kb, indicating the presence of a deletion mutation in *mecA* (Table 3).

We developed a reporter assay using COLnex to investigate whether smaller *mecA* mutations undetectable by PCR screening might also be present in apparently intact pYK20. Resistance is homogeneous upon transformation of COLnex with pYK20 carrying intact *mecA* (Fig. 1); therefore, COLnex transformed with pYK20 in which there was a mutation of *mecA* or its promoter that affected PBP2a production or activity should be detectable by a loss of resistance in COLnex transformants. As expected, 100% of the COLnex(pKY20) transformants purified from experienced host donors were resistant to 100 μg of nafcillin/ml (Table 4). A total of 96.9% of the COLnex (pYK20) transformants obtained with pYK20 isolated from naive hosts 83A(pYK20) and NewmanP(pYK20) were resistant to 100 μg of nafcillin/ml. In contrast, 3.7 to 76.8% of the COLnex(pYK20) transformants obtained with pYK20 isolated from naive hosts RN4220(pYK20), 1-63(pYK20), and 8325-4(pYK20) were resistant to 100 μg of nafcillin/ml.

We passaged four RN4220(pYK20) transformants and one COLnex(pYK20) transformant for 5 days in the absence of tetracycline and repeated the reporter assay. A total of 100% of the COLnex(pYK20) transformants originating from RN4220(pYK20) were susceptible to 2.5 μg of nafcillin/ml, whereas 100% of the COLnex(pYK20) transformants obtained from COLnex(pYK20) were resistant. We picked two passaged RN4220(pYK20) transformants and determined the *mecA* sequence. One had a point mutation in the *mecA* −35 promoter sequence (TTGACA → TTGAAA), and the other had a stop codon at the position of amino acid residue 95 in PBP2a. Thus, in the absence of β-lactam-selective pressure, naive strains to various degrees selected against the presence or expression of PBP2a.

Stability of plasmid pYK644 in naive hosts. In contrast to the genetic instability of unregulated *mecA* in the pYK20 construct, *mecA* under the strong repressor control (Fig. 3b) of *mecR1-mecI* in the pYK644 construct was faithfully maintained in naive hosts. A total of 100% of COLnex transformants with

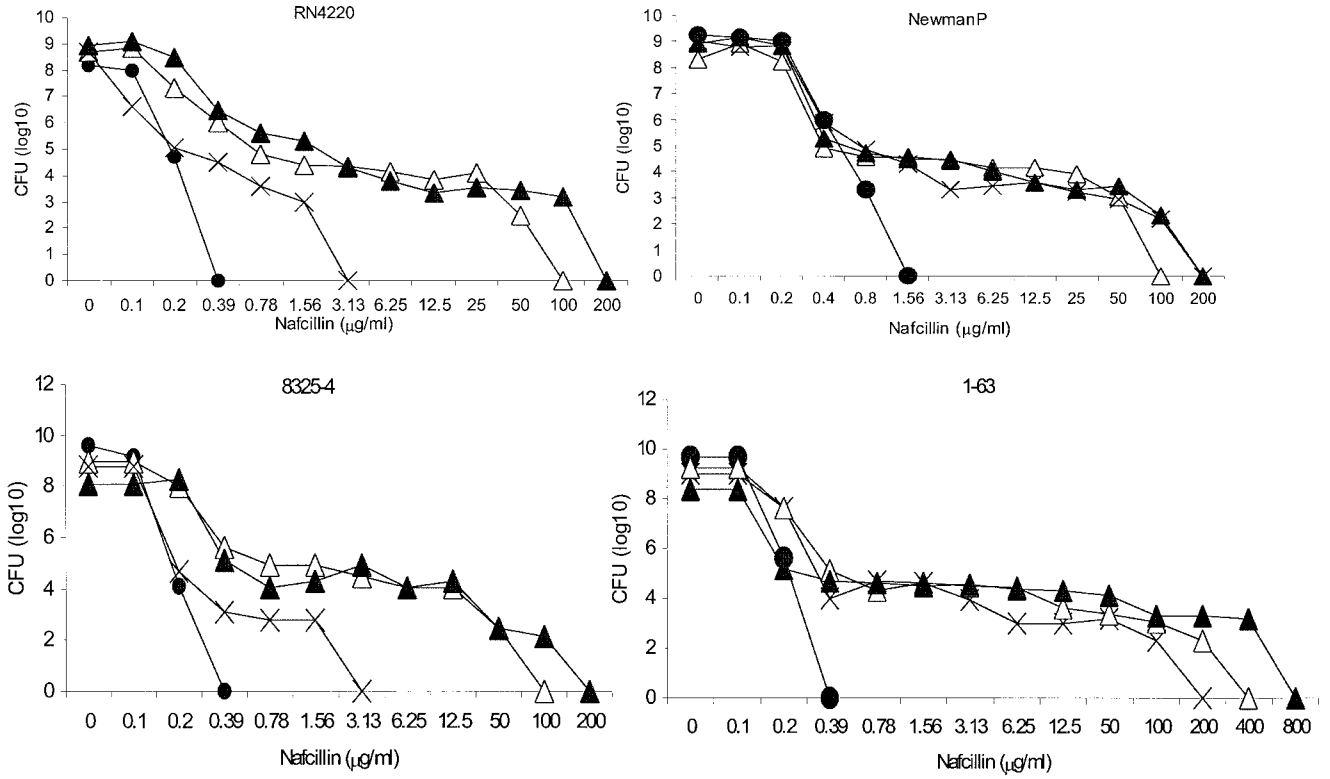

FIG. 2. Population analysis with nafcillin for four naive host methicillin-susceptible *S. aureus* transformants. Symbols: ●, wild-type naive host with cloning vector pAW8 only; △, naive host transformed with pYK20, carrying unregulated *mecA*; ×, naive host transformed with pYK644, carrying the intact *mecI-mecR1-mecA* complex; ▲, naive host cotransformed with pYK20 and pZRI, carrying the intact *blaI-blaR1-blaZ* complex.

plasmid pYK644 donated from naive hosts grew on agar containing 100 μg of nafcillin/ml. These results suggested that regulatory genes play a role in stabilizing *mecA* in a new host that is otherwise intolerant of its presence.

Effect of the *blaZ-blaR1-blaI* complex on PBP2a expression in a naive strain. In many, and perhaps most, clinical isolates, the expression of *mecA* is under the control of β-lactamase regulatory genes, *blaR1-blaI*, rather than *mecR1-mecI* genes, which either are deleted or mutated (26). The *blaR1-blaI* complex has significant amino acid homology to the *mecR1-mecI* complex (12), and the two systems function in the same way (18, 29). MecI and BlaI are repressors of both *mecA* transcription and *blaZ* transcription (19, 20), and MecR1 and BlaR1 are signal-transducing molecules which function as antirepressors for their cognate repressors and which cannot substitute for each other (20). To investigate whether the *bla* regulon also could stabilize *mecA*, we constructed double transformants of RN4220 possessing both the wild-type *blaZ-blaR1-blaI*-carrying plasmid pZRI (35) and pYK20. Reflecting the weaker repressor activity of *bla* than of *mec* regulatory elements, PBP2a was readily detectable in Western blots of RN4220 (pZRI)(pYK20), 8325-4(pZRI)(pYK20), and 1-63(pZRI)(pYK20) (Fig. 3b). RN4220(pZRI)(pYK20), 8325-4(pZRI)(pYK20), and 1-63(pZRI)(pYK20) double transformants and Newman(pYK20) yielded 95.8 or 100% of COLnex-(pYK20) transformants resistant to 100 μg of nafcillin/ml (Table 4). An RN4220 double transformant carrying the

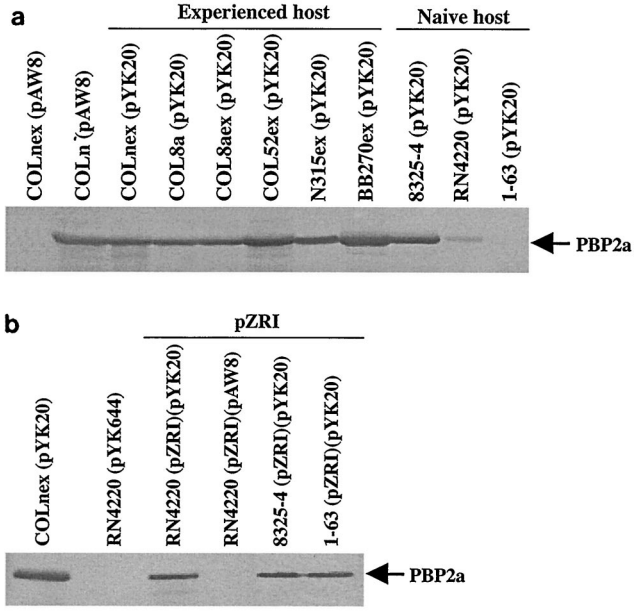

FIG. 3. Detection by Western blot analysis of PBP2a (78 kDa) encoded by *mecA*. COLn(pAW8) is the positive control; COLnex-(pAW8), RN4220(pAW8), and RN4220(pZRI)(pAW8) are the negative controls. (a) Comparison of experienced host and naive host transformants carrying pYK20. (b) Representative transformants with cloning vector pAW8 only, pYK644 (carrying intact *mecR1-mecI-mecA*), and pYK20 (carrying unregulated *mecA*) and cotransformants with pZRI (carrying *blaR1-blaI-blaZ*). All were grown under noninducing conditions.

TABLE 3. Detection by PCR of *mecA* insert in plasmid pYK20

Transformant	No. of transformants	Fragment ratio[a]	Relative amt of PBP2 (%)[b]
COLnex(pYK20)	125	0	96
RN4220(pYK20)	100	24.4	33
8325-4(pYK20)	53	4.4	107
1-63(pYK20)	26	70	18

[a] Ratio of unexpected <2.8-kb fragments in to total fragments screened transformants.
[b] Percent density of PBP2a in each transformant compared to PBP2a in COLn.

pRN5542 vector lacking *bla* genes yielded only 2.5% of COLnex(pYK20) transformants that were nafcillin resistant.

To determine which *bla* gene products were important for stabilizing *mecA*, we constructed RN4220 double transformants with pYK20 plus one of two β-lactamase mutant plasmids, $pZR_{E/A}I$ or pZRIc. $pZR_{E/A}I$ has a single amino acid substitution of alanine for glutamic acid in the metalloprotease motif of the BlaR1 sensory-transducer protein (35). This mutation blocks the proteolytic cleavage of BlaI and completely represses β-lactamase production (19, 35). The introduction of pYK20 from the RN4220($pZR_{E/A}I$)(pYK20) double transformant into COLnex yielded 100% of COLnex transformants that were resistant. pZRIc has a stop codon engineered into the β-lactamase repressor BlaI, so that only the inactive, cleaved 11-kDa form of the protein is expressed and β-lactamase is constitutively expressed. pYK20 from the RN4220 (pZRIc)(pYK20) double transformant failed to stabilize *mecA*, yielding 0.8% of COLnex transformants growing on nafcillin (Table 4). These results indicate that it is the β-lactamase repressor that stabilizes *mecA* in a naive background. PBP2a expression was not detected in either of these RN4220 double transformants by Western blotting, but for different reasons. Mutant *mecA* not expressing PBP2a was selected for in the absence of an active repressor with pZRIc. Unopposed repressor activity of $pZR_{E/A}I$, although it preserved *mecA*, as demonstrated in the COLnex reporter assay, did not allow for its expression in the RN4220 cotransformant.

TABLE 4. Assay of phenotype of COLnex transformants with pAW8, pYK20, or pYK644 and purified from control, experienced, and naive plasmid donors

Plasmid donor strain	No. of COLnex transformants				Ratio[a]
	Total	Replicated on plate containing at nafcillin at (μg/ml):			
		2.5	10	100	
Controls					
DH5α(pYK20)	157			157	100
COLnex(pYK20)	21			21	100[b]
COLnex(pAW8)	211	0	0	0	0[b]
RN4220(pZRI)(pAW8)	17	0	0	0	0[b]
COLnex(pYK644)	36			36	100
Experienced host					
COL8aex(pYK20)	775			775	100[b]
COL8a(pYK20)	212			212	100[b]
N315ex(pYK20)	195			195	100[b]
COL52ex(pYK20)	543			543	100[b]
BB270ex(pYK20)	45			45	100[b]
Naive host					
RN4220(pYK20)	62	6	6	6	9.7[b]
8325-4(pYK20)	168	168	168	129	76.8[b]
1-63(pYK20)	54	12	2	2	3.7[b]
83A(pYK20)	128	123	123	123	96.9
NewmanP(pYK20)	355	344	344	344	96.9
Naive host (*mecI-mecR1-mecA* complex in pYK644)					
RN4220(pYK644)	48			48	100[b]
8325-4(pYK644)	51			51	100
1-63(pYK644)	125			125	100
Naive host + *mecA* + β-lactamase plasmid					
RN4220(pYK20)(pZR1)	48	46	46	46	95.8[b]
RN4220(pYK20)(pRN5542)[c]	712	23	18	18	2.5
RN4220(pYK20)(pZRIc)	911	41	7	7	0.8
RN4220(pYK20)($pZR_{E/A}I$)	48			48	100
RN4220(pYK644)(pRN5542)[c]	48			48	100
RN4220(pYK644)(pZRI)	130			130	100
8325-4(pYK20)(pZRI)	354			354	100[b]
1-63(pYK20)(pZRI)	232			232	100[b]
Newman(pYK20)	285			285	100

[a] Ratio of number of transformants growing on agar containing 100 μg of nafcillin per ml to total number of transformants.
[b] Transformant used in Western blot analysis.
[c] pRN5542 is the vector into which the β-lactamase regulon, *blaI-blaR1-blaZ*, was cloned.

DISCUSSION

These experiments demonstrate that some MRSA strains select against the expression of PBP2a, presumably due to a fitness cost posing a barrier to the stability and maintenance of *mecA*. The unregulated *mecA* plasmid construct was stable in the experienced strains previously harboring *mecA*. The results underscore the important contribution of the genetic background to both the phenotype and the genotype of methicillin resistance and suggest that strains harboring *mecA* perhaps have adapted to it, compensated for it, previously experienced it, or are otherwise properly prepared for it. This instability of *mecA* in some genetic backgrounds may account in part for the relatively restricted clonal clustering of the mobile SCC*mec* element.

Particularly interesting is the ability of *mec* or *bla* regulatory genes to stabilize *mecA*, a finding which suggests a previously unrecognized role of these elements in facilitating the dissemination of *mecA*. The repression of *mecA* may be an important first step in its successful engraftment by a new host. Although in principle this role could be provided by either *mec* or *bla* regulatory genes, the latter is the more likely to do so, because the *mecR1-mecI* genes strongly repress *mecA* expression, and the latter could be a survival disadvantage in the presence of a β-lactam antibiotic (18, 29).

The relatively much stronger repression of *mecA* expression by *mec* than by *bla* regulatory genes is evident from the Western blots of RN4220(pYK644), in which *mecA* is paired with *mecR1-mecI*, compared to RN4220(pZRI)(pYK20), in which *mecA* is paired with *blaR1-blaI* (Fig. 3b). Strong repression can translate to a significant survival disadvantage in some (e.g., RN4220 and 8325-4) but not all (e.g., NewmanP and 1-63)

naive genetic backgrounds (Fig. 2). *mec*-regulated repression of *mecA* in experienced strains resulted in a very heterogeneous resistance pattern for COLn and BB270 compared to that for parents and transformants with unregulated *mecA* (Fig. 1). The effect of *mec* regulation on the phenotype of a naturally very heterogeneous strain, N315, was also quite apparent. The parent and the pYK644 transformant, in which the *bla* and *mec* regulatory genes both are present, exhibit a much more heterogeneous resistance pattern than the pYK20 transformant, in which the β-lactamase plasmid is present and the *mecR1-mecI* genes are absent. The important contribution of the genetic background to the resistance phenotype is again demonstrated by the behavior of strain COL52, the homogeneous phenotype of which is fundamentally unchanged by the imposition of *mec* regulation. On balance, β-lactamase regulatory genes seem to provide a compromise solution to the need for some control over PBP2a production to minimize the cost of maintaining *mecA* while also being able to express the protein in the presence of an antibiotic.

These experimental results correlate well with what others have found concerning the relationship between β-lactamase-mediated and PBP2a-mediated resistance. As would be predicted from the strong repression of *mecA*, *mecR1-mecI* elements in SCC*mec* typically are deleted or mutated (15, 24). The early observation that β-lactamase was a critical determinant for transduction of the methicillin resistance determinant (4, 9, 28) and reports that methicillin resistance tends to be unstable in clinical isolates when the penicillinase plasmid is absent are understandable in view of the ability of the β-lactamase repressor to stabilize *mecA* in some genetic backgrounds (13, 25, 27, 30). This interesting synergy between the two dissimilar resistance mechanisms can be thought of as a novel variation on the theme of compensatory mutations in drug resistance (3, 21), such that merely introducing a gene is often not enough. The genetic or biochemical basis of the barrier to *mecA* and or tolerance to it (aside from regulatory genes) is unknown but may have important implications for drug resistance and drug development, as the genes involved could be drug targets. We also propose that this barrier may account for the clonality that is typical of MRSA by restricting the element to certain backgrounds.

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REFERENCES

1. **Asheshov, E. H., and K. G. Dyke.** 1968. Regulation of the synthesis of penicillinase in diploids of *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. **30:**213–218.
2. **Berger-Bächi, B., and M. L. Köhler.** 1983. A novel site on the chromosome of *Staphylococcus aureus* influencing the level methicillin resistance: genetic mapping. FEMS Microbiol. Lett. **20:**305–309.
3. **Bjorkman, J., I. Nagaev, O. G. Berg, D. Hughes, and D. I. Andersson.** 2000. Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. Science **287:**1479–1482.
4. **Boyce, J. M., and A. A. Medeiros.** 1987. Role of beta-lactamase in expression of resistance by methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **31:**1426–1428.
5. **Brumfitt, W., and J. Hamilton-Miller.** 1989. Methicillin-resistant *Staphylococcus aureus*. N. Engl. J. Med. **320:**1188–1196.
6. **Chambers, H. F.** 1995. In vitro and in vivo antistaphylococcal activities of L-695,256, a carbapenem with high affinity for the penicillin-binding protein PBP2a. Antimicrob. Agents Chemother. **39:**462–466.
7. **Chambers, H. F., and C. J. Hackbarth.** 1987. Effect of NaCl and nafcillin on penicillin-binding protein 2a and heterogeneous expression of methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **31:** 1982–1988.
8. **Chambers, H. F., G. L. Archer, and M. Matsuhashi.** 1989. Low-level methicillin resistance in strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **33:**424–428.
9. **Cohen, S., and H. M. Sweeney.** 1973. Effect of the prophage and penicillinase plasmid of the recipient strain upon the transduction and the stability of methicillin resistance in *Staphylococcus aureus*. J. Bacteriol. **116:**803–811.
10. **Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt.** 2002. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc. Natl. Acad. Sci. USA **99:**7687–7692.
11. **Hartman, B. J., and A. Tomasz.** 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. J. Bacteriol. **158:**513–516.
12. **Hiramatsu, K., K. Asada, E. Suzuki, K. Okonogi, and T. Yokota.** 1992. Molecular cloning and nucleotide sequence determination of the regulator region of *mecA* gene in methicillin-resistant *Staphylococcus aureus* (MRSA). FEBS Lett. **298:**133–136.
13. **Hiramatsu, K., E. Suzuki, H. Takayama, Y. Katayama, and T. Yokota.** 1990. Role of penicillinase plasmids in the stability of the *mecA* gene in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **34:**600–604.
14. **Ito, T., Y. Katayama, and K. Hiramatsu.** 1999. Cloning and nucleotide sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. Antimicrob. Agents Chemother. **43:** 1449–1458.
15. **Ito, T., Y. Katayama, K. Asada, N. Mori, K. Tsutsumimoto, C. Tiensasitorn, and K. Hiramatsu.** 2001. Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **45:** 1323–1336.
16. **Katayama, Y., T. Ito, and K. Hiramatsu.** 2000. A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **44:**1549–1555.
17. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709–712.
18. **Kuwahara-Arai, K., N. Kondo, S. Hori, E. Tateda-Suzuki, and K. Hiramatsu.** 1996. Suppression of methicillin resistance in a *mecA*-containing pre-methicillin-resistant *Staphylococcus aureus* strain is caused by the *mecI*-mediated repression of PBP2′ production. Antimicrob. Agents Chemother. **40:**2680–2685.
19. **Lewis, R. A., and K. G. Dyke.** 2000. MecI represses synthesis from the beta-lactamase operon of *Staphylococcus aureus*. J. Antimicrob. Chemother. **45:**139–144.
20. **McKinney, T. K., V. K. Sharma, W. A. Craig, and G. L. Archer.** 2001. Transcription of the gene mediating methicillin resistance in *Staphylococcus aureus* (*mecA*) is corepressed but not coinduced by cognate *mecA* and beta-lactamase regulators. J. Bacteriol. **183:**6862–6868.
21. **Nagaev, I., J. Bjorkman, D. I. Andersson, and D. Hughes.** 2001. Biological cost and compensatory evolution in fusidic acid-resistant *Staphylococcus aureus*. Mol. Microbiol. **40:**433–439.
22. **Nicholas, P. J. D., E. M. Catrin, M. C. Enright, A. R. Berendt, J. M. Smith, M. F. Murphy, S. J. Peacock, B. G. Spratt, and E. J. Feil.** 2001. A link between virulence and ecological abundance in natural populations of *Staphylococcus aureus*. Science **292:**114–116.
23. **Novick, R. P.** 1991. Genetic systems in staphylococci. Methods Enzymol. **204:**587–636.
24. **Okuma, K., K. Iwakawa, J. D. Turnidge, W. B. Grubb, J. M. Bell, F. G. O'Brien, G. W. Coombs, J. W. Pearman, F. C. Tenover, M. Kapi, C. Tiensasitorn, T. Ito, and K. Hiramatsu.** 2002. Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. J. Clin. Microbiol. **40:**4289–4294.
25. **Richmond, M. H.** 1965. Dominance of the inducible state in strains of *Staphylococcus aureus* containing two distinct penicillinase plasmids. J. Bacteriol. **90:**370–374.
26. **Rosato, A. E., B. N. Kreiswirth, W. A. Craig, W. Eisner, M. W. Climo, and G. L. Archer.** 2003. *mecA-blaZ* corepressors in clinical *Staphylococcus aureus* isolates. Antimicrob. Agents Chemother. **47:**1460–1463.
27. **Ryffel, C., A. Strassle, F. H. Kayser, and B. Berger-Bächi.** 1994. Mechanisms of heteroresistance in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **38:**724–728.
28. **Ryffel, C., F. H. Kayser, and B. Berger-Bächi.** 1992. Correlation between regulation of *mecA* transcription and expression of methicillin resistance in staphylococci. Antimicrob. Agents Chemother. **36:**25–31.
29. **Sharma, V. K., C. J. Hackbarth, and T. M. Dickinson.** 1998. Interaction of

native and mutant MecI repressors with sequences that regulate *mecA*, the gene encoding penicillin binding protein 2a in methicillin-resistant staphylococci. J. Bacteriol. **180:**2160–2166.
30. **Stewart, G. C., and E. D. Rosenblum.** 1980. Transduction of methicillin resistance in *Staphylococcus aureus*: recipient effectiveness and β-lactamase production. Antimicrob. Agents Chemother. **18:**424–432.
31. **Tomasz, A., H. B. Drugeon, H. M. de Lencastre, D. Jabes, L. McDougall, and J. Bille.** 1989. New mechanism for methicillin resistance in *Staphylococcus aureus*: clinical isolates that lack the PBP2a gene and contain normal penicillin-binding proteins with modified penicillin-binding capacity. Antimicrob. Agents Chemother. **33:**1869–1874.
32. **Tomasz, A., S. Nachman, and H. Leaf.** 1991. Stable classes of phenotypic expression in methicillin-resistant clinical isolates of staphylococci. Antimicrob. Agents Chemother. **35:**124–129.
33. **Utsui, Y., and T. Yokota.** 1985. Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **28:**397–403.
34. **Wada, A., and H. Watanabe.** 1998. Penicillin-binding protein 1 of *Staphylococcus aureus* is essential for growth. J. Bacteriol. **180:**2759–2765.
35. **Zhang, H. Z., C. J. Hackbarth, K. M. Chansky, and H. F. Chambers.** 2001. A proteolytic transmembrane signaling pathway and resistance to β-lactams in staphylococci. Science **291:**1962–1965.