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Biochemical Characterization of Penicillin-Resistant and -Sensitive Penicillin-Binding Protein 2x Transpeptidase Activities of *Streptococcus pneumoniae* and Mechanistic Implications in Bacterial Resistance to β-Lactam Antibiotics

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To understand the biochemical basis of resistance of bacteria to β-lactam antibiotics, we purified a penicillin-resistant penicillin-binding protein 2x (R-PBP2x) and a penicillin-sensitive PBP2x (S-PBP2x) enzyme of *Streptococcus pneumoniae* and characterized their transpeptidase activities, using a thioester analog of stem peptides as a substrate. A comparison of the k_{cat}/K_m values for the two purified enzymes (3,400 M^{-1} s^{-1} for S-PBP2x and 11.2 M^{-1} s^{-1} for R-PBP2x) suggests that they are significantly different kinetically. Implications of this finding are discussed. We also found that the two purified enzymes did not possess a detectable level of β-lactam hydrolytic activity. Finally, we show that the expression levels of both PBP2x enzymes were similar during different growth phases.

Penicillin-binding proteins (PBPs), the target enzymes of β-lactam antibiotics, play an essential role in bacterial cell wall biosynthesis, cell division, and cell elongation (17, 18, 33, 46). PBPs are membrane-bound enzymes that are widespread in gram-negative and -positive bacteria (17, 18, 23, 29, 33, 44, 46). Two classes of PBPs were recognized mainly on the basis of their molecular sizes (17, 18). Low-molecular-mass PBPs exhibit DD-carboxypeptidase activity, and this class of PBPs appears to be important but not essential for cell growth (17, 18, 39, 41). High-molecular-mass PBPs constitute a class of essential transpeptidase and transglycosylase enzymes that catalyze the final steps of cell wall peptidoglycan cross-linking and elongation, respectively (17, 18, 33, 46). Transpeptidase and DD-carboxypeptidase activities of PBPs are inhibited by β-lactam antibiotics because of their structural similarity to the natural substrates (stem peptides) and ability to form a covalent penicilloyl complex via the active site serine residues (17, 18, 46). Formation of the penicilloyl-PBP enzyme complex leads to the functional inactivation of PBPs (17, 18, 46).

The emerging resistance of bacteria to penicillin is a frightening problem with a number of common pathogenic bacterial species (12, 35, 44, 45). *Streptococcus pneumoniae*, one of the major human pathogens of the upper respiratory tract, has developed resistance to many β-lactams, an important class of antibiotics (12–15, 19, 20, 30, 35, 44, 45). The intrinsic resistance of *S. pneumoniae* to β-lactam antibiotics has occurred by the development of altered high-molecular-mass PBPs that have reduced affinity for the antibiotics (12–15, 17–21, 30, 31, 44, 45). Five high-molecular-mass PBPs, 1a, 1b, 2x, 2a, and 2b, could be resolved in penicillin-resistant and -sensitive isolates of *S. pneumoniae* using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by their binding of radiolabeled penicillin (20, 21, 24, 28–31, 40, 43–45). Under laboratory conditions, however, only the *pbp1a*, *pbp2x*, and *pbp2b* genes were able to confer resistance to penicillin when they were transformed into penicillin-sensitive strains of *S. pneumoniae* (12–15, 28, 30, 31, 43–45). The *pbp2b* and *pbp2x* genes were shown to be the primary resistance determinants for different classes of β-lactam antibiotics (4, 10, 19). Furthermore, the penicillin-resistant *pbp1a*, *pbp2b*, and *pbp2x* genes of *S. pneumoniae* were shown to have evolved by interspecies recombinational events that replaced portions of the sensitive *pbp* genes with parts of the *pbp* genes of closely related streptococcal species (12–15, 30, 31, 44, 45).

Like other high-molecular-mass PBP enzymes, PBP2x appears to be multifunctional. The crystal structure of PBP2x revealed the presence of three structural domains, one of which corresponds to the transpeptidase domain (37). The function of the other two domains is not known (18, 37). However, unlike PBP1a and PBP1b, PBP2x does not appear to contain a transglycosylase domain (18, 28, 37). Nevertheless, the PBP2x enzyme of *S. pneumoniae* appears to be essential for cell growth (24).

The mechanisms of bacterial resistance to penicillin have been subjected to extensive molecular and genetic studies, yet little is known about the biochemical basis of resistance. A substantial amount of evidence shows that the cell wall composition of *S. pneumoniae* penicillin-resistant isolates differs drastically from that of penicillin-sensitive strains (16, 40, 42, 43). On the basis of this observation, it was proposed that an alteration in the substrate specificity of penicillin-resistant PBPs might have resulted in the heterogeneity of cell wall composition (16, 40, 42, 43). This hypothesis, however, has not been tested biochemically.

In this study, we report the purification of the penicillin-resistant PBP2x (R-PBP2x) and the penicillin-sensitive PBP2x (S-PBP2x) enzymes of *S. pneumoniae* 328 and R6 and the biochemical characterization of their transpeptidase activities, using a thioester analog of stem peptides as a probe substrate.

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The results of our kinetic studies show that the two purified PBP2x enzymes exhibited significantly different kinetic properties. These results are consistent with the proposed hypothesis that the penicillin-resistant and -sensitive PBP enzymes utilize different substrates in vivo. We also show that both purified enzymes did not possess a detectable level of β-lactam hydrolytic activity and that their cellular amounts were similar during different growth phases.

MATERIALS AND METHODS

Materials. *N*-benzoyl-D-alanylmercaptoacetic acid (S2d) (1, 23, 25), *N*-benzoyl-glycylmercaptoacetic acid (S2a) (2, 23, 25), C-benzoyl-D-Ala, and C-benzoyl-D-Ala-D-Ala were provided by Jean-Marie Frère (Université de Liege, Belgium). *p*-Nitrophenylacetate, Ala-D-isoglutaminyl-lys-D-Ala-D-Ala, Ala-D-γ-glu-lys-D-Ala-D-Ala, 4,4′-dithiodipyridine, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and molecular mass standards for gel filtration (cytochrome *c*, 12.4 kDa; carbonic anhydrase, 29 kDa; bovine serum albumin [BSA], 66 kDa; and alcohol dehydrogenase, 150 kDa) were purchased from Sigma Chemical Company (St. Louis, Mo.). Nitrocefin was obtained from Difco Laboratories (Detroit, Mich.), and clavulanic acid was purchased from Smith-Kline Beecham (Bala Cynwyd, Pa.). Macro-Prep ceramic hydroxyapatite type I (80 μm) was obtained from Bio-Rad Laboratories (Hercules, Calif.). Prestained molecular mass standards for SDS-PAGE (myosin, 250 kDa; BSA, 98 kDa; glutamic dehydrogenase, 64 kDa; alcohol dehydrogenase, 50 kDa; carbonic anhydrase, 36 kDa; myoglobin, 30 kDa; and lysozyme, 16 kDa) were obtained from Novex (San Diego, Calif.). LY303834 (a thioester compound), LY359210 (a β-lactam compound), and penicillin G were obtained from Eli Lilly and Company. Source-Q (15 and 30 μm), Source-S (15 μm), and chelating Sepharose fast flow resin were obtained from Pharmacia LKB Biotechnology (Alameda, Calif.).

Bacterial strains and culture conditions. The following strains of *Escherichia coli* and *S. pneumoniae* were used in this study: *E. coli* BL21(DE3) pLysS [F$^-$ *dcm ompT hsdS* (r_B^- m_B^-) *gal* λ (*DE3*) (pLysS Cam^r)] (Stratagene, La Jolla, Calif.); *E. coli* XL2-Blue {*rec1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q}*ZΔM15 Tn10* (*tet*^{r})] (Stratagene), *E. coli* BL21(DE3) pLysS(pRCX1) (R-PBP2x$^+$} Amp^r [this study]); *E. coli* BL21(DE3) pLysS(pRCX2) (his-R-PBP2x$^+$ Amp^r [this study]); *E. coli* BL21(DE3) pLysS(pRCX3) (S-PBP2x$^+$, Amp^r [this study]); *E. coli* BL21(DE3) pLysS(pRCX4) (his-S-PBP2x$^+$ Amp^r [this study]); *S. pneumoniae* R6 (MIC of penicillin = 0.006 μg/ml [22]), an unencapsulated derivative of the Rockefeller University strain R36A (3, 22) that was kindly provided by A. Tomasz (Rockefeller University); and *S. pneumoniae* 328 (MIC of penicillin = 4 μg/ml), a penicillin-resistant clinical isolate that was kindly provided by R. Moellering (New England Deaconess Hospital).

Luria-Bertani (LB) (5 g of NaCl per liter), C medium (25a) plus 0.1% yeast extract (CY), and Todd-Hewitt media were purchased from Difco Laboratories. Ampicillin (Amp; 100 μg/ml), chloramphenicol (Cam; 30 μg/ml), penicillin G (1 μg/ml), and isopropyl-1-thio-β-galactopyranoside (IPTG, 1 mM) were added to media as indicated in each experiment.

Cloning of genes encoding PBP2x from *S. pneumoniae* R6 and 328. Chromosomal DNA was prepared from *S. pneumoniae* R6 and 328 that were grown in CY medium at 37°C in the presence of 5% CO_2 and harvested at logarithmic phase. The cells were washed once with 50 mM sodium phosphate (pH 7.5), resuspended in a solution containing 1 mM EDTA, 3.3% deoxycholate, and 0.01% SDS, and incubated at 37°C until cell lysis. After incubation, 3 ml of 0.03 mM sodium citrate, pH 8.5, was added to the preparations, followed by proteinase K (80 μg/ml), and then the preparations were incubated at 37°C for 30 min. The resulting DNA preparations were phenol extracted twice, precipitated with ethanol, and resuspended in 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

The genes encoding PBP2x from the penicillin-sensitive laboratory strain, *S. pneumoniae* R6, and the penicillin-resistant clinical isolate, *S. pneumoniae* 328, designated S-PBP2x and R-PBP2x genes, respectively, were cloned by PCR amplification of their chromosomal DNA with the primers: 5′-CGGAATTCAT ATGAAGTGGACAAAAAGAGTAATCCG-3′ and 5′-GCTCTAGACCAGC ACTGATGGAAATAAACATA-3′. Vent (*exo*$^+$) DNA polymerase, a high-fidelity enzyme (New England Biolabs, Beverly, Mass.), was used following the manufacturer's instructions. An expected 2.1-kb DNA fragment was amplified under these conditions. The amplified DNA fragment was purified from an agarose gel by electroelution and cloned into pCR-Script SK(+) (Stratagene) in accordance with the manufacturer's instructions. The resulting ligation mixture was transformed into supercompetent cells of *E. coli* XL1-Blue (Stratagene). Plasmids were purified from several white transformants with a Wizard miniprep DNA purification kit (Promega, Madison, Wis.), digested with *Not*I and *Bam*HI, and shown to carry a 2.1-kb DNA fragment as expected. Sequencing analyses confirmed that the sequence of the S-PBP2x gene was identical to that published previously (28 and data not shown). For the R-PBP2x gene, two independent PCR amplification reactions generated fragments with an identical sequence, which was concluded to be the sequence of the R-PBP2x gene (see GenBank accession no. below). The sequence of this R-PBP2x gene is nearly identical to that of a previously published R-PBP2x gene cloned from *S. pneumoniae* 577 (30). The only difference in the transpeptidase domain is that the R-PBP2x gene cloned from *S. pneumoniae* 328 contains an Ala codon at 572, but the PBP2x gene sequenced from *S. pneumoniae* 577 has a Val codon instead (30). The mutations in the R-PBP2x gene from *S. pneumoniae* 328 are typical of those in other PBP2x genes from penicillin-resistant clinical isolates (18, 19, 30).

To create water-soluble forms of PBP2x enzymes, the hydrophobic transmembrane domain coding region of each PBP2x gene was removed by PCR amplification of its downstream transmembrane domain coding region with the primers 5′-CCATCGATCATATGGGGAGTGGTAGTAAATTTGG-3′ (upstream) and 5′-CGCGGATCCCATGGAGCTGAGCTAGTCCG-3′ (downstream) for R-PBP2x and 5′-CCATCGATCATATGGGGACAGGCACTCGCTTTGG-3′ (upstream) and 5′-CGCGGATCCCATGGAGCTGAGCTAGGCCG-3′ (downstream) for S-PBP2x. The upstream primers each contained an *Nde*I site that was used to create an ATG start site which was followed by codons for R-PBP2x and S-PBP2x beginning at 49 when the gene was cloned into an expression vector. The downstream primers each contained *Blp*I and *Bam*HI sites to facilitate the cloning process. A 470-bp DNA fragment was PCR amplified with these primers. The amplified DNA fragment was purified from a gel by electroelution and cloned into pCR-Script SK(+) as described above. The DNA fragment was released by digestion with *Cla*I and *Blp*I. This *Cla*I-*Blp*I DNA fragment was then ligated to the rest of each PBP2x gene that was carried on pCR-Script SK(+), which was digested with the same enzymes. Analysis of this 470-bp region confirmed its sequence identity with the original gene except for the lack of the 48 codons at its 5′ end including the coding region for the hydrophobic transmembrane domain.

A polylinker was created to facilitate the cloning of the gene into pET vectors that would generate an *Not*I and *Eco*RI overhang: 5′-GGCCGCGTCGACG GTACCG-3′ and 5′-CGCAGCTGCCATGGCTTAA-3′. The R-PBP2x and S-PBP2x genes lacking their hydrophobic transmembrane domain coding regions were cloned into the expression vectors pET5a and pET16b (Novagen, Madison, Wis.). The resulting constructs were designated pRCX1 (the R-PBP2x gene was cloned into pET5a), pRCX2 (the R-PBP2x gene was cloned into pET16b and the resulting gene was designated the his-R-PBP2x gene), pRCX3 (the S-PBP2x gene was cloned into pET5a), and pRCX4 (the S-PBP2x gene was cloned into pET16b and the resulting gene was designated the his-S-PBP2x gene). The PBP2x proteins expressed from the pRCX1 and pRCX3 constructs were designated R-PBP2x and S-PBP2x, respectively. The PBP2x proteins expressed from the pRCX2 and pRCX4 constructs that contained histidine tags at their N termini were designated his-R-PBP2x and his-S-PBP2x, respectively.

Enzyme assay, kinetics, and ^{125}I-labeled penicillin V binding assay. Transpeptidase activities of the R-PBP2x, S-PBP2x, his-R-PBP2x, and his-S-PBP2x enzymes were assayed by measuring their ability to hydrolyze S2d, S2a, and phenylmethoxyl-carbonyl-amino-acetyl-mercaptoacetic acid (LY303834) thioester analogs of cell wall stem peptides (1, 2, 23, 25). The thio-group release that was coupled to 4,4′-dithiodipyridine was measured by monitoring the increase in optical density at 325 nm (molar extinction coefficient = 19,800 M^{-1} cm^{-1}) as described before (23, 25, 29), with minor modifications. Reaction mixtures (1 ml each) contained 52 mM potassium phosphate (pH 7.0), 2.0 mM each of thioester substrate, 0.8 mM 4,4′-dithiodipyridine, and 2 to 4 μg of S-PBP2x and his-S-PBP2x or 75 to 180 μg of R-PBP2x and his-R-PBP2x. The increase in A_{325} was monitored at 37°C for 3 min with a double-beam BioSpec-1601 spectrophotometer (Shimadzu Instruments, Inc.). Protein concentrations were determined with a Bradford protein assay kit (Bio-Rad) with BSA as a standard (7).

The binding of penicillin to purified PBP2x enzymes was measured as described previously (38) with ^{125}I-labeled penicillin V (5). For the determination of binding constants of S-PBP2x and R-PBP2x enzymes for ^{125}I-labeled penicillin V and of 50% inhibitory concentrations of other antibiotics, 0.2 μg of each purified S-PBP2x and R-PBP2x enzyme was used. Binding intensities of individual protein bands were quantified with an Ultroscan XL Laser Densitometer (Pharmacia LKB Biotechnology) after exposure of the gels (12%) to X-ray films.

Purification of the soluble R-PBP2x and S-PBP2x enzymes of *S. pneumoniae* 328 and R6. *E. coli* BL21(DE3) pLysS(pRCX1) and (pRCX3) were grown in a 10-liter fermentor containing a minimal salt medium supplemented with Cam and Amp and induced with 1 mM IPTG (47). Approximately 30 g of cell pellet was used in each purification, and all steps of purification were carried out at 4°C. For the purification of the S-PBP2x enzyme, the cell pellet was suspended in 150 ml of 20 mM Tris-HCl (pH 8.0)—1 mM EDTA (buffer A) and disrupted by passage through a 20K French press cell (Aminco Laboratories, Inc.) twice at 20,000 lb/in^2. The resulting suspension was centrifuged at 180,000 × *g* for 60 min (Beckman Ti 70 rotor). The supernatant (crude extract) was collected and loaded onto a Source-Q (30-μm diameter) column (5 by 11 cm) that was previously equilibrated with buffer A. The column was washed with 600 ml of buffer A and eluted with a linear gradient of 0 to 1,000 mM KCl in buffer A. The presence of S-PBP2x in fractions was monitored by assaying its S2d hydrolysis activities and also followed by SDS-PAGE (26). The fractions containing S-PBP2x were pooled and dialyzed in dialysis tubing (50-kDa cutoff; Spectrum Medical Industries, Houston, Tex.) at 4°C overnight against 5 liters (no change) of 20 mM potassium phosphate (pH 7.0) and 1 mM EDTA (buffer B). The dialyzed enzyme preparation was applied to a Macro-Prep ceramic hydroxyapatite type I (80-μm diameter) column (2.6 by 12 cm) that was equilibrated with buffer B. The column was washed with 200 ml of buffer B and then eluted with a linear gradient of 20

to 700 mM potassium phosphate (pH 7.0) and 1 mM EDTA. The fractions containing S-PBP2x were pooled, dialyzed as described before in 20 mM potassium phosphate (pH 5.2) and 1 mM EDTA (buffer C), and then loaded onto a Source-S (15-μm diameter) column (1.6 by 13 cm) equilibrated with buffer C. The column was washed with 140 ml of buffer C, and the fractions containing S-PBP2x that was not retained by Source-S were pooled. The pooled fractions were dialyzed in buffer A as described before and stored in buffer A containing 15% (vol/vol) glycerol at −70°C in small aliquots.

R-PBP2x was purified as described for S-PBP2x except that the last step of purification was omitted.

Purification of the soluble his-R-PBP2x and his-S-PBP2x enzymes of *S. pneumoniae* 328 and R6. *E. coli* BL21(DE3) pLysS(pRCX2) and (pRCX4) were first grown overnight with vigorous shaking at 37°C in LB medium supplemented with Cam and Amp. The overnight cultures (2%) were inoculated into 2.5 liters of fresh LB medium containing Cam and Amp. Cultures were induced with 1 mM IPTG at an optical density at 600 nm of 0.5 to 0.6 for 3 h. Cells were harvested by centrifugation at 4,400 × *g* for 10 min, washed with 20 mM potassium phosphate (pH 7.5), and resuspended in 10 ml of 20 mM Tris-HCl (pH 7.9)–0.5 M NaCl–5 mM imidazole. The resulting suspension was disrupted by passage through a French press cell and centrifuged as described before. The supernatant collected was loaded onto a 4-ml chelating Sepharose column charged with 50 mM NiCl. The column was washed and eluted following the manufacturer's instructions (Novagen). The fractions containing his-PBP2x were pooled as judged by SDS-PAGE analysis of each fraction. The pooled fractions were dialyzed overnight in 5 liters of 20 mM Tris-HCl (pH 8.0–1 mM EDTA (buffer D) as described before and then loaded onto a Source-Q (15-μm diameter) column (1.6 by 3 cm) that was previously equilibrated with buffer D. The column was washed with 10 ml of buffer D and eluted with a linear gradient of 0 to 1,000 mM KCl in buffer D. The fractions containing his-PBP2x were pooled as determined by SDS-PAGE and their S2d hydrolysis activities. The pooled enzyme preparation was concentrated with a Centriprep 50 (50-kDa cutoff; Amicon, Inc., Beverly, Mass.) and diluted 2 to 3 times in buffer D. The enzyme preparation was adjusted to 15% (vol/vol) glycerol and stored at −70°C in small aliquots.

Biochemical characterization of purified PBP2x enzymes. The native molecular mass of each purified PBP2x enzyme was determined on a calibrated Superdex 200 HR10/30 column (Pharmacia LKB Biotechnology) that was equilibrated with 50 mM Tris-HCl (pH 7.5) and 100 mM KCl. The subunit molecular mass of each purified protein was determined by SDS-PAGE (26).

N-terminal sequences of purified PBP2x enzymes were determined with a Procise protein sequencer equipped with a Model 140C microgradient delivery system (Applied Biosystems, Inc.). The purified R-PBP2x and S-PBP2x enzymes were first dialyzed in 2 liters of water as described before and then subjected to N-terminal sequencing.

Temperature optima of purified PBP2x enzymes were determined in reaction mixtures (1 ml each) containing 52 mM potassium phosphate (pH 7.0), 2.0 mM S2d, 0.8 mM 4,4′-dithiodipyridine, and either 3.2 μg of S-PBP2x, 2.7 μg of his-S-PBP2x, 140 μg of R-PBP2x, or 55 μg of his-R-PBP2x at various temperatures for 3 min. pH optima of purified PBP2x enzymes were determined at various pH values in reaction mixtures (1 ml each) containing 52 mM potassium phosphate, 0.8 mM 4,4′-dithiodipyridine, 2.0 mM S2d, and either 3.2 μg of S-PBP2x, 175 μg of R-PBP2x, 2.7 μg of his-S-PBP2x, or 82.5 μg of his-R-PBP2x at 37°C for 3 min.

Electrospray ionization mass spectrometry was carried out in a PE-Sciex API III triple quadrupole mass spectrometer equipped with a pneumatically assisted electrospray source (Perkin-Elmer Sciex Instruments, Inc.). Protein samples (3.5 μg of R-PBP2x per μl and 2.0 μg of S-PBP2x per μl) were dialyzed in 2 liters of water as described before, and then 50 μl of each sample was mixed with or without penicillin G at a final concentration of 0.5 mM (for R-PBP2x) or 0.2 mM (for S-PBP2x) at 37°C for 15 min. The samples were diluted 1:1 (vol/vol) with 1% (vol/vol) acetic acid in acetonitrile and were then directly injected into the electrospray ionization source at 10 μl/min with a syringe pump. Analyses were performed in the positive ion detection mode, and data were acquired over a range of 1,000 to 2,000 u in 0.1-u intervals, with a dwell time of 1 ms per interval. A total of 20 scans were averaged for each sample.

Antibody preparation and quantitative Western blotting analysis of the S-PBP2x and R-PBP2x enzymes in *S. pneumoniae*. Polyclonal antibodies against SDS-denatured R-PBP2x were prepared by HRP, Inc. (Denver, Pa.). Purified R-PBP2x enzyme was denatured by SDS and subjected to SDS-PAGE (10% Tris-glycine gel from Bio-Rad). Protein bands were visualized by incubating the gel in a solution containing 0.5 M KCl and 50 mM potassium phosphate (pH 7.2) (48) and were cut out with a razor blade. Each protein band, which contained approximately 100 μg of R-PBP2x enzyme, was injected into two New Zealand White rabbits from which preimmune sera had been taken.

S. pneumoniae R6 and 328 were grown overnight in Todd-Hewitt broth medium at 37°C in the presence of 5% CO_2. The overnight cultures (2%) were inoculated into fresh Todd-Hewitt medium and grown to early logarithmic, middle logarithmic, and early stationary phases by monitoring cell densities at 600 nm. *S. pneumoniae* 328 was also grown to the three phases in Todd-Hewitt medium containing 1 μg of penicillin G per ml. The cultures were harvested at 8,000 × *g* for 10 min, washed with 20 mM potassium phosphate (pH 7.5), and resuspended in SDS-denaturing solution (26). These protein samples were heated at 100°C for 2 min and were then subjected to Western blotting that was carried out essentially as described previously (48). The protein concentrations were determined with a D_c protein assay kit from Bio-Rad.

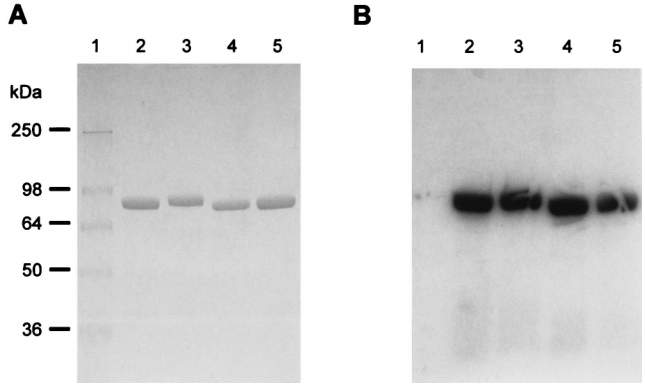

FIG. 1. SDS-PAGE of purified S-PBP2x, R-PBP2x, his-S-PBP2x, and his-R-PBP2x enzymes and their ability to bind ^{125}I-labeled penicillin V. PBP2x enzymes were purified as described in Materials and Methods. The purified proteins (2.3 μg each) were labeled with ^{125}I-labeled penicillin V and separated on a 10% gel. The gel was stained with Coomassie blue (A), dried, and exposed to an X-ray film (B). (A and B) Lane 1, prestained molecular mass standards; lane 2, his-S-PBP2x; lane 3, his-R-PBP2x; lane 4, S-PBP2x; and lane 5, R-PBP2x.

Nucleotide sequence accession number. The nucleotide sequence of the *S. pneumoniae* 328 R-PBP2x gene has been deposited in GenBank and assigned accession no. U87092.

RESULTS

Purification, identification, and electrospray mass spectrometric analysis of the R-PBP2x, S-PBP2x, his-R-PBP2x, and his-S-PBP2x enzymes. To compare the enzymatic properties of S-PBP2x and R-PBP2x enzymes, we created four expression constructs that produced soluble forms of PBP2x enzymes and purified both enzymes to apparent electrophoretic homogeneity (Fig. 1A). Approximately 100 mg each of S-PBP2x and R-PBP2x enzymes was purified from 30 g of cells (data not shown). The purified proteins had subunit molecular masses of 75 kDa as determined by SDS-PAGE, which are in reasonable agreement with those of 77,085 for R-PBP2x and 76,734 for S-PBP2x calculated from their amino acid sequences. Both purified proteins bound ^{125}I-labeled penicillin V (Fig. 1B). The sequence of 40 residues at the N terminus of each purified protein was determined and exactly matched the sequences predicted for the S-PBP2x and R-PBP2x enzymes except that the first methionine residue was absent from each protein. Together, these data show that the purified proteins were the S-PBP2x and R-PBP2x enzymes of *S. pneumoniae* R6 and 328, respectively.

Using the thioester substrate of S2d, an analog of stem peptides that has been utilized as a colorimetric substrate for several PBPs (1, 18, 23, 25, 29), the purified S-PBP2x enzyme showed more than a 100-fold-higher level of hydrolysis activity than did the purified R-PBP2x enzyme (Table 1). The hydrolysis activities of R-PBP2x and S-PBP2x were completely inhibited by penicillin G (Table 1). When tested against S2d as a substrate, the purified his-R-PBP2x and his-S-PBP2x enzymes (Fig. 1A) exhibited specific activities virtually identical to those of R-PBP2x and S-PBP2x, respectively (data not shown and Table 1). The two purified proteins were shown to bind ^{125}I-labeled penicillin V and had the expected molecular masses (Fig. 1).

To rule out the possibility that the purified R-PBP2x enzyme was inactive, we determined its penicillin-binding ability by electrospray mass spectrometry. In the absence of penicillin G,

TABLE 1. Enzymatic activities of the purified S-PBP2x and R-PBP2x enzymes of *S. pneumoniae* tested against different thioester analogs of stem peptides[a]

Substrate	Hydrolysis activity (nmol/min/mg)[b] for:	
	S-PBP2x	R-PBP2x
2 mM S2d	3608	20
2 mM S2d + 0.25 mM Pen G[c]	0.0	0.0
2 mM S2a	161	6.2
2 mM S2a + 0.25 mM Pen G	0.0	0.0
2 mM LY303834	110	2
2 mM LY303834 + 0.25 mM Pen G	0.0	0.0

[a] Assay conditions are described in Materials and Methods.
[b] D-Alanine release activity was not detected in reaction mixtures containing 2 mM C-benzoyl-D-Ala, 2 mM C-benzoyl-D-Ala-D-Ala, 1 mM Ala-D-isoglutaminyl-lys-D-Ala-D-Ala, and 2 mM Ala-D-γ-glu-lys-D-Ala-D-Ala, and *p*-nitrophenyl group release activity was not detected at 402 nm in reaction mixtures containing 2 mM *p*-nitrophenylacetate (data not shown).
[c] Pen G, penicillin G.

the purified R-PBP2x protein exhibited only one major peak and was shown to have a molecular mass of 76,961, which is in good agreement with the predicted value of 76,953 (excluding the first methionine residue) (Fig. 2A). In the presence of excess penicillin G, the purified R-PBP2x protein again exhibited only one major peak but had a molecular mass of 77,301 (Fig. 2B). Penicillin G was shown by mass spectrometry to have a molecular mass of 335 (data not shown). Therefore, the major peak detected in the presence of penicillin G must be the R-PBP2x-penicillin G covalently bound complex. These binding results clearly established that the purified R-PBP2x enzyme was active, and therefore the much lower hydrolysis activities observed were a result of its intrinsic property. Similar results were obtained with S-PBP2x in the presence or absence of penicillin G except that there was a minor peak (≈20% of the total) which exhibited a slightly higher molecular mass but also bound penicillin G (data not shown). This heterogeneous behavior of S-PBP2x revealed by mass spectrometry is consistent with previous observations (23, 29, and data not shown). S-PBP2x enzyme preparations derived from different peak fractions of the Mono-S column were shown to have similar kinetic properties (23).

Biochemical properties of the purified S-PBP2x, his-S-PBP2x, R-PBP2x, and his-R-PBP2x enzymes. All four purified enzymes hydrolyzed the thioester substrates, S2a and LY303834 (Table 1), but the purified R-PBP2x and his-R-PBP2x enzymes showed much lower activities than those of S-PBP2x and his-S-PBP2x (Table 1 and data not shown). All four purified enzymes, however, did not hydrolyze C-benzoyl-D-Ala, C-benzoyl-D-Ala-D-Ala, Ala-D-isoglutaminyl-lys-D-Ala-D-Ala, and Ala-D-γ-glu-lys-D-Ala-D-Ala (data not shown) when D-alanine release was monitored (47), a result that is consistent with previous findings for other PBPs (17, 18, 23).

The purified S-PBP2x, his-S-PBP2x, R-PBP2x, and his-R-PBP2x enzymes had native molecular masses of 86, 82, 86, and 82 kDa, respectively, as determined by gel filtration, and thus appeared to be monomeric enzymes. The S2d hydrolysis activities of all four purified PBP2x enzymes increased slowly, with temperature between 25 and 45°C at pH 7.0 (data not shown). The S2d hydrolysis activities of the four purified enzymes rose sharply between pH 5.0 and 5.5 and then dropped steadily between pH 5.5 and 8.0 (data not shown). Thus, the optimal pH for the PBP2x enzymes was 5.5.

The kinetic parameters of the purified PBP2x transpeptidases were determined by standard steady-state methods with S2d as a substrate. All assays were carried out at pH 7.0. The apparent K_m values obtained for the purified S-PBP2x, his-S-PBP2x, R-PBP2x, and his-R-PBP2x enzymes were 2.5, 2.3, 25, and 25 mM, respectively. The apparent V_{max} (k_{cat}) values obtained for the purified S-PBP2x, his-S-PBP2x, R-PBP2x, and his-R-PBP2x enzymes were 6.7 μmol/min/mg (8.5 s^{-1}), 6.5 μmol/min/mg (8.3 s^{-1}), 0.22 μmol/min/mg (0.28 s^{-1}), and 0.25 μmol/min/mg (0.32 s^{-1}), respectively. The kinetic properties determined for the purified S-PBP2x enzyme in this study are two- to threefold lower than those determined before (K_m = 5.6 mM and k_{cat} = 30 s^{-1}) (23). The apparent discrepancy is probably due to the fact that we monitored the hydrolysis reaction by measuring thiol-group release that was coupled to 4,4′-dithiodipyridine (see Materials and Methods and reference 25), while Jamin et al. directly measured the hydrolyzed product by monitoring UV absorption decrease (23). The measurement of the thiol-group release by coupling to 4,4′-dithiodipyridine is approximately 15-fold more sensitive than the direct measurement of the hydrolyzed product by monitoring the decrease in UV absorption (25).

When assays were done at pH 5.5, a severe substrate inhibition of the S-PBP2x and R-PBP2x hydrolysis activities was observed when the S2d concentrations used in the assay were

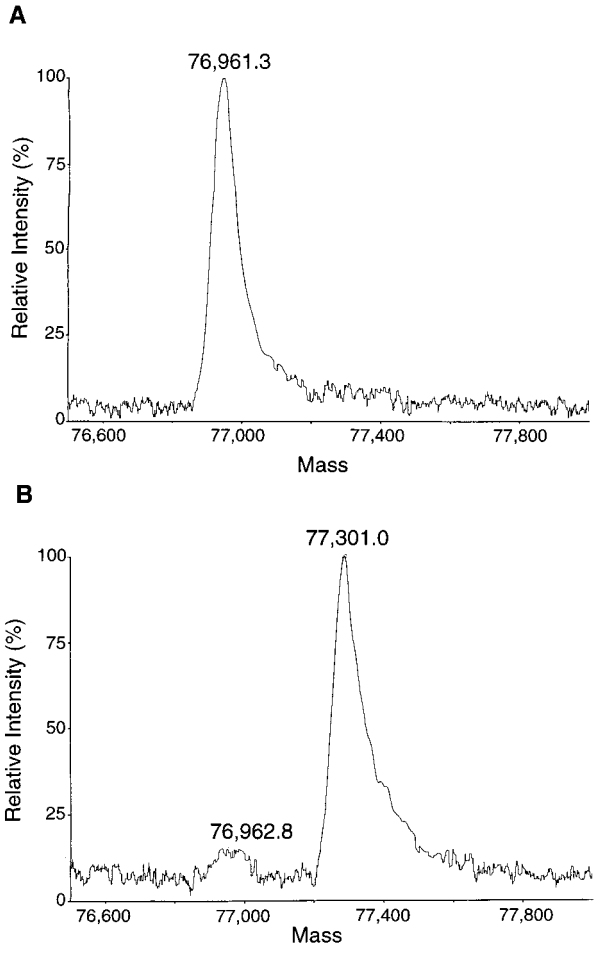

FIG. 2. Electrospray mass spectrometric analysis of purified R-PBP2x and S-PBP2x enzymes. The purified R-PBP2x and S-PBP2x enzymes were dialyzed in water, incubated with or without penicillin G at 37°C for 15 min, and injected into a mass spectrometer (see Materials and Methods). Panel A, R-PBP2x; panel B, R-PBP2x + penicillin G.

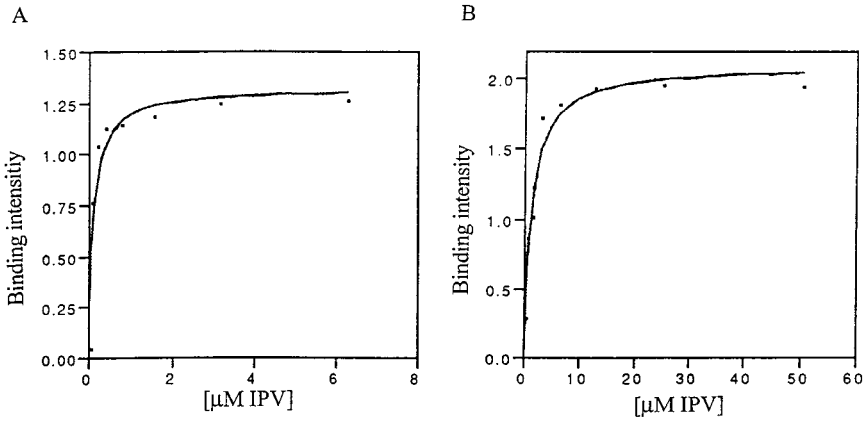

FIG. 3. Determination of binding constants (K_d) of R-PBP2x and S-PBP2x enzymes for ^{125}I-labeled penicillin V. Protein samples (0.2 μg each) were incubated with various concentrations of ^{125}I-labeled penicillin at 37°C for 15 min, separated on a 12% gel, and exposed to X-ray films after drying. Penicillin binding intensities were quantified with a laser scanner as described in Materials and Methods. Panel A, S-PBP2x; panel B, R-PBP2x.

higher than 2 and 3 mM, respectively (data not shown). The mechanism of this inhibition is unknown and probably warrants further investigation. The apparent K_m values determined for S-PBP2x and R-PBP2x were 1.6 and 16 mM, respectively. The apparent V_{max} (k_{cat}) values determined for S-PBP2x and R-PBP2x were 8.3 μmol/min/mg (10.5 s^{-1}) and 0.13 μmol/min/mg (0.17 s^{-1}), respectively. Thus, the K_m and V_{max} values determined for both enzymes at pH 5.5 did not differ significantly from those determined at pH 7.0.

Determination of the binding affinity of S-PBP2x and R-PBP2x for ^{125}I-labeled penicillin V gave K_d values of 0.11 and 1.28 μM, respectively (Fig. 3). The 12-fold-lower K_d value of S-PBP2x for ^{125}I-labeled penicillin V is in good agreement with our kinetic results showing that the K_m value of S-PBP2x for S2d is 10-fold-lower than that of R-PBP2x. We also determined 50% inhibitory concentration (IC_{50}) values of nitrocefin and LY359210 for R-PBP2x and S-PBP2x enzymes assayed with ^{125}I-labeled penicillin V (3 μM) as a probe substrate. The IC_{50} values of nitrocefin and LY359210 were 2.5 and 0.7 μM, respectively, for S-PBP2x and 12 and 8.5 μM, respectively, for R-PBP2x. Thus, the two drugs tested did not effectively inhibit the purified R-PBP2x enzyme.

Lack of β-lactam hydrolytic activities of S-PBP2x and R-PBP2x enzymes. To test whether the lower binding affinity of R-PBP2x for ^{125}I-labeled penicillin V was due to an intrinsic ability to hydrolyze penicillin, we tested both S-PBP2x and R-PBP2x enzymes for their ability to hydrolyze nitrocefin, a β-lactam antibiotic that has been routinely used as a colorimetric substrate for β-lactamases (8, 32, 36). Hydrolysis activity was measured at 480 nm in reaction mixtures (1 ml each) containing 50 mM potassium phosphate (pH 7.0), 0.5 to 1.0 mM nitrocefin, and 7 to 20 μg of the enzymes (36). Under these conditions, neither the R-PBP2x nor the S-PBP2x enzyme showed a detectable level of nitrocefin hydrolysis activity (data not shown).

To further confirm this result, we carried out ^{125}I-labeled penicillin V dilution experiments with R-PBP2x and S-PBP2x. First, both enzymes (2 μg of each) were labeled with 16 μg of ^{125}I-labeled penicillin V per ml (see Materials and Methods), and the reaction mixtures were diluted with equal volumes of 430 mg of cold penicillin G per ml. Immediately after dilution, the reaction mixtures were incubated at 37°C for 0, 10, and 90 min, denatured by boiling at 100°C in SDS-denaturing solution, and subjected to SDS-PAGE. The binding intensities of penicillin to PBP2x enzymes remained unchanged after the 0-, 10-, and 90-min incubations (data not shown). Taken together, these data suggest that neither R-PBP2x nor S-PBP2x possessed a detectable level of penicillin or nitrocefin hydrolysis activity under the assay conditions. Therefore, the resistance of *S. pneumoniae* to β-lactams did not result from the ability of R-PBP2x to hydrolyze the antibiotics.

Cellular amounts of PBP2x enzymes in *S. pneumoniae*. We wanted to know whether the levels of R-PBP2x and S-PBP2x enzymes differed in penicillin-resistant and -sensitive strains of *S. pneumoniae*, whether penicillin would induce expression of R-PBP2x, and finally, whether levels of R-PBP2x and S-PBP2x in penicillin-resistant and -sensitive isolates of *S. pneumoniae* would change during growth. As shown in Fig. 4, the amounts of R-PBP2x and S-PBP2x appeared to vary in different growth phases, but the amount of S-PBP2x did not differ significantly from that of R-PBP2x. Further, the expression pattern and the amount of R-PBP2x in *S. pneumoniae* 328 did not change when grown in Todd-Hewitt medium alone or supplemented with 1 μg of penicillin G per ml (data not shown).

DISCUSSION

In this study, we found that R-PBP2x and his-R-PBP2x enzymes exhibited 10-fold-lower affinity for S2d and approximately 30-fold-lower activity (V_{max}) than those of S-PBP2x and his-S-PBP2x enzymes. The much lower activities of R-PBP2x and his-R-PBP2x enzymes were not due to enzyme contamination or inactivation. First, R-PBP2x and his-R-PBP2x enzymes were purified by different column chromatographic methods, yet they exhibited similar kinetic properties. Second, the relative affinity of the R-PBP2x enzyme for ^{125}I-labeled penicillin V was approximately 12-fold lower than that of S-PBP2x. Third, electrospray mass spectrometric analysis of the purified R-PBP2x enzyme confirmed that virtually 100% of the R-PBP2x enzyme molecules were active with respect to penicillin binding.

It is well established that the intrinsic resistance of *S. pneumoniae* to β-lactam antibiotics is due to the alteration of PBPs that have reduced affinity for these antibiotics (see Introduction). As a result, bacteria become resistant to the antibiotics. However, the underlying biochemical basis of resistance is not well understood. Studies have shown that the cell wall peptidoglycan composition of penicillin-resistant clinical isolates of *S. pneumoniae* differs significantly from that of penicillin-sensitive strains (16, 40, 42, 43). The cell wall peptidoglycan of

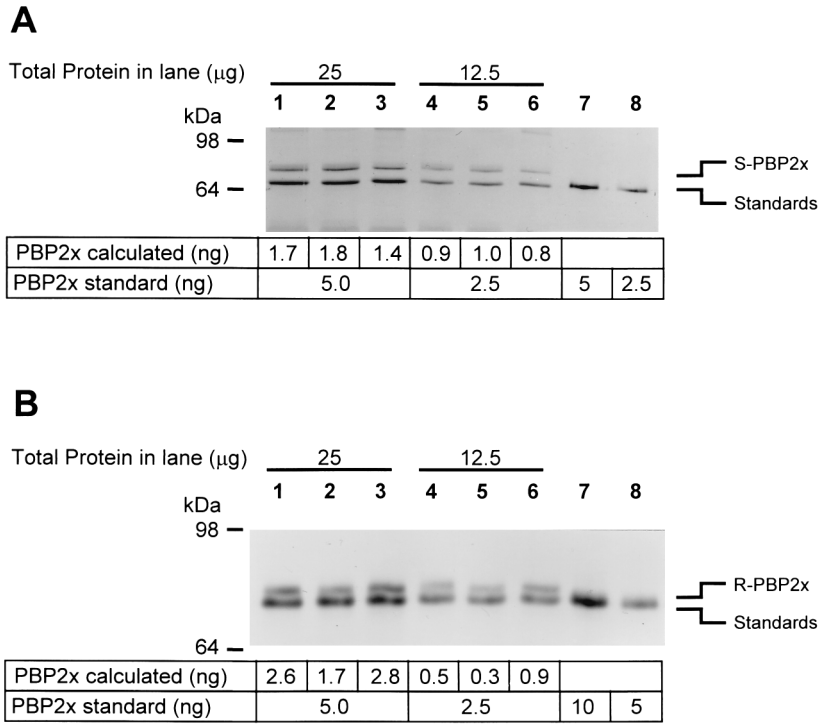

FIG. 4. Quantitative Western blotting analysis of cellular amounts of S-PBP2x and R-PBP2x in *S. pneumoniae* harvested at different growth phases. Cells were grown under the conditions described in Materials and Methods. (A) Lanes 1 to 3, 5.0 ng of purified S-PBP2x mixed with 25 μg of crude extract of *S. pneumoniae* R6 harvested at early stationary, middle logarithmic, and early logarithmic phases, respectively; lanes 4 to 6, 2.5 ng each of purified S-PBP2x mixed with 12.5 μg of crude extract of *S. pneumoniae* R6 harvested at early stationary, middle logarithmic, and early logarithmic phases, respectively; lanes 7 to 8, 5.0 and 2.5 ng of purified S-PBP2x, respectively. (B) Lanes 1 to 3, 5.0 ng of purified R-PBP2x mixed with 25 μg of crude extract of *S. pneumoniae* 328 harvested at early stationary, middle logarithmic, and early logarithmic phases, respectively; lanes 4 to 6, 2.5 ng of purified R-PBP2x mixed with 12.5 μg of crude extract of *S. pneumoniae* 328 harvested at early stationary, middle logarithmic, and early logarithmic phases, respectively; and lanes 7 and 8, 10 and 5 ng of purified R-PBP2x, respectively.

penicillin-sensitive strains contains a majority of monomeric and oligomeric forms of linear-stem peptides (16, 40, 42, 43). On the other hand, the cell wall peptidoglycan of penicillin-resistant clinical isolates contains mainly abnormal branched-stem peptides (16, 40, 42, 43). These studies suggest that the abnormal branched-stem peptides might be the preferred substrates for penicillin-resistant PBPs in vivo (16, 40, 42, 43). We do indeed show that R-PBP2x differed drastically from S-PBP2x kinetically when it was assayed with the thioester S2d as a substrate. A comparison of their k_{cat}/K_m values, 3,400 and 11.2 M^{-1} s^{-1} for S-PBP2x and R-PBP2x, respectively, shows that the S-PBP2x enzyme is approximately 300-fold more active than the R-PBP2x enzyme. Therefore, these kinetic results clearly demonstrate that the purified enzymes are significantly different. This observation may indicate that the two purified enzymes utilize two different physiological substrates in vivo. Together, our kinetic studies along with the cell wall composition studies suggest a working hypothesis that these altered PBPs utilize the abnormal branched-stem peptides rather than the linear-stem peptides as substrates for the cell wall biosynthesis in penicillin-resistant clinical isolates of *S. pneumoniae*. This hypothesis can certainly be tested with the linear- and branched-stem peptides as substrates if they are available.

Recently, experiments have shown that the abnormal cell wall composition of penicillin-resistant strains of *S. pneumoniae* is separable from their resistance to penicillin by genetic transformation (40). These results are consistent with the well-established fact that *pbp* genes are essential for bacterial resistance to penicillin (12, 44, 45) and also suggest that genetic elements responsible for the production of the abnormal-stem peptides are dispensable for the resistance, at least under laboratory conditions. However, these genetic elements may play an important role in the long-term survival of penicillin-resistant clinical isolates in nature. Three lines of evidence seem to support this view. First, penicillin-resistant clinical isolates of *S. pneumoniae* are often faster growers compared with the penicillin-resistant mutants generated in the laboratory (16, 27, 28, 43). Second, the degree of cross-linking in the peptidoglycan of penicillin-resistant clinical isolates of *S. pneumoniae* was shown to be similar to that of penicillin-sensitive strains, but a drastic reduction in the cross-linking of the peptidoglycan was observed for penicillin-resistant laboratory mutants (16, 40, 42, 43). Third, penicillin-resistant mutants generated in the laboratory were usually associated with a low level rather than a high level of resistance to the drug (21, 22, 27, 28).

Our direct measurement of R-PBP2x and S-PBP2x proteins in the cell demonstrated that their expression levels varied at different growth phases and that the cellular amount of R-PBP2x was similar to that of S-PBP2x during growth. The fact that the R-PBP2x enzyme was not significantly overproduced in *S. pneumoniae* 328, a high-level penicillin-resistant clinical isolate, suggests that the resistance of *S. pneumoniae* to β-lactam antibiotics may have arisen from the alteration of the substrate specificity of PBP enzymes rather than their overproduction. This result also implies that altered PBP enzymes can efficiently utilize the branched-stem peptides as substrates even in the presence of penicillin and the normal-stem peptides in the cell that are potential inhibitors of the altered PBPs.

As shown in Fig. 4, 25 μg each of the crude extracts of *S.*

pneumoniae R6 and 328 harvested at the middle logarithmic phase contained approximately 2 ng each of S-PBP2x and R-PBP2x enzymes. The amounts of S-PBP2x and R-PBP2x enzymes present in the cell, therefore, represent 0.008% of the total cellular protein. Recently, PBP1a and PBP1b of *E. coli* K-12 were shown to be present at 221 and 127 molecules per cell, respectively, when *E. coli* cells were grown exponentially in LB medium (11). Since the molecular masses of *E. coli* PBP1a and PBP1b were calculated to be 93.6 and 88.9 kDa, respectively (9), and the average total protein of an *E. coli* K-12 cell was estimated to be 4.4×10^{-7} μg (6), the amounts of *E. coli* PBP1a and PBP1b represent 0.0078 and 0.0043% of the total cellular protein, respectively. Since the molecular masses of S-PBP2x and R-PBP2x were predicted to be 82.4 and 82.5 kDa (28 and this study), which are similar to those of the *E. coli* PBP1a and PBP1b, the cellular amounts of the *S. pneumoniae* R-PBP2x and S-PBP2x enzymes are thus virtually the same as that of *E. coli* PBP1a but approximately twofold higher than that of the *E. coli* PBP1b. This comparison suggests that the PBP enzymes from gram-positive and -negative bacteria are present at a low level.

Finally, the results of our kinetic studies have an important practical implication in drug development. On the basis of the kinetic property that the R-PBP2x enzyme may utilize the branched peptides as substrates, it should be possible to design a molecule with the features of the branched-stem peptides and penicillin. This molecule should have an antimicrobial activity against both penicillin-resistant and -sensitive organisms.

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REFERENCES

1. **Adam, M., C. Damblon, M. Jamin, W. Zorzi, V. Dusart, M. Galleni, A. El Kharroubi, G. Piras, B. G. Spratt, W. Keck, J. Coyette, J.-M. Ghuysen, M. Nguyen-Disteche, and J.-M. Frere.** 1991. Acyltransferase activities of the high-molecular-mass essential penicillin-binding proteins. Biochem. J. **279:** 601–604.
2. **Adam, M., C. Damblon, B. Plaitin, L. Christiaens, and J.-M. Frere.** 1990. Chromogenic depsipeptide substrates for β-lactamases and penicillin-sensitive DD-peptidases. Biochem. J. **270:**525–529.
3. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. J. Exp. Med. **79:**137–158.
4. **Barcus, V. A., K. Ghanekar, M. Yeo, T. J. Coffey, and C. G. Dowson.** 1995. Genetics of high level penicillin resistance in clinical isolates of *Streptococcus pneumoniae*. FEMS Microbiol. Lett. **126:**299–304.
5. **Blaszczak, L. C., N. G. Halligan, and D. E. Seitz.** 1989. Radioiododestannytation: convenient synthesis of a stable penicillin derivative for rapid penicillin-binding protein (PBP) assay. J. Labelled Compd. Radiopharm. **27:**401–406.
6. **Boye, E., M. G. Marinus, and A. Løbner-Olesen.** 1992. Quantitation of Dam methyltransferase in *Escherichia coli*. J. Bacteriol. **174:**1682–1685.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
8. **Bradford, P. A., C. Urban, A. Jaiswal, N. Mariano, B. A. Rasmussen, S. J. Projan, J. J. Rahal, and K. Bush.** 1995. SHV-7, a novel cefotaxime-hydrolyzing β-lactamase, identified in *Escherichia coli* isolates from hospitalized nursing home patients. Antimicrob. Agents Chemother. **39:**899–905.
9. **Broome-Smith, J. K., A. Edelman, S. Yousif, and B. G. Spratt.** 1985. The nucleotide sequences of the *ponA* and *ponB* genes encoding penicillin-binding protein 1A and 1B of *Escherichia coli* K12. Eur. J. Biochem. **147:**437–446.
10. **Coffey, T. J., M. Daniels, L. K. McDougal, C. G. Dowson, F. C. Tenover, and B. G. Spratt.** 1995. Genetic analysis of clinical isolates of *Streptococcus pneumoniae* with high-level resistance to expanded-spectrum cephalosporins. Antimicrob. Agents Chemother. **39:**1306–1313.
11. **Dougherty, T. J., K. Kennedy, R. E. Kessler, and M. J. Pucci.** 1996. Direct quantitation of the number of individual penicillin-binding proteins per cell in *Escherichia coli*. J. Bacteriol. **178:**6110–6115.
12. **Dowson, C. G., T. J. Coffey, and B. G. Spratt.** 1994. Origin and molecular epidemiology of penicillin-binding protein-mediated resistance to β-lactam antibiotics. Trends Microbiol. **2:**361–366.
13. **Dowson, C. G., A. Hutchison, J. A. Brannigan, R. C. George, D. Hansman, J. Linares, A. Tomasz, J. Maynard Smith, and B. G. Spratt.** 1989. Horizontal transfer of penicillin-binding protein genes in penicillin resistant clinical isolates of *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **86:**8842–8846.
14. **Dowson, C. G., A. Hutchison, and B. G. Spratt.** 1989. Extensive remodeling of the transpeptidase domain of penicillin-binding protein 2B of a penicillin-resistant South African isolate of *Streptococcus pneumoniae*. Mol. Microbiol. **3:**95–102.
15. **Dowson, C. G., A. Hutchison, N. Woodford, A. P. Johnson, R. C. George, and B. G. Spratt.** 1990. Penicillin-resistant viridans streptococci have obtained altered penicillin-binding proteins genes from penicillin-resistant strains of *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **87:**5858–5862.
16. **Garcia-Bustos, J., and A. Tomasz.** 1990. A biological price of antibiotic resistance: major changes in the peptidoglycan structure of penicillin-resistant pneumococci. Proc. Natl. Acad. Sci. USA **87:**5414–5419.
17. **Ghuysen, J.-M.** 1991. Serine β-lactamases and penicillin binding proteins. Annu. Rev. Microbiol. **45:**37–67.
18. **Ghuysen, J.-M., and G. Dive.** 1994. Biochemistry of the penicilloyl serine transferases, p. 103–129. *In* J.-M. Ghuysen and R. Hekenbeck (ed.), Bacterial cell wall. Elsevier Science B.V., Amsterdam, The Netherlands.
19. **Grebe, T., and R. Hakenbeck.** 1996. Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of β-lactam antibiotics. Antimicrob. Agents Chemother. **40:**829–834.
20. **Hakenbeck, R., M. Tarpay, and A. Tomasz.** 1980. Multiple changes of penicillin-binding proteins in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **17:**364–371.
21. **Handwerger, S., and A. Tomasz.** 1986. Alterations in kinetic properties of penicillin-binding proteins of penicillin-resistant *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **30:**57–63.
22. **Handwerger, S., and A. Tomasz.** 1986. Alterations in penicillin-binding proteins of clinical and laboratory isolates of pathogenic *Streptococcus pneumoniae* with low levels of penicillin resistance. J. Infect. Dis. **153:**83–89.
23. **Jamin, M., C. Damblon, S. Millier, R. Hakenbeck, and J.-M. Frere.** 1993. Penicillin-binding protein 2x of *Streptococcus pneumoniae*: enzymic activities and interactions with β-lactams. Biochem. J. **292:**735–741.
24. **Kell, C. M., U. K. Sharma, C. G. Dowson, C. Town, T. S. Balganesh, and B. G. Spratt.** 1993. Deletion analysis of the essentiality of penicillin-binding proteins 1A, 2B and 2X of *Streptococcus pneumoniae*. FEMS Microbiol. Lett. **106:**171–176.
25. **Kelly, J. A., S. G. Waley, M. Adam, and J.-M. Frere.** 1992. Crystalline enzyme kinetics: activity of the *Streptomyces* R61 D-alanyl-D-alanine peptidase. Biochim. Biophys. Acta **1119:**256–260.

25a. **Lacks, S., and R. D. Hotchkiss.** 1960. A study of the genetic material determining an enzyme activity in pneumococcus. Biochim. Biophys. Acta **39:**508–517.

26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
27. **Laible, G., and R. Hakenbeck.** 1987. Penicillin-binding proteins in β-lactam-resistant laboratory mutants of *Streptococcus pneumoniae*. Mol. Microbiol. **1:**355–363.
28. **Laible, G., R. Hakenbeck, M. A. Slcard, B. Joris, and J.-M. Ghuysen.** 1989. Nucleotide sequences of the *pbpx* genes encoding the penicillin-binding proteins 2x from *Streptococcus pneumoniae* R6 and a cefotaxime-resistant mutant, C506. Mol. Microbiol. **3:**1337–1348.
29. **Laible, G., W. Keck, R. Lurz, H. Mottl, J.-M. Frere, M. Jamin, and R. Hakenbeck.** 1992. Penicillin-binding protein 2x of *Streptococcus pneumoniae*: expression in *Escherichia coli* and purification of a soluble enzymatically active derivative. Eur. J. Biochem. **207:**943–949.
30. **Laible, G., B. G. Spratt, and R. Hakenbeck.** 1991. Interspecies recombinational events during the evolution of altered PBP 2x genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. Mol. Microbiol. **5:** 1993–2002.
31. **Martin, C., C. Sibold, and R. Hakenbeck.** 1992. Relatedness of penicillin-binding protein 1a genes from different clones of penicillin-resistant *Streptococcus pneumoniae* isolated in South Africa and Spain. EMBO J. **11:**3831–3836.
32. **Matagne, A., A.-M. Misselyn-Bauduin, B. Joris, T. Erpicum, B. Granier, and J.-M. Frere.** The diversity of the catalytic properties of class A β-lactamases. Biochem. J. **265:**131–146.
33. **Matsuhashi, M.** 1994. Utilization of lipid-linked precursors and the formation of peptidoglycan in the process of cell growth and division: membrane enzymes involved in the final steps of peptidoglycan synthesis and the mechanism of their regulation, p. 55–72. *In* J.-M. Ghuysen and R. Hekenbeck

(ed.), Bacterial cell wall. Elsevier Science B.V., Amsterdam, The Netherlands.
34. **Maynard Smith, J. M., C. G. Dowson, and B. G. Spratt.** 1991. Localized sex in bacteria. Nature (London) **349:**29–31.
35. **Neu, H. C.** 1992. The crisis in antibiotic resistance. Science **257:**1064–1073.
36. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1:**283–288.
37. **Pares, S., N. Mouz, Y. Petillot, R. Hakenbeck, and O. Dideberg.** 1996. X-ray structure of *Streptococcus pneumoniae* PBP2x, a primary penicillin target enzyme. Nat. Struct. Biol. **3:**284–289.
38. **Preston, D. A., C. Y. E. Wu, L. C. Blaszczak, D. E. Seitz, and N. G. Halligan.** 1990. Biological characterization of a new radioactive labeling reagent for bacterial penicillin binding proteins. Antimicrob. Agents Chemother. **34:** 718–721.
39. **Schuster, C., B. Dobrinski, and R. Hakenbeck.** 1990. Unusual septum formation in *Streptococcus pneumoniae* mutants with an alteration in the D,D-carboxypeptidase penicillin-binding protein 3. J. Bacteriol. **172:**6499–6505.
40. **Severin, A., A. M. Figueiredo, and A. Tomasz.** 1996. Separation of abnormal cell wall composition from penicillin resistance through genetic transformation of *Streptococcus pneumoniae*. J. Bacteriol. **178:**1788–1792.
41. **Severin, A., C. Schuster, R. Hakenbeck, and A. Tomasz.** 1992. Altered murein composition in a DD-carboxypeptidase mutant of *Streptococcus pneumoniae*. J. Bacteriol. **174:**5152–5155.
42. **Severin, A., and A. Tomasz.** 1996. Naturally occurring peptidoglycan variants of *Streptococcus pneumoniae*. J. Bacteriol. **178:**168–174.
43. **Severin, A., M. V. Vaz Pato, A. M. S. Figueiredo, and A. Tomasz.** 1995. Drastic changes in the peptidoglycan composition of penicillin resistant laboratory mutants of *Streptococcus pneumoniae*. FEMS Microbiol. Lett. **130:**31–35.
44. **Spratt, B. G.** 1994. Resistance to β-lactam antibiotics, p. 517–534. *In* J.-M. Ghuysen and R. Hekenbeck (ed.), Bacterial cell wall. Elsevier Science B.V., Amsterdam, The Netherlands.
45. **Tomasz, A., and R. Munoz.** 1995. β-lactam antibiotic resistance in Gram-positive bacterial pathogens of the upper respiratory tract: a brief overview of mechanisms. Microb. Drug Resist. **1:**103–109.
46. **Van Heijenoort, J.** 1996. Murein synthesis, p. 1025–1034. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. C. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
47. **Zhao, G.** 1996. Unpublished results.
48. **Zhao, G., and M. E. Winkler.** 1995. Kinetic limitation and cellular amount of pyridoxine (pyridoxamine) 5′-phosphate oxidase of *Escherichia coli* K-12. J. Bacteriol. **177:**883–891.