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Characterization of Mechanisms of Resistance to β-Lactam Antibiotics in Methicillin-Resistant Strains of *Staphylococcus saprophyticus*

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The resistance mechanisms of methicillin-resistant strains of *Staphylococcus saprophyticus* were characterized. Penicillin-binding protein (PBP) studies demonstrated an inducible PBP identical to PBP 2a in the membranes of these isolates. The amount of β-lactamase produced was minimal. PBP 2a is responsible for the methicillin resistance observed in these strains of *S. saprophyticus*.

Staphylococcus saprophyticus causes urinary tract infections (11–13, 18, 22, 31), including pyelonephritis and sepsis (8, 9, 20, 26). *S. saprophyticus* is susceptible to most antibiotics (6, 21, 24, 29). Methicillin resistance (27) and β-lactamase production (19) have been described in *S. saprophyticus*, although the mechanisms of resistance have not been investigated; these mechanisms were characterized in this study.

Methicillin-resistant *S. saprophyticus* was isolated from three patients with urinary tract infections. Identification was done by standard methods (1, 16). *Staphylococcus aureus* strains included strain 209P (β-lactamase negative, methicillin susceptible), strain 67-0 (β-lactamase positive, heterogeneously methicillin resistant), and strain 27R (β-lactamase negative, homogeneously methicillin resistant) (4, 10).

Methicillin (Bristol-Myers) was used to induce β-lactamase. Nafcillin and clavulanic acid (SmithKline Beecham) were used to saturate penicillin-binding proteins (PBPs) and to inactivate β-lactamase, respectively. Nitrocefin (BBL Microbiology Systems, Cockeysville, Md.), cephaloridine (Sigma Chemical Co., St. Louis, Mo.) and cefazolin (Eli Lilly & Co., Indianapolis, Ind.) were used for kinetic determinations. Penicillin G (Sigma), methicillin (Bristol-Myers), nafcillin, oxacillin (SmithKline Beecham), cephalothin, and cefazolin (Eli Lilly) were used for the determination of MICs by macrodilution methodology (23).

Mueller-Hinton broth (BBL) was used for susceptibility testing. Trypticase soy broth (TSB; BBL) was used for membrane preparations. Modified 1% CY broth and CY agars for β-lactamase typing were prepared as previously described (14).

Exponentially growing cells in TSB with 4% NaCl were harvested, washed, and then mechanically disrupted (3, 4). Membranes were separated by centrifugation and suspended to a final protein concentration of 10 mg/ml as in the Bio-Rad assay (2). To label PBP 2a, samples were preincubated with 10 μg of nafcillin per ml plus 100 μg of clavulanic acid per ml for 15 min at 37°C. [^{3}H]penicillin (20 μg/ml) was then added for 15 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17) and stained by Coomassie blue. Radiolabeled PBP 2a was detected by fluorography (3).

Peptide maps were prepared as follows (5, 28). [^{3}H] penicillin-labeled PBP 2a in membrane samples were subjected to SDS-PAGE. PBP 2a was identified, sliced from the gel, and digested with staphylococcal V8 protease (ICN Immunobiologicals, Lisle, Ill.). Radiolabeled peptide fragments were detected by fluorography.

Polyclonal antibody to PBP 2a was raised in rabbits as follows. Membrane protein (strain 67-0) was applied to an affinity column to extract PBPs 1 through 3 (32). The element containing PBP 2a was subjected to SDS-PAGE; PBP 2a was cut from the gel, homogenized in saline, and injected subcutaneously at biweekly intervals. Antibody was purified from immune serum by protein A affinity chromatography.

Western blotting (immunoblot) was done by standard methods (25) with modifications. Membranes from SS1 and SS3 were radiolabeled and subjected to SDS-PAGE. Membrane proteins and prestained protein standards were transferred to nitrocellulose paper. This was blocked overnight at 4°C with 5% casein in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween [pH 8.0]) and then for 4 h at 37°C with 10% human serum in TBST. Either preimmune rabbit antibody or anti-PBP 2a rabbit antibody diluted 1:1,000 with TBST was added for 10 min at room temperature. The paper was washed in TBST and then reacted with goat anti-rabbit immunoglobulin G alkaline-phosphatase conjugate (ProtoBlot; Promega Biotec, Madison, Wis.) diluted 1:7,500 in TBST. The nitrocellulose paper was washed and then reacted with 100 mM Tris hydrochloride (pH 9.5), 100 mM NaCl, 5 nN $MgCl_2$ plus Nitro Blue Tetrazolium plus 5-bromo-4-chloro-3-3-indolyl phosphate. After 10 min, the reaction was stopped with 20 mM Tris and 5 mM EDTA (pH 8.0). Specificity of antibody binding was confirmed by fluorography and use of preimmune serum controls.

Whole-cell suspensions of each isolate were prepared for β-lactamase typing and assays as previously described (14). The whole-cell preparations were used to hydrolyze 100 μM solutions of nitrocefin, cephaloridine, and cefazolin; hydrolysis was monitored at 37°C by using a DU-70 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Relative rates of hydrolysis were calculated by comparing the rates of degradation of two different cephalosporins as a ratio (14, 15).

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TABLE 1. MICs for three methicillin-resistant strains of *S. saprophyticus*[a]

Antibiotic	MIC (μg/ml) for strain:		
	SS1	SS2	SS3
Penicillin G	8	16	8
Methicillin	128	128	128
Nafcillin	32	16	16
Oxacillin	128	128	128
Cefazolin	64	128	128

[a] MICs were determined in Mueller-Hinton broth by using macrodilution techniques and an inoculum of 5×10^5 CFU/ml.

For quantitative assays of β-lactamase activity, each hydrolysis rate was divided by the measured A_{272} of the corresponding whole-cell suspension and reported in units of micromoles of antibiotic degraded per minute per standard cell mass. Induction ratios were determined as previously described (14).

For all three isolates of *S. saprophyticus*, methicillin MICs were ≧16 μg/ml. MICs of penicillin, methicillin, nafcillin, cephalothin, and cefazolin are summarized in Table 1.

A single PBP, PBP 2a, was radiolabeled when membranes prepared from *S. aureus* 27R were preincubated with nafcillin and clavulanic acid (Fig. 1). An inducible PBP with the same molecular weight as PBP 2a was present in membranes of *S. saprophyticus* (SS1 and SS3; SS2 not tested) when these strains were grown in medium containing methicillin. No PBP was detected when these strains were grown in broth without methicillin.

Peptide maps after limited digestion demonstrated that the digestion patterns associated with the *S. saprophyticus* strains were identical to that observed with *S. aureus* 27R (Fig. 2).

A Western blot of membrane proteins from strains SS2, SS3, and 27R combined with anti-PBP 2a antibody demonstrated binding to a 78-kilodalton membrane protein in each of these methicillin-resistant strains. In contrast, no binding occurred with membrane proteins from a methicillin-susceptible isolate (*S. aureus* 209P). Antibody from preimmune serum did not bind to protein of any strain.

Two *S. saprophyticus* isolates (SS1 and SS2) exhibited β-lactamase activity below the lower limit of sensitivity of the spectrophotometer. With extended incubation in broth, however, both strains were able to cause some degradation of nitrocefin. The third isolate (SS3) degraded nitrocefin at a rate of 0.56 and cephaloridine at a rate of 0.32 nmol per min per standard cell mass. The induction ratio was low, being only 3. The nitrocefin/cephaloridine ratio of 1.75 was similar to that of *S. aureus* strains making type B or C β-lactamases (14, 15).

We characterized resistance mechanisms of methicillin-resistant *S. saprophyticus* and found minimal β-lactamase activity whereas PBP 2a was detected. This altered PBP is responsible for methicillin resistance. PBP 2a in these strains appears to be the same as PBP 2a in other methicillin-resistant staphylococci (3, 4, 7, 10, 30, 32).

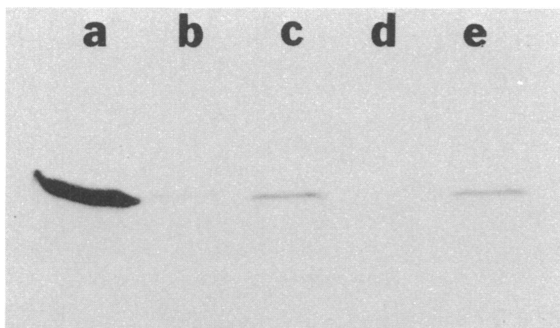

FIG. 1. Fluorograph of [^{3}H]penicillin-labeled PBP 2a in membrane preparations. Lanes: a, membrane protein from the methicillin-resistant strain of *S. aureus*, 27R; b and c, membranes prepared from the *S. saphrophyticus* SS3, grown in broth and in broth plus 10 μg of methicillin per ml for 90 min, respectively; d and e, membranes prepared from *S. saprophyticus* SS1, grown in broth and in broth plus 10 μg of methicillin per ml, respectively.

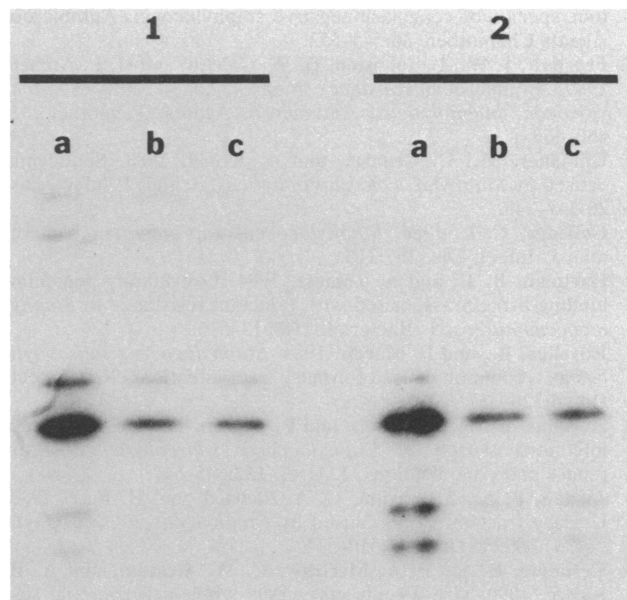

FIG. 2. Peptide maps of [^{3}H]penicillin-labeled PBP 2a digested with staphylococcal V8 protease. Set 1 shows digestion by 0.1 μg of V8 protease; set 2 shows digestion by 0.5 μg of V8 protease. For each set, the methicillin-resistant strain of *S. aureus*, 27R (lane a), *S. saprophyticus* SS3 (lane b), and *S. saprophyticus* SS1 (lane c) were used.

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