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β-Lactam-Specific Resistant Mutants of *Staphylococcus aureus*

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In an approach to understanding the origin of methicillin resistance in clinical isolates of staphylococci, a series of *Staphylococcus aureus* mutants resistant to various beta-lactam antibiotics were isolated in the laboratory by antibiotic selection. Mutants with low- and intermediate-level resistance showed considerable specificity for the particular antibiotic used in the selection process (methicillin, cefotaxime, cephalexin, and amdinocillin), and resistance in such mutants also showed alterations in the antibiotic binding capacities of penicillin-binding proteins (PBPs). In each case the isolation of mutants resistant to high concentrations of antibiotics required sequential passage in gradually increasing concentrations of the drug. The acquisition of increasing levels of methicillin resistance was paralleled by a gradual decrease in the binding capacities of PBPs 2, 3, and, possibly, 1. In a highly methicillin-resistant mutant (MIC, 150 μg/ml), PBPs 2 and 3 were no longer detectable by the penicillin binding assay. Instead, a new PBP of poor binding capacity and anomalous molecular size (about 78 kilodaltons [kDa]) appeared in these cells. This corresponds to the molecular size of PBP 2a, the unique PBP that appears to be the biochemical correlate of resistance in clinical isolates of methicillin-resistant *S. aureus*. Also, similar to the case of resistant clinical isolates, high-level beta-lactam resistance was highly pH dependent in the laboratory mutants. We compared the patterns of radioactive peptides generated by partial proteolysis from the penicillin-labeled PBP 2 of antibiotic-susceptible staphylococci and from the 78-kDa PBP 2a of a resistant clinical strain. Although the patterns were clearly different, seven of the eight characteristic peptides generated from PBP 2 of the susceptible strain were also detectable among the peptides released from PBP 2a. The results suggest that the 78-kDa PBP 2a of the resistant clinical strain evolved from PBP 2 of antibiotic-susceptible staphylococci and that in PBP 2a of the clinical isolate mutational changes have resulted in extensive alterations near the beta-lactam binding site.

Shortly after the clinical introduction of methicillin for the control of penicillin-resistant (beta-lactamase-producing) staphylococci, the isolation of the first methicillin-resistant *Staphylococcus aureus* was described (18). Over the ensuing two-and-a-half decades, methicillin-resistant *S. aureus* has been encountered with varying frequency as a significant cause of serious human disease worldwide. Recent surveys indicate that methicillin-resistant *S. aureus* has become a major nosocomial pathogen in the United States (13), Japan (29), and Australia (13) and has also reemerged in England, after a temporary decline in frequency in the 1970s (4, 28).

The mechanism of methicillin resistance is not fully understood. Clearly, it does not involve inactivation of the antibiotic. Penicillin-binding proteins (PBPs) with altered antibiotic binding capacity have been described in clinical isolates of methicillin-resistant *S. aureus* in several laboratories (3, 9, 12, 15, 17, 25, 29). A common feature of most such isolates from the United Kingdom, United States, France, Italy, Australia, and Japan appears to be the presence of an extra PBP (PBP 2a or PBP 2′) of about 78 kilodaltons (kDa) molecular size and unusually low affinity for a wide range of beta-lactam antibiotics. In addition to this PBP, most clinical strains examined seem to retain a full complement of the four PBPs characteristic of antibiotic-susceptible isolates of *S. aureus*, although lowered affinity or changed cellular concentration of PBP 3 or both have also been reported in some isolates (25). The 78-kDa, low-affinity PBP was reported to migrate in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis either with a smaller (3, 9, 12, 15) or with a somewhat larger (29) molecular size than the normal PBP 2 of these strains.

Intrinsic resistance to beta-lactam antibiotics based on altered PBPs is not unique to methicillin-resistant *S. aureus* but has been detected among clinical isolates of most of the major invasive human pathogens, such as pneumococci (31), gonococci (1, 8), *Haemophilus influenzae* (22, 23), and *Streptococcus faecium* (10, 30). In addition, it was recently demonstrated that penicillin-resistant mutants of pneumococci generated in the laboratory by antibiotic selection contained altered PBPs similar to the PBPs detectable in clinical isolates with comparable penicillin susceptibilities (14).

In an attempt to elucidate the origin of the low-affinity, 78-kDa PBP characteristic of clinical methicillin-resistant *S. aureus* strains, we isolated a series of beta-lactam-resistant mutants from an antibiotic-susceptible parental strain of *S. aureus* by serial passage on gradually increasing concentrations of beta-lactam antibiotics. In this report, we describe the biochemical and physiological properties of several such mutant strains.

MATERIALS AND METHODS

Strains and culture conditions. Isogenic strains of *S. aureus*, 27s and 27r, were kindly supplied by R. Novick, Public Health Research Institute, New York, N.Y. 27r was obtained by transduction with phage 80a of the methicillin resistance determinants of a clinical isolate into susceptible strain 27s. *S. aureus* resistant mutants M5, M25, M50, and M100 were obtained from 27s by the procedure described below. All strains were stored at −70°C in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) containing 10% (vol/vol) glycerol. For overnight cultures, 0.1 ml of the frozen stock was inoculated in 10 ml of TSB and incubated

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with aeration at 37°C. All experiments were done with exponentially growing cultures in mid-log phase obtained by inoculating 0.2 ml of overnight culture into fresh, prewarmed TSB (10 ml) and cultivation at 37°C under aerobic conditions. Bacterial growth was measured with a nephelometer (Coleman Instruments, Oak Brook, Ill.).

Selection of mutant strains resistant to methicillin. Strain 27s was used as the parental strain in the isolation of all mutants. Mutants resistant to methicillin were obtained by growing 27s in the presence of increasing amounts of antibiotic for several passages. At each passage, the cells grown in the previous culture at the highest concentration of methicillin were used as inocula for cultures to be incubated in the presence of higher concentrations of the beta-lactam antibiotic. Thus, in step 1 of the procedure, 0.5 ml of an overnight culture of 27s (about 5×10^8 viable cells) was inoculated into flasks containing 50 ml of TSB supplemented with concentrations of methicillin ranging from 0.4 μg/ml (0.5 × the MIC) to 1.6 μg/ml (2 × the MIC). After 24 h of incubation at 37°C, bacterial cells grew in all the inoculated flasks, and the culture grown in the presence of 1.6 μg of methicillin per ml was used as the inoculum for a new series of culture flasks containing TSB plus methicillin. At each step, the culture grown in the presence of the highest concentration of antibiotic was frozen at −70°C. At the end of the selection procedure, nine strains resistant to methicillin concentrations ranging from 1.6 to 88 μg/ml were obtained; from these, five strains with distinct levels of resistance were selected for further studies. Resistance classes were purified in the following manner. Twenty-four colonies of each strain were grown on TSA plates and transferred by replica plating to a series of test plates (TSA containing 5, 10, 25, 50, 100, and 150 μg of methicillin per ml). This procedure enabled us to obtain homogeneous populations of resistant strains M5, M25, M50, and M100 which were able to grow in the presence of a maximum of 20, 50, 100, and 150 μg of methicillin per ml. Strains resistant to cefotaxime (CTX), cephalexin (CFL), and amdinocillin (Ci) were obtained by using an analogous procedure of selection, except that these antibiotics, instead of methicillin, were used for selection.

Antibiotic MIC determination. Organisms in mid-log phase were inoculated into TSB containing twofold dilutions of the antibiotic; 10^4 to 10^5 CFU/ml was used as the inoculum. After 24 h of incubation at 37°C, MICs were determined as the lowest antibiotic concentrations not allowing bacterial growth.

The homogeneity of methicillin resistance was verified by determining the efficiency of plating on TSB agar (2% Difco agar) plates containing various concentrations of methicillin. Efficiency of plating = (the number of CFU on methicillin-containing plates/the number of CFU on methicillin-free plates) × 100.

Membrane preparation and analysis of PBPS. For membrane preparation and PBP analysis (15), each strain was grown to log phase in 1 liter of TSB. The cultures were centrifuged at 12,000 × *g* for 15 min at 4°C, washed, and suspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.9) containing lysostaphin (100 μg/ml; Sigma Chemical Co., St. Louis, Mo.), DNase (10 μg/ml; Sigma), and RNase (5 μg/ml; Sigma). Cell suspensions were incubated at 37°C for 30 min and then centrifuged at 12,000 × *g* for 15 min; membranes were collected from the supernatant by centrifugation at 200,000 × *g* for 45 min at 4°C. The pellet was washed twice with phosphate buffer (50 mM; pH 7.2), resuspended in 1 ml of the same buffer, and stored at −70°C. The protein concentration, determined by the method of Lowry et al., was adjusted to 7 mg/ml (20). For PBP analysis, 10 μl of membrane suspension was mixed with 10 μl of [^{3}H]penicillin (ethylpiperidinium salt; specific activity, 25 Ci/mmol; Merck & Co., Inc., Rahway, N.J.). After 10 min of incubation at 37°C, the reaction was stopped by adding 5 μl of nonradiolabeled penicillin (12 mg/ml). Upon the addition of 30 μl of sample buffer, each sample was boiled for 2 min, and membrane proteins were separated on discontinuous SDS-polyacrylamide slab gels (10% acrylamide and 0.13% bis-acrylamide) (19). PBPs were detected by fluorography (2) using presensitized Kodak X-Omat XR-2 film and 5 days of exposure at −70°C. For competition assays, the membranes were treated with the test antibiotic for 10 min at 37°C followed by treatment with [^{3}H]penicillin, as described above.

PBP peptide maps. The chemical structure of PBPs 2 of the susceptible and intermediately resistant strains and of PBPs 2a of the highly methicillin-resistant strains was compared by peptide mapping after limited proteolysis (6). Membranes were prepared from strains 27s, M50, M100, and 27r. To restrict the binding of [^{3}H]penicillin to the PBP 2 (or 2a) component, membranes (100 to 200 μg) were preincubated with cephalexin (0.1 μg/ml) in the case of 27s or nafcillin (10 μg/ml) in the case of 27r before labeling with [^{3}H]penicillin (10 μg/ml) (5). Strain M50 had no detectable PBP 3 (only PBP 2), and in M100 the only detectable high-molecular-weight PBP was PBP 2a. After labeling, protein separation was performed in dimension 1 on polyacrylamide gels (7 cm in length and 0.75 mm in thickness). After a short staining with Coomassie brilliant blue and 30 min of destaining with methanol:acetic acid:water (30:5:65), the gels were washed five times in distilled water and twice in Tris hydrochloride buffer (pH 6.8; 0.1 M), and then they were soaked for 20 min in the same buffer containing 2 μg of *S. aureus* V8 protease (Miles Laboratories, Inc., Naperville, Ill.). The gel was cut into lanes containing the electrophoretically separated PBPs; the lanes were placed lengthwise on top of a second gel (15% acrylamide, 0.2% bis-acrylamide) from which SDS was omitted and in which 2 μg of V8 protease per ml was included in the stacking gel (5% acrylamide, 0.65% bis-acrylamide). The loaded lanes were sealed with 1% agarose and the dimension 2 migration was done at 70 V through the stacking gel and at 120 V through the separating gel. The gels were stained, destained, fluorographed, and processed after a 20-day exposure, as previously described (2).

DNA extraction. For extraction of DNA (21), 5 ml of overnight cultures of strains M100 and 27r were inoculated into 500 ml of TSB and incubated at 37°C until mid-log phase. The cultures were centrifuged, washed in TES (50 mM Tris hydrochloride, 9 mM EDTA, 50 mM NaCl [pH 8.0]) and suspended in 5 ml of the same buffer containing lysostaphin (100 μg/ml) and RNase (20 μg/ml). After 30 min of incubation at 37°C, the cells were lysed by adding 2.5 ml of 3% SDS. Pronase (100 μg/ml) was added, and the mixture was incubated at 37°C for 30 min. DNA was extracted with chloroform and precipitated by adding 0.7 ml of 3 M ammonium acetate and 20 ml of cold ethanol. Precipitated DNA was collected by spooling it onto a glass rod and then resuspended in diluted TES buffer.

Competent cells and transformation. For transformation of competent cells (24, 26), 0.1 ml of an overnight culture of strain 27s was inoculated into 10 ml of TSB and incubated until mid-log phase; 5 ml of this culture was inoculated into 500 ml of prewarmed TSB and shaken at 37°C until early log phase. The cells were collected by centrifugation, suspended into 0.75 ml of TSB, and heated for 2 min at 56°C; then, 1.5

TABLE 1. Susceptibility of *S. aureus* laboratory mutants to a variety of beta-lactam antibiotics

Mutant	Selection	MIC (μg/ml)[a]			
		Methicillin (PBP 3)	Cephalexin (PBP 3)	Amdinocillin (PBP 3)	Cefotaxime (PBP 2)
27s	Susceptible parent strain	0.8	1.0	15	1.0
M5	Methicillin	20 (25×)	20 (20×)	50 (3.3×)	3.0 (3×)
M25		50 (62.5×)	70–100 (100×)	200 (13×)	3.0 (3×)
M50		100 (125×)	~150 (150×)		4.0 (4×)
M100		150 (188×)	150 (150×)	500 (33×)	4.0 (4×)
CFL10	Cephalexin	2 (2.5×)	50 (50×)		3.0 (3×)
CFL100		5 (6.2×)	100–150 (150×)	100–150 (10×)	4.0 (4×)
Ci100	Amdinocillin	10 (12×)	100 (100×)	500 (33×)	20.0 (20×)
CTX20	Cefotaxime	1.5 (2×)	50 (50×)	50 (3.3×)	50.0 (50×)
CTX50		4.0 (5×)	300 (300×)		100 (100×)
CTX100		10.0 (12×)	200 (200×)	300 (20×)	400–500 (500×)
M100/t	Transformant[b]	20 (25×)	20 (20×)	40 (2.5×)	1.0
27r/t	Transformant	20 (25×)	20 (20×)	50 (3×)	4.0 (4×)
27r	Transductant[c]	600–1,000 (1,200×)	250	>1,000	>500
COL	Clinical isolate (United Kingdom)[d]	500	>100	>500	>100
DU	Clinical isolate (United States)	500	>100	>500	>100
AC	Clinical isolate (France)	1,000	>100	>500	>100

[a] MICs were determined by the tube dilution method. The PBP for which the particular antibiotic has the highest affinity is indicated in parentheses. The numbers in parentheses next to the MICs indicate the multiples of the parental MICs.

[b] Transformants were obtained by treating competent 27s cells with DNA from M100 or 27r and selecting for methicillin-resistant colonies, as outlined in Materials and Methods.

[c] Strain 27r was obtained by transduction (15).

[d] These clinical isolates are described in more detail in reference 16.

ml of phage 55 (10^{10} CFU/ml) and then 0.45 ml of normal rabbit serum (diluted 1:10 with TES) were added. After 5 min at room temperature, the cells were centrifuged, washed with Tris-maleate buffer (0.05 M; pH 7.0), and suspended in 5 ml of the same buffer containing $CaCl_2$ (0.1 M). Transformation was performed by adding 0.25 ml of DNA (200 μg/ml) to 0.75 ml of competent cells, and the reaction mixture was maintained on ice for 5 min and then at 37°C for 25 min. The cells were centrifuged, suspended in 1 ml of TSB, and incubated at 37°C for 2 h before being plated on TSB agar plates containing methicillin.

RESULTS

Physiological properties of beta-lactam-resistant laboratory mutants. The antibiotic susceptibilities of the parental *S. aureus* strain (27s) and several of the mutants are shown in Table 1. The choice of the particular beta-lactam antibiotic tested was based on the known selective affinity of several beta-lactam antibiotics for PBP 2 or PBP 3 in *S. aureus* (11, 12). The antibiotic susceptibilities of the strains were determined both by tube dilution and by plating on antibiotic-containing agar. Table 1 also shows the antibiotic susceptibility profiles of several clinical methicillin-resistant *S. aureus* isolates that have been characterized (15), as well as two genetic transformants, strains M100/t and 27r/t. Strain M100/t was constructed by transforming 27s with DNA isolated from M100, and 27r/t was constructed with DNA isolated from clinical resistant strain 27r.

Selectivity for resistance in the mutants is illustrated by the data in Table 1. For instance, the very substantial increase in the MIC of cephalexin (150 ×) for mutant CFL100 was not accompanied by more than modest changes in susceptibility to cefotaxime or methicillin. Similarly, the substantial increase in the cephalexin and cefotaxime MICs for mutant CTX100 was not paralleled by a comparable increase in methicillin resistance. Comparison of the cross resistance of mutants with low-level resistance to various antibiotics suggests a pattern that parallels the relative affinities of the corresponding antibiotics for PBPs 2 and 3 of *S. aureus* (12). In M5, M25, M50, and M100, mutants isolated by selection with methicillin, an antibiotic with an approximately 100-fold preferential affinity for PBP 3 over PBP 2, methicillin resistance was paralleled by comparable degrees of cross resistance to cephalexin and amdinocillin (also PBP 3-specific antibiotics) but not to cefotaxime, a drug with preferential binding to PBP 2 (12). A similar situation appears to be at least partially true in the case of mutants CFL10 and CFL100 (selected by cephalexin), which are cross resistant to amdinocillin but not to cefotaxime. On the other hand, these mutants show no cross resistance to methicillin, suggesting a highly specific alteration of PBP 3. None of the laboratory mutants with low-level resistance listed in Table 1 had changes in susceptibility to benzylpenicillin that were comparable to the degree of resistance of these strains to the antibiotics used in the selection process.

The highly resistant laboratory isolates (MIC increase of 50-fold or more) were found to be unstable during cultivation in the absence of the antibiotic, and observation of such cultures by phase-contrast microscopy revealed the frequent presence of aberrant cell morphology (swollen cells, incomplete septa). Another property of the laboratory isolates, particularly noticeable in the highly resistant mutants, was the presence of a prolonged lag in the resumption of exponential growth when stationary-phase cultures were diluted

TABLE 2. Dependence of antibiotic MICs on pH value of culture medium[a]

Strain	MIC (μg/ml)					
	Methicillin		Cephalexin		Cefotaxime	
	pH 7	pH 5.2	pH 7	pH 5.2	pH 7	pH 5.2
27s	0.8	0.8	4.0	4.0	1.0	1.0
M100	125	4.0	60	2.0	4.0	0.5
CTX50	15.0	0.5	>500	0.2	>500	8.0
27r	1,000	1.5	>250	4–6	>250	1–2

[a] MICs were determined in liquid culture in TSB adjusted to the appropriate pH values by the addition of alkali (NaOH) or acid (HCl), as described in Materials and Methods.

into fresh growth medium. Population analyses indicated that all the mutants were homogeneously resistant (16). Incubation of resistant bacteria at 30°C on agar plates containing methicillin increased the MIC by a factor of about 2 over that observed at 37°C. A remarkable property of all the highly resistant laboratory isolates was the pH dependence of the MIC: incubation in growth medium adjusted to pH 5.2 (instead of pH 7.0) resulted in the virtually complete loss of resistance (Table 2). This property is reminiscent of the behavior of clinical methicillin-resistant *S. aureus* isolates (15, 16).

PBPs of resistant laboratory mutants. In several of the mutants tested, selective resistance to certain antibiotics was paralleled by selective changes in the binding capacity of PBPs. For instance, Fig. 1 shows the binding capacities of the PBPs of mutant CTX50 for benzylpenicillin relative to those of the parental susceptible strain. Saturation of PBPs 1 and 3 by benzylpenicillin was complete at a concentration of 0.005 μg of penicillin per ml in susceptible strain 27s, and somewhat higher concentrations (0.1 and 0.25 μg/ml) were required to saturate the corresponding PBPs in mutant CTX50. There was no apparent change in the binding capacity for penicillin in PBP 2 of the mutant. In contrast to these relatively minor changes in penicillin binding capacity, both PBPs 2 and 3 of mutant CTX50 showed a massive decrease in binding capacity for cefotaxime, as indicated by the results of the competition assay shown in Fig. 2. Whereas PBP 2 of susceptible strain 27s was completely bound by as little as 0.1 μg of cefotaxime per ml and PBP 3 was bound by 5 μg of cefotaxime per ml, in CTX50 even 100 μg of cefotaxime per ml showed no apparent binding to either PBP 2 or 3. PBPs 2 and 3 may have greatly decreased affinity for cefotaxime; alternatively, the cefotaxime complexes of these PBPs may have greatly increased rates of deacylation.

The [^{3}H]penicillin binding patterns of three methicillin-resistant mutants with different levels of resistance are shown in Fig. 3 and Fig. 4. Mutant 5, with low-level methicillin resistance, appears to have a modest decrease in binding capacity (affinity) of PBP 2 for benzylpenicillin (saturation at 0.5 μg/ml in M5 instead of 0.1 μg/ml in the parent strain). A similar PBP profile was seen in the transformant M100/t (MIC, 20 μg/ml for M5 and M100/t; results not shown). However, a striking additional change in penicillin binding appeared in the more resistant mutants M50 and M100 (MIC, 100 and 150 μg/ml, respectively). In M50, no PBP 3 was detectable even upon exposure to as much as 10 μg of [^{3}H]penicillin per ml (i.e., almost 100 × the concentration needed to saturate PBP 3 of the parent strain; Fig. 3). In M100, neither PBP 3 nor PBP 2 was detectable at 10 μg of [^{3}H]penicillin per ml. Presumably, the loss of detectable PBPs involved a drastic decrease in antibiotic affinity. Upon exposure of M100 membranes to still higher concentrations (25 μg/ml and above), a faint band was detected with a mobility between those of PBPs 2 and 3 of the susceptible bacteria (Fig. 4). This relative mobility, more clearly seen in Fig. 5, corresponds to about 78 kDa, which is the molecular size of the unique PBP 2a demonstrated in many methicillin-resistant clinical isolates and which ap-

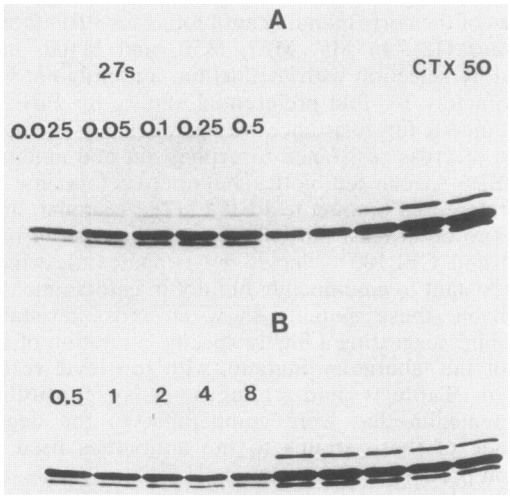

FIG. 1. Binding capacity of PBPs of a cefotaxime-resistant mutant (CTX50) and its parent strain (27s) for benzylpenicillin. Membrane preparations were exposed to [^{3}H]penicillin at the low (A) and high (B) concentrations (in micrograms per milliliter) indicated by the numbers over the lanes of the fluorograms of the strain 27s PBPs. The same concentrations (from left to right) were used with the membrane preparation from CTX50. Incubation and conditions for the penicillin binding assay were as described in Materials and Methods.

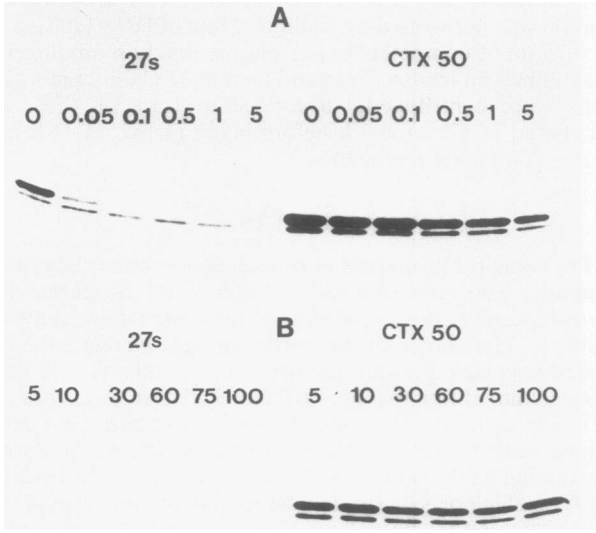

FIG. 2. Binding capacity of PBPs of a cefotaxime-resistant mutant (CTX50) and its parent strain (27s) for cefotaxime. Membrane preparations were exposed to cefotaxime at the concentrations (in micrograms per milliliter) indicated by the numbers. After 10 min of preincubation with this drug, each membrane preparation received an identical concentration (2 μg/ml) of [^{3}H]penicillin and was further incubated for 10 min, followed by analysis of PBPs, as described in Materials and Methods.

pears to be a biochemical correlate of beta-lactam antibiotic resistance in these bacteria (3, 9, 12, 15, 17, 25, 29).

Partial proteolysis of the [^{3}H]penicillin-labeled PBPs of susceptible and resistant staphylococci. Membrane preparations from susceptible (27s) and laboratory mutant (M50, M100) strains, as well as clinical strain 27r, were labeled with [^{3}H]penicillin and then analyzed by two-dimensional electrophoresis after partial proteolytic digestion with the staphylococcal protease V8. The results are shown in Fig. 6. As seen in lanes A, B, and C, PBP 2 of both the susceptible strain and strain M50, with low-level resistance, generated a peptide pattern virtually identical to that of PBP 2a of strain M100. This suggested a high degree of relatedness between PBP 2 and 2a. The peptide pattern of PBP 2 of 27s and PBP 2a of isogenic clinical resistant strain 27r again shared the same eight peptides, but two important differences were noted (lanes D and G versus E and F). First, the relative amount of the peptides was dramatically altered (e.g., cf. peptide 8 in lanes D and E). Secondly, four new peptides were detectable in the resistant strain (open arrowheads, lane E).

DISCUSSION

The isolation of penicillin-resistant *S. aureus* mutants in the laboratory by exposure of susceptible bacteria to gradually increasing concentrations of the antibiotic was first described by Demerec in 1945 (7). However, the mechanism of these mutations was not determined. We have now shown that resistant staphylococcal mutants isolated in this manner contain altered PBPs. Several features of these resistant

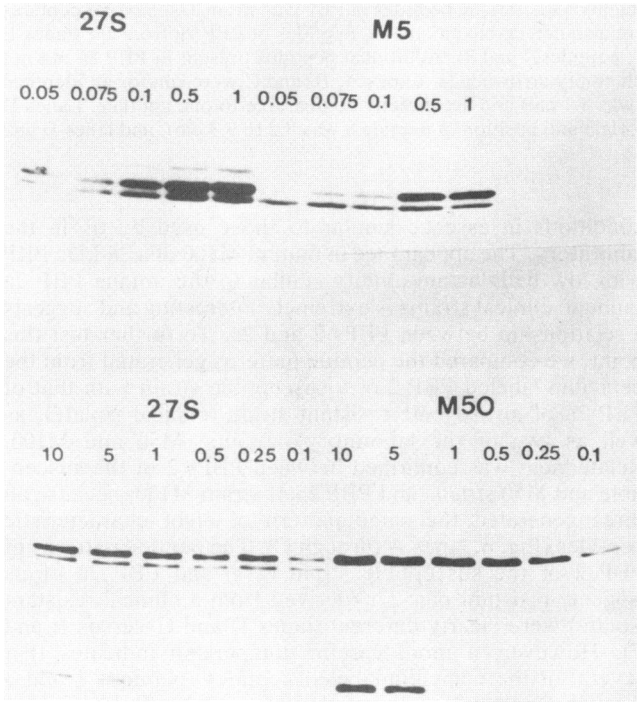

FIG. 3. Penicillin binding capacities of PBPs of mutants with low- (M5) and intermediate (M50)-level methicillin resistance. Membrane preparations from the parent (27s) and mutant (M5 and M50) strains were exposed to different concentrations (in micrograms per milliliter) of [^{3}H]penicillin under the standard conditions of the PBP assay. In the upper panel (27s and M5), about 280 μg of protein was applied to the gels, whereas only about 100 μg of protein was used in the gels in the lower panel.

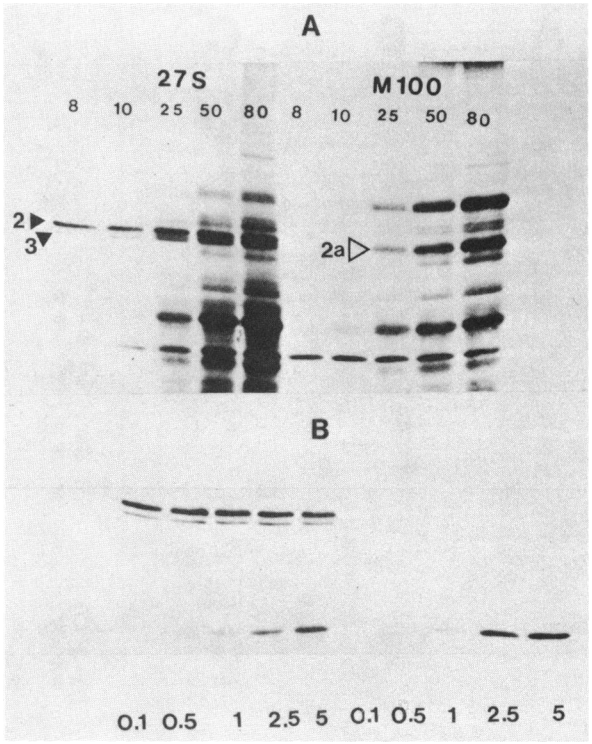

FIG. 4. Penicillin binding capacity of highly methicillin-resistant mutant M100. Membrane preparations were exposed to high (A) and low (B) [^{3}H]penicillin concentrations (in micrograms per milliliter), as indicated by the numbers. Only PBP 4 could be detected in M100 at up to 10 μg of [^{3}H]penicillin per ml. At concentrations of 25 μg/ml and above, an additional PBP (PBP 2a) could be visualized (open arrowhead). PBPs 2 and 3 of the susceptible strain are indicated by the solid arrowheads. The high concentrations of [^{3}H]penicillin needed for the detection of PBP 2a resulted in the appearance of a considerable number of extra bands in both the susceptible and mutant membrane preparations, presumably representing nonspecific binding.

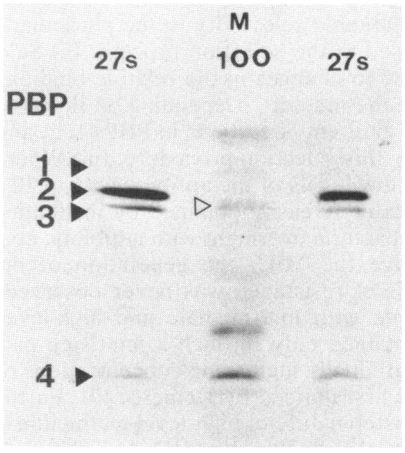

FIG. 5. PBPs of strains 27s and M100. Membrane preparation from methicillin-susceptible strain 27s were treated with 2 μg of [^{3}H]penicillin per ml, and membranes from strain M100 were treated with 25 μg of [^{3}H]penicillin per ml, under the standard conditions of the PBP assay. Preparations were run in alternate lanes of the same gel with empty lanes in between to minimize cross diffusion of material and to allow a better definition of the molecular size of PBP 2a (open arrowhead) of strain M100 relative to PBPs 1, 2, and 3 of strain 27s.

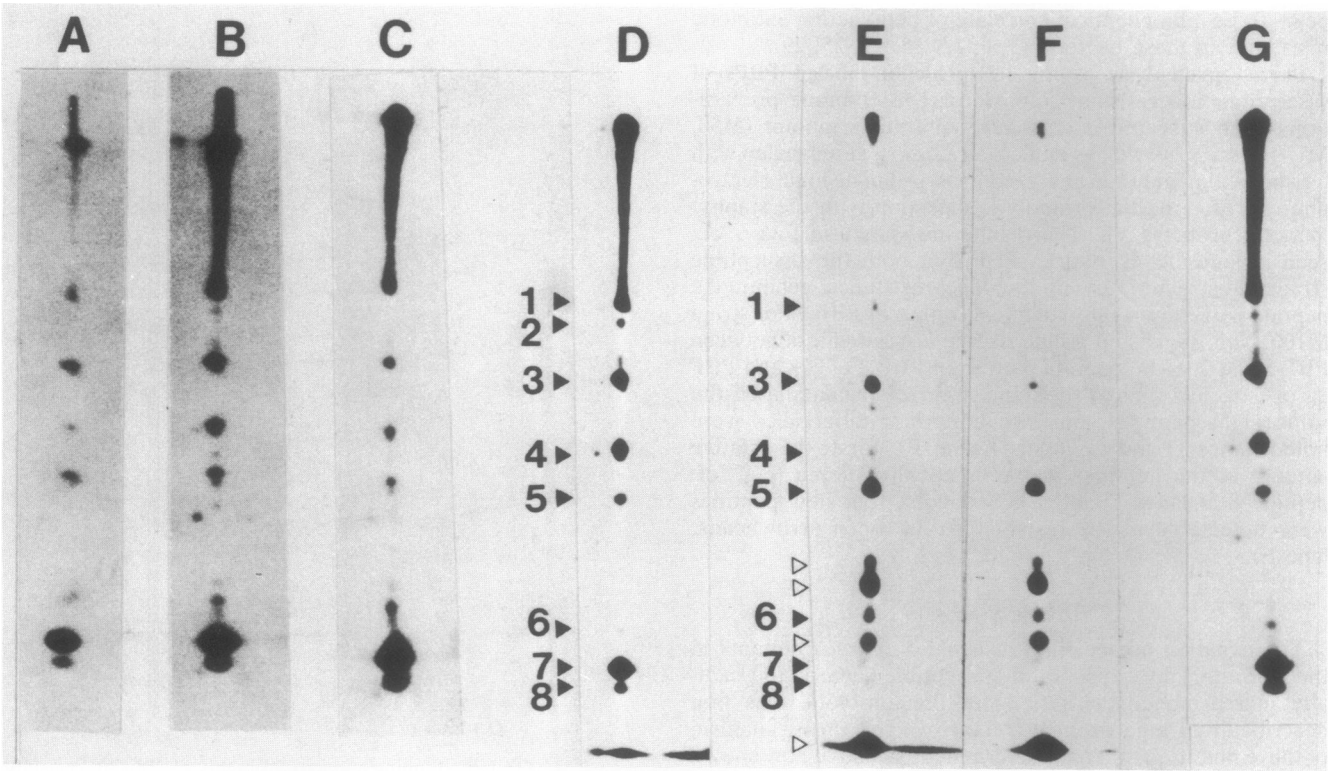

FIG. 6. [^{3}H]penicillin-labeled peptides generated from the PBP 2 and 2a components of susceptible and resistant strains by partial proteolysis. The pattern of radioactive penicillin-labeled peptides from the PBP 2 components of 27s (A) and M50 (B) and PBP 2a of M100 (C) are shown. The peptide patterns of PBP 2 of strain 27s (D and G) and PBP 2a of 27r (E and F) are aligned to allow comparison of radioactive peptides with similar mobilities. Solid arrowheads indicate the eight characteristic peptides of PBP 2 of strain 27s. Several peptides corresponding in mobility to those of peptides 3, 4, 5, 6, 7, and 8 of 27s are also detectable among the peptides of PBP 2a from 27r (lanes E and F), although with different relative amounts of radioactive label (e.g., peptides 7 and 8). Additional peptides present in PBP 2a but not apparent among the peptides generated from PBP 2 of 27s are labeled with empty arrowheads. Lanes A, B, and C were run for an identical length of time (the distance between the origin and position of peptide 8 was 8.1 cm) and are directly comparable to one another. Lanes D through G were run for somewhat longer times (the distance between the origin and position of peptide 8 was 9.2 to 9.3 cm), and lanes D and E were run on the same dimension 2 gel system.

mutants require comment. (i) The resistance mutations showed remarkable selectivity to the particular beta-lactam antibiotic used in the selection process. (ii) Selectivity was also reflected in changes in the relative binding capacity of the PBPs in the mutants. Depending on the antibiotic used, a variety of different alterations in PBPs 1, 2, and 3 could be produced by this selection procedure. (iii) Whereas mutants resistant to low levels of the antibiotics (5 to 10 × the MIC) could be relatively easily obtained by incubating 10^7 to 10^8 susceptible bacteria overnight with antibiotic concentrations slightly above the MIC, the generation of mutants with higher levels of resistance was never observed in a single step. Mutants with intermediate and high-level resistance could be obtained only through a multistep passage of the bacteria in gradually increasing concentrations of the antibiotic, as was first noticed by Demerec (7). Further evidence for the multistep nature of high-level methicillin resistance is provided by the finding that DNA isolated from highly methicillin-resistant strain M100 could transform only a low level of antibiotic resistance in a single round of transformation (M100/t; Table 1). This is reminiscent of the findings obtained in the genetic transformation of penicillin resistance in pneumococci (31).

The ultimate origin of methicillin resistance in *S. aureus* clinical specimens is most likely related to antibiotic selection in the natural environment of these bacteria, i.e., under conditions in essence similar to those used by us in the laboratory. The appearance in mutant M100 of a 78-kDa PBP with low beta-lactam affinity similar to the unique PBP 2a found in clinical strains is extremely interesting and suggests a relationship between PBPs 2 and 2a. To further test this point, we compared the peptide patterns generated from the penicillin-labeled PBP 2 of a susceptible strain with that of PBP 2a of an isogenic resistant strain (clinical isolate), as well as two of the laboratory mutants, M50 and M100. Relatedness was confirmed between PBPs 2 of the susceptible and M50 strains and PBP 2a of strain M100, because all three generated the same pattern of eight characteristic peptides (Fig. 6, lanes A through C). The peptide patterns of PBP 2 of the susceptible strain (27s) and PBP 2a of its isogenic resistant pair 27r (derived from a clinical resistant isolate) were clearly different (lanes D and G versus E and F). However, a more careful comparison indicates that several of the penicillin-labeled peptides (peptides 1 and 3 through 8) characteristic of the susceptible strain are also present among the peptides generated from PBP 2a of strain 27r, although in very different relative amounts. In addition, partial proteolysis of PBP 2a of strain 27r generated at least four lower-molecular-weight peptides not present in PBP 2 of strain 27s. The most abundant of these four peptides ran faster than peptide 8 of strain 27s. These findings suggest that PBP 2a of clinical methicillin-resistant *S. aureus* may

have originated from PBP 2 of susceptible bacteria, presumably by the introduction of a series of point mutations which resulted in a gradual decrease in affinity for beta-lactam antibiotics. Clearly, some of the amino acid replacements resulted in the generation of new cutting sites for the V8 protease (glutamic acid residues) within the mutationally altered PBP 2 near the beta-lactam binding site of the protein. The relatively faster migration of PBP 2a (apparent size, 78 kDa) compared with that of PBP 2 (80 kDa) could result from changes in the composition of charged amino acid residues affecting binding to SDS during electrophoresis. Alternatively, one of the amino acid substitutions could have resulted in the generation of a new proteolytic cutting site within PBP 2 which is subsequently processed to the slightly smaller PBP 2a. This type of mechanism has been postulated to explain the relationship between PBPs 1a and 1c in penicillin-resistant pneumococci (27). Interestingly, PBP 2a of resistant laboratory mutant M100 still has the same peptide pattern as the susceptible strain and does not produce the four extra peptides characteristic of PBP 2a of the clinical resistant strain. Because the methicillin resistance level of strain M100 (MIC, 150 μg/ml) is still far below that of strain 27r (MIC, >1 mg/ml), alteration in molecular size from 80 to 78 kDa may be associated with intermediate-level resistance, whereas extensive protein alterations near the penicillin binding site may occur only during the acquisition of extremely high resistance levels.

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