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Point Mutations in *Staphylococcus aureus* PBP 2 Gene Affect Penicillin-Binding Kinetics and Are Associated with Resistance

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In *Staphylococcus aureus*, penicillin-binding protein 2 (PBP 2) has been implicated in non-PBP 2a-mediated methicillin resistance. The PBP 2 gene (*pbpB*) was cloned from an expression library of a methicillin-susceptible strain of *S. aureus* (209P), and its entire sequence was compared with that of the *pbpB* gene from strains BB255, BB255R, and CDC6. Point mutations that resulted in amino acid substitutions near the conserved penicillin-binding motifs were detected in BB255R and CDC6, two low-level methicillin-resistant strains. Penicillin binding to PBP 2 in both BB255R and CDC6 is altered, and kinetic analysis indicated that altered binding of PBP 2 by penicillin was due to both lower binding affinity and more rapid release of bound drug. These structural and biochemical changes may contribute to the strains' resistance to β-lactam antibiotics.

Penicillin-binding proteins (PBPs) catalyze the cross-linking of the peptidoglycan subunits in the bacterial cell wall, and their transpeptidase activity is essential for the cell's structural integrity (31). With β-lactamases, PBPs form a superfamily of penicillin-interacting serine D,D-peptidases. These enzymes have a lysine residue downstream from the active site serine that is required for their catalytic activity (14). This S-X-X-K sequence is a conserved motif in the transpeptidase domain of this class of enzymes. S-X-N and K(H)-T(S)-G sequences downstream of the active site serine are also conserved and are instrumental to the penicillin-binding reaction. β-Lactam antibiotics are structural analogs of the PBP substrate, and their mechanism of action involves covalently binding the active site of the enzyme, thus preventing its function. The sensitivity of prokaryotes to these antibiotics is primarily due to their PBPs having a high affinity for the drugs.

Seven PBPs have been identified in *Escherichia coli*, and most have been sequenced. The low-M_r *E. coli* PBPs (no. 4, 5, and 6) appear to have carboxypeptidase, rather than transpeptidase, activity and to be nonessential for growth (29, 31). The high-M_r PBPs (1A, 1B, 2, and 3) are bifunctional enzymes with a penicillin-insensitive transglycosylase domain as well as a penicillin-sensitive transpeptidase domain (15–17). β-Lactams exert their lethal effects by inactivating one or more of these high-M_r PBPs.

The genes from *Streptococcus pneumoniae* PBP 1A (21), *Streptococcus oralis* PBP 1A (21), and *Bacillus subtilis* PBP F (11, 25) were recently cloned, and the peptides from their translated nucleotide sequences are homologous to the transpeptidase domains of PBPs 1A and 1B from *E. coli*. Unlike the bifunctional enzymes in *E. coli*, these high-M_r PBPs from gram-positive organisms do not appear to have transglycosylase activity.

Four PBPs (no. 1 to 4) with M_rs of 85, 81, 75, and 45, respectively, have been identified in susceptible strains of *Staphylococcus aureus* (8). In addition to PBPs no. 1 to 4, *mecA*-encoded PBP 2a is produced by methicillin-resistant staphylococci, and its production mediates most methicillin resistance in staphylococci. This protein has decreased binding affinity for β-lactam antibiotics and continues to function when the other PBPs are bound by drug (13). Recently, non-PBP 2a-producing methicillin-resistant strains of *S. aureus* have also been identified (3, 30). Modification of PBPs has been suggested as the mechanism that mediates resistance in these strains, but the structural basis of these modifications and how they affect penicillin binding are unknown.

To understand how structural modifications affect penicillin binding of altered PBPs, the PBP 2 gene from two resistant strains was amplified and the sequence was compared with that of a susceptible strain's cloned gene. The kinetics of penicillin binding to PBP 2 were determined and discussed in reference to the mutations obtained.

MATERIALS AND METHODS

Strains and susceptibilities. All *S. aureus* strains used in this study are *mecA* negative and do not produce β-lactamase. *S. aureus* 209P (ATCC 6538P), a well-characterized methicillin-susceptible laboratory strain, was used for the gel purification of PBP 2 and the molecular cloning of its gene. It has a penicillin MIC of 0.04 μg/ml. BB255R was derived from strain BB255 (NCTC 8325) by in vitro selection on methicillin (3). These strains have penicillin MICs of 8.0 and 0.04 μg/ml, respectively. Strain CDC6 is a clinical isolate (30) and has a penicillin MIC of 0.7 μg/ml. Both CDC6 and BB255R have modified binding affinity of PBP 2 for β-lactam antibiotics, which has been proposed to contribute to their enhanced resistance to β-lactam antibiotics.

Kinetic analysis of PBP 2. Binding kinetics of PBP 2 for 209P, BB255, BB255R, and CDC6 were calculated from relative densitometry values obtained from fluorographs of radiolabeled PBPs by the methods of Frère and Joris (7) and Ghuysen et al. (10). (For further explanation and extensive analyses, see reference 5.)

The model reaction between PBP and β-lactam antibiotic (penicillin [PEN]) obeys Michaelis-Menten kinetics and is described below:

$$\mathrm{PBP} + \mathrm{PEN} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \mathrm{PBP \cdot PEN} \xrightarrow{k_2} \mathrm{PBP - PEN} \xrightarrow{k_3} \mathrm{PBP} + \mathrm{PEN_i}$$

(1) (2) (3)

In reaction 1, the penicillin substrate reversibly binds to the enzyme's active site serine to form the Michaelis complex for which the dissociation constant $K = k_{-1}/k_1$. In the acylation reaction (reaction 2), penicillin becomes covalently bound to the active site serine. The rate of acyl-enzyme formation is described by

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the second-order rate constant k_2/K; the larger this value, the higher the affinity and the more rapid the rate of binding to the PBP. In the deacylation reaction (reaction 3), the acyl-enzyme intermediate is hydrolyzed, generating inactive drug (PEN_i) and regenerating active enzyme. This reaction is described by the first-order rate constant, k_3; the larger this value, the more rapidly the antibiotic is hydrolyzed and released. Because PBPs are relatively stable once they are acylated with penicillins and breakdown of the penicilloyl-PBP bond is rate limiting (i.e., $k_3 << k_2$), the concentration at which PBP 2 is 50% bound at steady state can be calculated by the Michaelis-Menten constant, $K_m = k_3 \div (k_2/K)$.

Detection and purification of PBPs. PBPs from *S. aureus* 209P membrane preparations were affinity purified with 6-amino-penicillanic acid (Sigma, St. Louis, Mo.) coupled to Sepharose 4B (Pharmacia LKB, Piscataway, N.J. [9, 33]). The PBPs eluted from the affinity column were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4, 18) in a mini-gel apparatus (Bio-Rad, Hercules, Calif.), and the affinity-purified PBP 2 was transferred to Immobilon-P membrane (Millipore Corp., Bedford, Mass.) for gas phase amino acid microsequence analysis. Affinity-purified PBP 2 was also used for the production of polyclonal antiserum in New Zealand White rabbits. Gel slices that contained PBP 2 were homogenized and used as immunogen.

DNA manipulations. DNA manipulations were performed according to standard procedures (26). DNA modifying and restriction enzymes obtained from New England Biolabs (Beverly, Mass.), Promega (Madison, Wis.), and Gibco-BRL (Baltimore, Md.) were used according to manufacturers' instructions. Hybridization probes were end labeled with [γ-^{32}P]ATP or radiolabeled with [α-^{32}P]ATP and -TTP (New England Nuclear, Boston, Mass.) by random priming (Multiprime DNA Labeling System; Amersham Corp., Northbrook, Ill.).

Library construction and screening. Chromosomal DNA from *S. aureus* 209P was used to construct a genomic expression library in λZapII (Stratagene, La Jolla, Calif.). The library packaging, amplification, and subsequent cloning experiments were performed in *E. coli* XL-1 Blue (Stratagene). Polyclonal antiserum directed against affinity-purified PBP 2 was used to screen the library, and PBP 2-expressing clones were detected on nitrocellulose membranes with an alkaline phosphatase detection system (Protoblot; Promega). Prior to screening of the library, the antiserum was preabsorbed with *E. coli* phage lysate (12).

Sequencing *pbpB* from *S. aureus* 209P. Plasmid DNA was purified by CsCl gradient centrifugation or with QIA-Prep Spin Plasmid Kit (Qiagen, Studio City, Calif.). Nucleotide sequencing was by the dideoxy chain termination method (27) with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) and [α-^{35}S]ATP (Amersham Corp.). The gene was sequenced on both strands with universal or empirically derived primers synthesized at the Biomedical Resource Center at UCSF.

Nucleotide and deduced amino acid sequences were analyzed with DNA Strider 1.1 (20) and the University of Wisconsin Genetics Computer Group (Madison, Wis.) software package (Program Manual for the GCG Package, Version 7 [1991]).

PCR and cycle sequencing of *pbpB* from other strains. The *pbpB* gene from strains BB255, BB255R, and CDC6 was PCR amplified from genomic DNA with primers empirically derived from the nucleotide sequence of *pbpB* in 209P. For each reaction, the template was denatured for 3 min at 96°C, and denaturation was followed by 30 amplification cycles (94°C, 50°C, and 72°C; 1 min each) with AmpliTaq (Perkin-Elmer Cetus, Norwalk, Conn.). The amplified fragments were purified with a spin column and directly sequenced with the AmpliTaq Cycle Sequencing Kit (Perkin-Elmer) and 5′-end-labeled primers ([γ-^{33}P]ATP; Amersham) that were derived from the cloned *pbpB* sequence. The sequenced genes were compared with the cloned gene, and whenever nucleotide discrepancies were detected, the complementary strand was also sequenced to confirm the change.

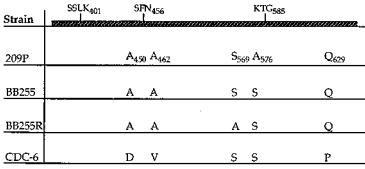
Nucleotide sequence accession number. The sequence reported here is available through GenBank (accession no. L25426).

RESULTS

Three clones that specifically reacted with the anti-PBP 2 antiserum were identified from the initial screening of the *S. aureus* expression library. The recombinant phagemids excised from their plaque-purified clones were sequenced with universal primers. An open reading frame that was similar to the 3′ end of the *ponA* gene from *S. pneumoniae* (21) was identified and used as a probe to clone the remaining upstream portion of the gene. Overlapping fragments of the gene were subcloned into pBluescript and sequenced on both strands.

DNA sequence analysis of the cloned region revealed an open reading frame of 2,181 nucleotides that was translated into a 727-amino-acid protein with a predicted M_r of 80,348. As predicted, consensus sequences S-F-N-456 and K-T-G-585 are located downstream from the S-S-L-K-401 motif. Residues no. 2 to 11 in the translated protein (T-E-N-K-G-S-S-Q-P-K) were identical to the amino acids obtained from the N-terminal microsequence analysis of SDS-PAGE gel-purified PBP 2. Contour-clamped homogeneous electric field analysis (32) indicated that the gene mapped to the *Sma*I A fragment of chromosomal DNA from 209P (data not shown).

A



B

Strain	Km	k2/K	k3 (t 1/2)
209P	0.01	14.2 ± 0.3	1.8 ± 0.2 (64)
BB255	0.02	5.9 ± 1.6	1.2 ± 0.2 (96)
BB255R	0.22	1.4 ± 0.2	3.3 ± 0.3 (33)
CDC-6	0.45	0.7 ± 0.1	3.2 ± 0.7 (36)

FIG. 1. Comparison of amino acid sequence (A) and penicillin-binding kinetics (B) of *S. aureus* PBP 2. (A) The three consensus penicillin-interacting motifs (S-S-L-K, S-F-N, and K-T-G) found in the penicillin-binding domain of *pbpB* and the location of amino acid differences between the strains are indicated. (B) At a concentration of K_m (μM), PBP 2 is 50% saturated with penicillin. k_2/K (10^3 M^{-1} s^{-1}) defines the acylation rate of PBP 2 by penicillin. k_3 (10^{-4} s^{-1}) defines the rate of deacylation, and the number in parentheses is the half-life (minutes) of the acylated PBP. (The kinetic data are summarized from reference 5.)

The nucleotide and translated amino acid sequences were almost identical to those obtained from the PBP 2 gene from methicillin-resistant strain 705, which was simultaneously cloned and whose sequence was recently reported (22). There was one amino acid difference, and *pbpB* from strain 209P encoded an additional 11 residues at the C terminus immediately upstream of the stop codon.

The cloning and sequencing of *pbpB* from strain 209P provided a means to identify and sequence the gene in resistant strains with a modified PBP 2. The PCR-amplified gene from CDC6 and BB255R was directly sequenced, and the nucleotide and translated amino acid sequences were compared with those for the cloned gene. Because BB255R was derived from BB255, the gene from the parent strain was also sequenced for comparative analysis.

S. aureus 209P and BB255 are both susceptible to β-lactam antibiotics, and their *pbpB*-encoded peptides differed by one amino acid (A-576→S, Fig. 1A); there were five additional third-position nucleotide differences that did not result in a residue change. This one amino acid difference for PBP 2 in BB255 was associated with a slightly slower rate of both acylation and deacylation, which together had a neutral effect on the K_m (0.01 versus 0.02 μM; Fig. 1B).

In BB255R, the resistant strain that was selected in vitro by growth on methicillin, *pbpB* had one nucleotide difference compared with its parent strain, BB255. This resulted in one amino acid difference (S-569→A), 14 residues upstream from the K-T-G penicillin-binding motif, and there was a decreased affinity (k_2/K = 1.4 versus 5.9) and more rapid deacylation of the bound drug (Fig. 1). In fact, the half-life of the acylated PBP in BB255R was 33 min compared with 96 min for PBP 2 in BB255. Associated with this one mutation there was also a 10-fold increase in the concentration of penicillin required to bind 50% of PBP 2 (0.02 versus 0.22).

PBP 2 in CDC6 differed from that in 209P at four residues (A-450→D; A-462→V; A-576→S; Q-629→P). S-576 was the same altered residue that affected both acylation and deacylation rates of PBP 2 in BB255 compared with 209P. Two of the other amino acid changes were near the S-F-N motif, and one was 25 residues downstream from the K-T-G sequence (Fig. 1). With these additional residue changes, PBP 2 in CDC6 had an even slower acylation rate and more rapid deacylation and

required a 40-fold increase in the concentration of penicillin for 50% binding of PBP 2.

We do not have kinetic analysis for PBP 2 in *S. aureus* 705, but the amino acid sequence of its penicillin-binding domain was identical to that obtained for BB255, and at the nucleotide level, there were four additional third-position differences which did not result in codon changes.

DISCUSSION

In this study, we report the sequence of the PBP 2 gene from a susceptible strain of *S. aureus*. The location of the conserved penicillin-binding domains in the translated peptide confirmed that it was a penicillin-reactive protein and the fact that residues 2 to 11 were identical to the amino acid sequence obtained from purified PBP 2 verified that the gene encoded that specific protein.

PBP 2a, the additional 76-kDa PBP unique to methicillin-resistant staphylococci, is not closely related to PBP 2. When the entire protein was analyzed, PBP 2a appeared to be more closely related to *B. subtilis* PBP 2B (52% similarity [6]) and *E. coli* PBP 3 (48% similarity [24]) than to *S. aureus* PBP 2 (43% similarity).

Low-affinity binding of β-lactam antibiotics by target PBPs is the proposed mechanism of penicillin resistance in PBP 2a-mediated resistance in staphylococci (13) as well as in pneumococci and gonococci (28). Laible and Hakenbeck demonstrated that cefotaxime-resistant laboratory mutants of pneumococci had a series of accumulated mutations near the conserved consensus motifs in the penicillin-binding domain of PBP 2x (19). These mutations were responsible for the PBP's reduced penicillin binding. Our kinetic analysis of penicillin binding by staphylococcal PBP 2 provided additional insight into how the mutations may affect the penicillin binding. For example, when one compared the PBP 2 gene for the two susceptible strains they differed by only one residue (A-576→S). This change had little or no effect on overall binding as measured by K_m; however, in BB255, affinity of PBP 2 was reduced approximately 2.5-fold. Since the half-life of the bound drug was also increased, this offset the effect of the lower binding affinity. The additional mutation detected in BB255R altered the amount of PBP that was bound (K_m increased 10-fold), because of a further decrease in affinity as well as an increased rate of deacylation. These are examples of how binding affinity and rate of deacylation of bound drug can both contribute to the overall net effect of bound drug. Intuitively, it makes sense that alterations to either of these activities are to the organism's advantage when under antibiotic pressure because their survival depends upon the function of these enzymes.

The net binding of PBP 2 to penicillin was similar for CDC6 and BB255R, yet the penicillin MICs for these strains differed appreciably (0.7 μg/ml versus 8 μg/ml, respectively). In both strains, PBP 2 was 90% bound at 0.7 μg/ml (5). This correlated well with the penicillin MIC for CDC6 but not with that for BB255R. However, PBP 4 also had altered binding in BB255R, and the penicillin MIC correlated with the concentration at which PBP 4 was >90% bound. Additional mutations are also required for the expression of PBP 2a-mediated methicillin resistance (1, 2, 23). Because there is no single target PBP, accumulated mutations in other proteins involved in cell wall synthesis may be required for phenotypic expression of resistance to penicillin.

This study provided direct evidence that selective pressure can result in point mutations of native PBP genes in staphylococci. Identifying mutations in PBPs is the first step in determining their effects on the enzymatic function and how resistance to this class of antibiotics develops in staphylococci.

Resistance due to altered binding of resident PBPs in *S. aureus* is difficult to assess, but the clinical relevance of non-PBP 2a-mediated methicillin resistance is of growing concern. Moreover, when β-lactam antibiotics that also bind PBP 2a are developed for clinical use, modification of resident PBPs may become the primary mechanism by which these organisms develop resistance. Because these alterations result from single point mutations, the differences can be biochemically analyzed to kinetically characterize the specific changes, and new drugs that take into account these changes can be modeled.

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