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Borderline Susceptibility to Antistaphylococcal Penicillins Is Not Conferred Exclusively by the Hyperproduction of β-Lactamase

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Staphylococcus aureus **strains bearing the 17.2-kb β-lactamase plasmid pBW15 and belonging to phage group 94/96 exhibit borderline susceptibility to the antistaphylococcal penicillins. Borderline susceptibility within phage group 94/96 is thought to be mediated by the hyperproduction of type A staphylococcal β-lactamase. Evaluation of 84 non-94/96 phage type *S. aureus* strains that also produced the type A enzyme identified 7 additional hyperproducing strains. However, none of these isolates contained pBW15, and only one met the criteria for borderline susceptibility. To determine the role of pBW15 and the 94/96 phage type in the expression of borderline susceptibility, pBW15 was transformed in two plasmid-free, penicillin-susceptible strains, one of which belonged to phage group 94/96. Penicillin MICs for both transformants and quantitative β-lactamase activity were comparable to those for the parent pBW15-containing strain. A fourfold difference in the oxacillin MICs for the 94/96 and non-94/96 phage type transformants (1.0 and 0.25 μg/ml, respectively) was identified, and only the 94/96 phage type transformant met the criteria for borderline susceptibility. Chromosomal DNA from borderline-susceptible phage group 94/96 strains did not hybridize with a probe for *mecA*, and the beta-lactam binding affinity of PBPs 1, 2, 3, and 4 from a penicillin-susceptible 94/96 phage type strain and a non-94/96 phage type strain were comparable. Although hyperproduction of the type A β-lactamase appears to be necessary for the expression of borderline susceptibility within certain phage group 94/96 strains, β-lactamase production of a comparable magnitude by a group of *S. aureus* strains belonging to other phage types does not confer borderline susceptibility. These data suggest that borderline susceptibility is not solely due to the hyperproduction of β-lactamase.**

Simple, reproducible criteria separate strains of *Staphylococcus aureus* into three groups on the basis of their susceptibilities to beta-lactam antimicrobial agents: strains that are penicillin susceptible, strains that are penicillin resistant but methicillin susceptible, and strains that are methicillin resistant. Penicillin resistance for strains defined in this manner is conferred by elaboration of β-lactamase, and methicillin resistance is conferred by the elaboration of PBP 2a as well as other factors (6, 13). Recently, strains with intermediate susceptibilities to methicillin and other antistaphylococcal penicillins have been identified. This phenotype has been reported to be mediated by an altered binding affinity of normal penicillin-binding proteins (PBPs) (20) and by hyperproduction of β-lactamase (10, 11). Intermediately susceptible strains that hyperproduce β-lactamase have been termed borderline methicillin susceptible, while other strains have been called low-level methicillin resistant.

We have described a widespread strain of borderline-susceptible *S. aureus* that is phage type 94/96 and that produces very large amounts of the type A variant of staphylococcal β-lactamase. These strains also harbor a novel β-lactamase plasmid, pBW15 (1, 11). In the present investigation, we examined the association of these determinants (hyperproduction of β-lactamase, plasmid pBW15, and phage type 94/96) with the borderline-susceptible phenotype.

MATERIALS AND METHODS

Strains. Phage complex 94/96 *S. aureus* SA5003 harboring pBW15 (9) was used as a standard borderline-susceptible isolate. Both *S. aureus* SA2076 (phage complex 94/96), which was obtained during a colonization study of hospital personnel, and RN450, which was obtained from R. Novick, New York, N.Y., were plasmid-free and penicillin susceptible. Reference type A-producing isolates RN11 and PC1 were also obtained from R. Novick. Other type A β-lactamase-producing strains were clinical and surveillance cultures from the St. Thomas Hospital and Vanderbilt University. These strains were phage typed as part of a previous study (11). *S. aureus* ATCC 25923 was used as a penicillin-susceptible control. A clinical isolate, MRSA47 (methicillin MIC, ≥128 μg/ml), was used as a methicillin-resistant control strain (Table 1).

Antimicrobial agents and media. Antibiotic disks and sterile paper disks were purchased from BBL Microbiology Systems, Cockeysville, Md. Standard powders of penicillin G (Pfizer, New York, N.Y.), cefotaxime (Hoechst-Roussel, Nutley, N.J.), nitrocefin (BBL), and oxacillin and methicillin (Bristol-Myers, Syracuse, N.Y.) were used to prepare sterile antimicrobial solutions for MIC determinations and β-lactamase and membrane affinity assays. Mueller-Hinton agar, cation-supplemented Mueller-Hinton broth, tryptic soy agar, and brain heart infusion (BHI) agar and broth were obtained from Difco Laboratories, Detroit, Mich.

Susceptibility testing. Broth microdilution and macrodilution MIC determinations and oxacillin zone diameters were determined by the guidelines recommended by the National Committee for Clinical Laboratory Standards (14). MIC determinations were performed by using broth supplemented with 2% NaCl. Macrodilution MBCs were performed by published methods (19).

Definitions. All *S. aureus* isolates were classified as susceptible to penicillin G (MIC, ≤0.125 μg/ml) or penicillin resistant but methicillin susceptible (penicillin G MIC,

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TABLE 1. Strains and plasmids used in this study

Plasmid or strain	Description[a]	Source or reference
Plasmids		
pBW15	Pen^r Cad^r	11
pC194	Cam^r	7
pWN101	Pen^r Cam^r	24
pPMF13	Amp^r, *Pst*I-*Xba*I fragment of the 4.1-kb *Hin*dIII fragment containing the *mecA* gene cloned in pTZ18	P. Mathews, New York, N.Y.
Strains		
SA5003	BSSA, phage type 94/96	11
SA2076	PSSA, phage type 94/96	Colonization Study Isolate
RN450	PSSA	R. Novick, New York, N.Y.
PC1	PRSA, constitutive production of β-lactamase	15
RN11	PRSA, inducible production of β-lactamase	15; R. Novick, New York, N.Y.
25923	PSSA	ATCC[b]
MRSA47	MRSA	Clinical isolate

[a] Amp, ampicillin; Cad, cadmium; Cam, chloramphenicol; Pen, penicillin; BSSA, borderline-susceptible *S. aureus*; PSSA, penicillin-susceptible *S. aureus*; PRSA, penicillin-resistant *S. aureus*; MRSA, methicillin-resistant *S. aureus*.

[b] ATCC, American Type Culture Collection, Rockville, Md.

>0.125 μg/ml; methicillin MIC, ≤8 μg/ml) by using recommended criteria (14). Penicillin-resistant but methicillin-susceptible strains were designated BSSA if (i) microdilution MICs of penicillin G were ≥64 μg/ml, (ii) microdilution MICs of oxacillin were 0.5 to 4.0 μg/ml, and (iii) zone diameters were 6 to 13 mm using 1-μg oxacillin disks and Mueller-Hinton agar.

Transformation. Large-scale isolation of plasmid DNA was accomplished by the method of Townsend (22) and purified by density gradient centrifugation in $CsCl_2$. After protoplast transformation, RN450 transformants were selected on DM3 medium containing cadmium sulfate (6 to 8 μg/ml; Sigma, St. Louis, Mo.) (24) and subcultured on BHI agar containing ampicillin at 20 μg/ml. Strain SA2076 was transformed with pBW15 and pC194 (2.9 kb) by congression (16). Transformants were selected on DM3 medium containing chloramphenicol at 6 μg/ml and were then subcultured onto ampicillin plates as described above. All ampicillin-resistant transformants were tested for β-lactamase production by incubating a suspension of 10^6 CFU of each strain in 100 μl of nitrocefin (500 μg/ml in 0.05 M phosphate buffer) for 30 min and were screened for plasmids by an alkaline lysis method and with lysostaphin (18).

Plasmid curing. An overnight BHI broth culture (10 μl) of the desired strain was inoculated into 10 ml of fresh BHI broth and incubated at 20°C with vigorous shaking for 24 h. After five such cycles, 100 μl of serial dilutions were plated onto BHI agar and incubated overnight. One hundred random colonies were picked onto BHI agar and onto BHI agar with ampicillin at 10 μg/ml. Ampicillin-susceptible colonies were examined by Gram stain, coagulase tested, assayed for β-lactamase production, and screened for plasmids.

β-Lactamase typing and quantitation. Induced suspensions of each strain were prepared, after 14 h of incubation, on 1% CY Tris agar containing methicillin at 0.5 μg/ml (8). The rates of nitrocefin hydrolysis by β-lactamase were measured by using a DU-70 recording spectrophotometer (Beckman Instruments, Fullerton, Calif.) and were recorded as micromoles of nitrocefin degraded per minute per standard cell mass (A_{272} = 1.0, or 10^8 CFU/ml).

Hybridizations. Overnight broth cultures (3 ml) were harvested by centrifugation, resuspended in TES buffer containing 12 U of lysostaphin (Applied Microbiology, New York, N.Y.) per ml, and incubated at 37°C for 30 min. Lysate (10 μl) was spotted in a grid pattern onto Hybond-N (Amersham, Arlington Heights, Ill.) filters and air-dried. Filters were saturated with 0.5 M NaOH for 5 min and then with 1 M Tris (pH 7) for 5 min and washed in 1 M Tris for 3 min and allowed to dry. A probe for the β-lactamase gene (*blaZ*) was prepared from the 840-bp *Hin*dIII-*Xba*I fragment of plasmid pWN101 (23) by excision from low-melting-point agarose and radiolabeled with [α-^{32}P]dCTP (Dupont NEN Research Products, Boston, Mass.) by an oligoextension method (5). In a similar manner, a probe for the methicillin resistance gene (*mecA*) was prepared from an intragenic 900-bp *Pst*I-*Xba*I fragment of plasmid pPMF13 kindly supplied by P. Mathews, New York, N.Y.

Blots were prehybridized for 4 h in a solution of 50% formamide (Sigma)–1% sodium dodecyl sulfate (SDS)–1 M NaCl–10% dextran sulfate (Sigma)–100 μg of salmon sperm DNA (Sigma) per ml. Radiolabeled probe was added, incubated at 42°C overnight, and then washed in 0.1% SDS–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) twice at 55°C for 30 min and twice in 0.1% SDS–0.1× SSC at 55°C for 30 min. Filters were air-dried and were exposed on X-OMAT film (Eastman Kodak, Rochester, N.Y.) for 24 h.

Northern hybridization. Strains were grown and total cellular RNA was isolated by the method of Kornblum et al. (9) and was transferred to Hybond-N filters (Amersham). β-Lactamase production was induced by adding sufficient 2-(2′-carboxyphenyl)-benzoyl-6-aminopenicillanic acid (CBAP; Sigma) to one of three successive tubes at 15-min intervals for a final concentration of 0.5 μg/ml. No CBAP was added to a fourth tube. Filters were prehybridized in 50% formamide (Sigma)–5× Denhardt's solution–5× SSC–10% dextran sulfate (Sigma)–25 mM KPO_4–50 μg of salmon sperm DNA per ml. The labeled *blaZ* probe was added and hybridized overnight. Filters were washed in 0.1% SDS–1× SSC twice for 30 min at 42°C and then in 0.1% SDS–0.25× SSC twice for 30 min at 42°C, dried, and applied to X-OMAT (Kodak) film for 48 to 72 h.

Assay of PBP binding affinity. Whole cells were used for the detection of PBPs by fluorography (3). In competition assays, nonradioactive antibiotics were used at concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 50 μg/ml. A final concentration of 5 μg of [^{3}H]penicillin per ml was used to label PBPs.

A direct PBP assay was performed by using [^{3}H]penicillin at concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 μg/ml. The method was similar to that used in the competition assays, except that cells were collected by centrifugation before [^{3}H]penicillin was added.

Binding affinity was expressed in terms of the antibiotic concentration (the 50% saturating concentration) that resulted in a PBP band density in the fluorograph that was 50% relative to its density in a radiolabeled reference. The PBP band density at each sample concentration was measured by scanning densitometry (Quick-Scan, Jr., Helena Instruments, Beaumont, Tex.). Percent density was calculated as follows: % density = (1 − PBP density$_{sample}$/PBP density$_{reference}$) × 100. For the competition assay, the reference was a sample incubated with 5 μg of [^{3}H]penicillin per

ml for 15 min at 37°C. For direct assays, the reference sample was incubated with 1 μg of [^{3}H]penicillin per ml (a lower concentration was chosen to reduce background). Percent PBP density was plotted versus concentration. The 50% saturating concentration was calculated by using the equation derived by the least-squares method for the linear portion of the density-concentration curve.

RESULTS

Phenotypic and genetic comparisons of 94/96 phage type versus non-94/96 phage type strains that elaborate large amounts of the type A β-lactamase. In examining the role of β-lactamase hyperproduction in mediating borderline susceptibility, we first determined whether isolates producing comparable amounts of the type A β-lactamase from other phage groups either exhibited the BSSA phenotype or contained pBW15. Among 84 non-94/96 type A β-lactamase-producing strains, 7 strains were identified that hydrolyzed nitrocefin at greater than or equal to the lowest rate of nitrocefin hydrolysis among 30 94/96 phage type BSSA strains (87 μmol/min per standard cell mass). Compared with the 30 94/96 phage type strains, the 7 non-94/96 phage type strains (including phage groups I, III, and nontypeable) were similar in their quantitative β-lactamase activities (mean of 145.4 versus 124.1 μmol of nitrocefin hydrolyzed per min per standard cell mass). In contrast, the seven non-94/96 phage type strains were more susceptible than the 94/96 phage type strains to penicillin G (MICs, 64 and 256 μg/ml, respectively; $P < 0.02$ by the Wilcoxon rank sum test) and oxacillin (MICs, 0.5 and 1 μg/ml, respectively; $P < 0.01$ by the Wilcoxon rank sum test). Only one of the seven non-94/96 phage type strains met all of the criteria for borderline susceptibility. Plasmid pBW15 was not present in any of the 7 non-94/96 phage type strains.

We also examined the regulation of the β-lactamase gene in borderline-susceptible strains. As determined by Northern hybridization, the β-lactamase gene in these strains is inducible (Fig. 1).

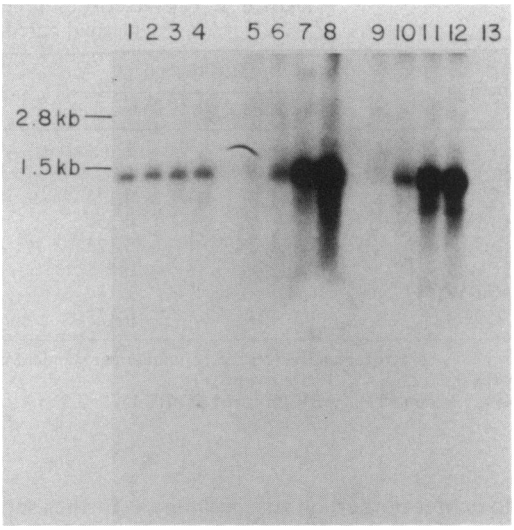

FIG. 1. Lanes 1 through 12 show *blaZ*-specific mRNA signals from three penicillin-resistant *S. aureus* strains. Induction times of 0, 15, 30, and 45 min are represented; the shortest induction time corresponds to the first lane in each set, and the longest induction time corresponds to the last lane in each set. Lanes 1 through 4, strain PC1, a constitutive producer of β-lactamase; lanes 5 through 8, RN11, a strain with inducible production of β-lactamase; lanes 9 through 12, borderline-susceptible strain SA5003. The signal from SA5003 increased over 45 min, indicating that the β-lactamase gene in this strain is inducible. No signal was observed in lane 13, representing RNA isolated from a penicillin-susceptible strain (RN450).

Does pBW15 confer hyperproduction of β-lactamase and borderline susceptibility? Since six of the seven β-lactamase hyperproducers that did not harbor pBW15 were not borderline susceptible and 96% of a select group of borderline-susceptible strains harbored pBW15 (11), perhaps plasmid pBW15 was crucial for the expression of this phenotype. This was tested by transforming pBW15 in RN450 (derived from phage group III) and SA2076 (phage group 94/96).

For transformant SA2076(pBW15), the oxacillin and cefotaxime MICs and oxacillin zone diameter were comparable to those for the borderline strain SA5003, in contrast to the values indicating greater susceptibility obtained for transformant RN450(pBW15). Oxacillin MBCs for SA5003 and SA2076(pBW15) were greater than those for the other strains tested, but the strains were not oxacillin tolerant (Table 2).

Although susceptibility results differed, RN450(pBW15) and SA2076(pBW15) produced β-lactamase at nearly the same level as SA5003 (BSSA), as measured by spectrophotometry (Table 2).

To be certain of the partial role of pBW15, plasmid-cured derivatives of the borderline-susceptible strain SA5003 were evaluated next. SA5003, when it was cured of the 17.2-kb β-lactamase plasmid, was susceptible to penicillin and oxacillin. However, the oxacillin and cefotaxime MICs were consistently higher for SA5003 cured of pBW15 than they were for the penicillin-susceptible strains ATCC 25923 and RN450. The oxacillin and cefotaxime MICs for SA2076 were similar to those for SA5003 cured of the β-lactamase plasmid (Table 2). Results for RN450(pBW15) cured of pBW15 were identical to those for RN450.

PBP studies. We next attempted to determine whether the relative resistance of the 94/96 phage type strains could be explained by the presence of PBP 2a or other novel PBPs or an altered affinity of the normal PBPs. The *mecA* gene probe did not hybridize with DNA from strain SA5003. Dot blots of all transformants hybridized with the *blaZ* probe but not the *mecA* probe. PBP 2a or other novel PBPs were not detected in the borderline-susceptible strain by fluorography. Comparisons of the beta-lactam-binding affinities of PBPs 1, 2, 3, and 4 from the penicillin-susceptible strain RN450 and SA5003 cured of plasmid pBW15 did not show marked differences for penicillin, methicillin, clavulanate, or cefotaxime (Table 3).

DISCUSSION

A new subgroup of penicillin-resistant *S. aureus* that elaborates large quantities of β-lactamase has been termed borderline methicillin susceptible by McDougal and Thornsberry (10). It was postulated that hyperproduction of β-lactamase determined this phenotype.

Transfer of β-lactamase hyperproduction was shown by the transformation of pBW15 in strain RN450. The transformants, however, did not meet the borderline susceptibility criteria, because these isolates were more susceptible to oxacillin as determined by microdilution MICs and disk diffusion zone size. Apparently, the production of β-lactamase in quantities equivalent to those produced by borderline-susceptible strains is not sufficient to confer borderline susceptibility. The inability of β-lactamase hyperproduction

TABLE 2. Characteristics of penicillin-susceptible and penicillin-resistant *S. aureus*, plasmid-cured strains, and pBW15 transformants

Strain	MIC (μg/ml)		Oxacillin MBC (μg/ml)	Cefotaxime MIC (μg/ml)	Oxacillin zone diam (mm)	β-Lactamase quantity[a]
	Penicillin	Oxacillin				
SA5003	64	2	8	2	11	115.7
SA5003(c)[b]	<0.125	1	4	1	22	<0.15
RN450	<0.125	0.125	2	0.5	21	<0.15
RN450(pBW15)	64	0.25	4	1	15	118.6
RN450(c)[c]	<0.125	0.125	2	0.5	21	<0.15
SA2076	<0.25	0.5	4	1	22	<0.15
SA2076(pBW15)	64	1	8	2	12	104.4
ATCC 25923	<0.125	0.125	2	0.5	22	<0.15

[a] Micromoles of nitrocephin hydrolyzed perminute per standard cell mass.
[b] SA5003(c), strain SA5003 cured of pBW15.
[c] RN450(c), strain RN450(pBW15) cured of pBW15.

alone to confer borderline susceptibility is further supported by the identification of other non-borderline-susceptible penicillin-resistant strains that produced β-lactamase in amounts equivalent to those produced by borderline-methicillin-susceptible isolates.

Although the amount of β-lactamase produced was similar for borderline-susceptible and selected non-borderline-susceptible strains, perhaps the regulation of expression of the β-lactamase gene in borderline-susceptible strains was different. Enzyme hyperproduction could be caused by a mutation in an inducible gene that causes constitutive high-level expression. However, the β-lactamase gene in the BSSA strains tested was inducible by Northern hybridization.

Since plasmid-mediated β-lactamase production alone did not determine borderline susceptibility, we postulated that an increase in intrinsic resistance might be involved. This was suggested by the slightly higher oxacillin MICs and MBCs for SA5003 cured of plasmid pBW15 and SA2076 compared with those for the penicillin-susceptible control strains (RN450, ATCC 25923). The relative resistance of these phage complex 94/96 strains, in combination with the hyperproduction of β-lactamase, may confer borderline susceptibility. This phenomenon may be analogous to the increased background resistance in β-lactamase negative, *mecA*-inactivated, methicillin-resistant *S. aureus* strains reported by de Lencastre et al. (4).

It is clear that PBP 2a was not involved in the borderline susceptibility of our strains. No isolates tested in this study hybridized to a *mecA* probe, nor was PBP 2a detected. Montanari et al. (12) have also examined borderline-methicillin-susceptible strains and have not shown the presence of PBP 2a or novel PBPs in membrane preparations. Our evaluation of three of these strains demonstrated that one strain harbored a single plasmid of 17.2 kb that comigrated with pBW15. BSSA strains, therefore, probably lack novel PBPs.

We can only speculate about the mechanism of this relative resistance. Altered PBPs have been postulated for strains that do not produce detectable quantities of β-lactamase but that are intermediately susceptible to oxacillin (MIC, 1 to 4 μg/ml) (17). Tomasz et al. (20) have also described low-level-resistant strains with altered PBPs. These strains ($1 \geq$ oxacillin MIC ≤ 4 μg/ml; $4 \geq$ cefotaxime MIC ≤ 16 μg/ml) contained the normal complement of PBPs, but with altered binding affinity for beta-lactams (20). PBPs 1, 2, and 4 are possibly altered in strains that express non-PBP 2a-mediated resistance (2, 20, 21). Although oxacillin and cefotaxime MICs for the borderline strains we tested were higher than those for penicillin-susceptible control strains, a decreased affinity for beta-lactam antibiotics to any of the PBPs could not be demonstrated. These strains could still produce PBPs but with altered functions that were not easily detected by the methods that we used. Several drugs at concentrations increased in increment by two- or fivefold were examined in the binding studies, so if altered binding is present, it probably occurs over a narrow range of concentrations. Such small differences are unlikely to be detectable by drug-binding assays, and if they are found, the importance of such small differences to the mechanism of resistance may be questioned.

Several mechanisms exist that confer intermediate susceptibility to antistaphylococcal penicillins. For the strains that we evaluated, borderline susceptibility is probably a function of β-lactamase plus a small increase in intrinsic resistance.

TABLE 3. The 50% saturating concentrations for PBPs of the β-lactamase-free variant of the borderline-susceptible strain SA5003 cured of pBW15 and susceptible control strain RN450

Drug	50% Saturating concn (μg/ml) for PBP[a]:							
	1		2		3		4	
	SA5003(c)	RN450	SA5003(c)	RN450	SA5003(c)	RN450	SA5003(c)	RN450
[^{3}H]penicillin	0.06	0.1	0.05	0.07	0.2	0.2	>1	>1
Methicillin	0.05	0.06	4	5	0.01	0.04	0.09	0.04
Clavulamic acid	5	2	0.1	0.1	2	5	2	0.8
Cefotaxime	0.2	0.2	0.06	0.04	0.2	0.4	0.2	2.0

[a] SA5003(c), strain SA5003 cured of plasmid pBW15.

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