

Crystal Structure of PBP2x from a Highly Penicillin-resistant *Streptococcus pneumoniae* Clinical Isolate

A MOSAIC FRAMEWORK CONTAINING 83 MUTATIONS*

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Penicillin-binding proteins (PBPs) are the main targets for β -lactam antibiotics, such as penicillins and cephalosporins, in a wide range of bacterial species. In some Gram-positive strains, the surge of resistance to treatment with β -lactams is primarily the result of the proliferation of mosaic PBP-encoding genes, which encode novel proteins by recombination. PBP2x is a primary resistance determinant in *Streptococcus pneumoniae*, and its modification is an essential step in the development of high level β -lactam resistance. To understand such a resistance mechanism at an atomic level, we have solved the x-ray crystal structure of PBP2x from a highly penicillin-resistant clinical isolate of *S. pneumoniae*, Sp328, which harbors 83 mutations in the soluble region. In the proximity of the Sp328 PBP2x* active site, the Thr³³⁸ \rightarrow Ala mutation weakens the local hydrogen bonding network, thus abrogating the stabilization of a crucial buried water molecule. In addition, the Ser³⁸⁹ \rightarrow Leu and Asn⁵¹⁴ \rightarrow His mutations produce a destabilizing effect that generates an "open" active site. It has been suggested that peptidoglycan substrates for β -lactam-resistant PBPs contain a large amount of abnormal, branched peptides, whereas sensitive strains tend to catalyze cross-linking of linear forms. Thus, *in vivo*, an "open" active site could facilitate the recognition of distinct, branched physiological substrates.

β -Lactam antibiotics, a vast group of molecules that includes penicillins and structurally related compounds, have been employed worldwide for over 6 decades (1). These drugs target bacterial species by acylating the active site serine of the transpeptidase domains of penicillin-binding proteins (PBPs)¹; as a defense mechanism, bacteria have developed β -lactamases

in an effort to hydrolyze these drugs (2). PBPs catalyze key steps in the synthesis of the peptidoglycan, a highly cross-linked mesh, which is not only essential in cell division but also protects bacteria from osmotic shock and lysis (3). PBP action involves interconnecting glycan chains (polymers of *N*-glucosamine and *N*-acetylmuramic acid residues) and cross-linking, or transpeptidation, of short stem peptides, which are attached to such glycan chains. Different bacterial species may have between four and eight distinct PBPs; high molecular mass PBPs are multidomain molecules, consisting of a short cytoplasmic region, a single transmembrane domain, and a large periplasmic region. High molecular mass PBPs are classified into class A molecules (containing both glycosyltransfer and transpeptidation activities) and class B, which contain a transpeptidase domain unit in addition to a N-terminal domain of unknown function. Low molecular mass PBPs function mostly as carboxypeptidases and are believed to regulate the amount of cross-linking within the peptidoglycan mesh (4–6).

The basis of β -lactam action reflects the fact that the transpeptidation substrate, namely the D-alanyl-D-alanine C terminus of peptidoglycan stem peptides, has close structural analogy to the β -lactam ring. The antibiotics thus act as pseudosubstrates and acylate the PBP transpeptidation active sites, which then deacylate very slowly and are subsequently incapable of performing regular cross-linking functions. The vastly successful employment of β -lactams for the treatment of infections relies on the fact that the PBP transpeptidase domains reside in the periplasmic space, allowing the drugs to have free access to the active sites without the need for traversing the cytoplasmic membrane.

Although *Streptococcus pneumoniae*, the causative agent of a variety of upper respiratory tract infections, has been treated successfully over time with increasing doses of penicillin, antibiotic resistance has been traced to low β -lactam affinity forms of its PBPs (7). *S. pneumoniae* has five high molecular mass PBPs (1a, 1b, 2a, 2b, and 2x, the first three of which possess both glycosyltransfer and transpeptidase domain activities) and a low molecular mass PBP3 (4). Notably, low β -lactam affinity forms of streptococcal PBPs are present as highly mosaic genes, especially PBP 1a, 2x, and 2b; PBP2x and PBP2b are primary resistance determinants, and their modification is an essential step in the development of high level resistance (8, 9). Pneumococci are naturally highly transformable, and such mosaic structures are believed to have arisen by interspecies homologous recombination, generating PBPs that have decreased affinity for almost all β -lactams, including third generation cephalosporins (10). Clinically resistant strains of *S. pneumoniae* may harbor PBP2x variants with over 100 mutations spread throughout highly divergent regions (9, 11).

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¹ The abbreviations used are: PBP, penicillin-binding protein; R6 PBP2x*, water-soluble form of PBP2x (residues 49–750) from penicillin-sensitive strain R6; PAGE, polyacrylamide gel electrophoresis; Sp328 PBP2x*, water-soluble form of PBP2x (residues 49–750) from penicillin-resistant clinical isolate Sp328.

Important insight into biological function and resistance mechanisms generated by mutant PBPs have been made available through the structural and biochemical studies of *S. pneumoniae* PBP2x (12–15). Crystallization and x-ray structure solution of a β -lactam-sensitive form of PBP2x (R6 PBP2x*), possible after solubilization by omission of the cytoplasmic region and the membrane anchor, revealed a three-domain macromolecule composed of an elongated, “sugar tong”-like N-terminal unit, a central transpeptidase domain, and a C-terminal domain. The transpeptidase domain has an active site reminiscent of class A β -lactamases and harbors the essential nucleophile Ser³³⁷ (12, 15).

The most frequent mutation observed in PBP2xs of clinically resistant pneumococci is that of Thr³³⁸ (consequence of a T \rightarrow A mutation at the genetic level (13, 14)). Thr³³⁸ is immediately adjacent to the catalytic Ser³³⁷ and is buried in a small cavity shielded from the active site (15). This cavity also harbors a buried water molecule, which is coordinated to the side chain hydroxyl group of Thr³³⁸, among others. The mutation of Thr³³⁸ to glycine, alanine, proline, or valine revealed that the absence of a hydroxyl group at this position drastically lowered the acylation efficiency of PBP2x for β -lactams, even though this residue is not in direct contact with the antibiotic (13).

Here we report the crystal structure of a mosaic form of PBP2x from a highly penicillin-resistant clinical strain of *S. pneumoniae*, Sp328. This enzyme contains 92 mutations when compared with the sensitive form (83 in the soluble region), and the structure reveals information relevant for an understanding of antibiotic affinity and efficiency of catalytic turnover. The structure is analyzed in light of biochemical results obtained with laboratory and clinical mutants in an effort to further decipher the mechanism of antibiotic resistance in streptococci.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids, Site-directed Mutagenesis, and Protein Purification—The gene corresponding to PBP2x from the highly penicillin-resistant *S. pneumoniae* strain Sp328 (minimum inhibitory concentration of penicillin = 4 μ g/ml) was a kind gift from Eli Lilly (Indianapolis, IN). A fragment corresponding to residues 49–750 was amplified by polymerase chain reaction and cloned into *EcoRI-XhoI* sites in plasmid pGEX-4T1 (Amersham Pharmacia Biotech, Uppsala, Sweden). The first 48 residues of Sp328 PBP2x, which correspond to the cytoplasmic region and the transmembrane helix, were not included in the studies to generate a soluble form of the protein, here identified with an *. The resulting construct, pGEX-Sp328-PBP2x*, was transformed into *Escherichia coli* MC1061 cells, and this strain was employed for protein expression as described below.

Sp328 PBP2x* was expressed as a fusion protein to glutathione S-transferase with a thrombin-cleavable linker positioned N-terminally to the initiator methionine. The protein was purified to homogeneity as described previously (13). Once crystallization trials for Sp328 PBP2x* were initiated, an SDS-polyacrylamide gel electrophoresis (PAGE) analysis revealed that the protein was unstable and had been cleaved during purification into two fragments. N-terminal sequencing showed that the cleavage was at the level of Phe³⁶⁴ (a mutant residue) and, hence, was the product of a chymotrypsin-like protease. To increase the stability of Sp328 PBP2x*, Phe³⁶⁴ was mutated back to leucine, which is the amino acid present in R6 PBP2x*.

To generate the Phe³⁶⁴ \rightarrow Leu mutation, vector pGEX-Sp328-PBP2x* was modified by introducing the replication origin of phage f1 as described in Ref. 14. Site-directed mutagenesis was performed using the single-stranded DNA produced from the resulting phagemid, pGEX-Sp328-PBP2x*-f1. The resulting construct was subsequently transformed into competent *E. coli* MC1061 cells. Introduction of the desired mutation was verified by DNA sequencing. Sp328 PBP2x*-Phe³⁶⁴ \rightarrow Leu was expressed and purified as described above for Sp328 PBP2x* (and shall henceforth carry this denomination). SDS-PAGE controls confirmed that the mutant protein was stable, presenting no cleavage products.

Crystallization, Cryoprotection, and Data Collection—Sp328 PBP2x* crystals were obtained by the hanging drop vapor diffusion method in

TABLE I
Data collection statistics

Cell dimensions (\AA)	$a = b = 146.56, c = 132.61$
Space group	$P3_2$
Resolution (last shell) (\AA)	25–3.2 (3.31–3.2)
No. of unique/total reflections	52,413/167,780
R_{merge} (last shell) (%)	11.1 (36.7)
$I/\sigma I$ (last shell)	10.2 (3.1)
Completeness (last shell) (%)	96.1 (97.8)
Refinement	
No. of residues	2237
No. of water molecules	21
R_{cryst} (%)	23.4
R_{free} (%)	31.1
No. of reflections in working set	41,859
No. of reflections in test set	4,672
Root mean square bond deviation (\AA)	0.009
Root mean square angle deviation ($^\circ$)	1.6
Mean B -factor (\AA^2)	31.3

0.2 M sodium acetate pH 4.8, 22–24% polyethylene glycol 2000, and 200 mM $(\text{NH}_4)_2\text{SO}_4$. Typical crystals did not grow larger than $50 \times 50 \times 100 \mu\text{m}$. Cryoprotection was performed by initially introducing 10 μl of 0.1 M sodium acetate, pH 4.5, 24% polyethylene glycol 4000, 200 mM $(\text{NH}_4)_2\text{SO}_4$, and 5% ethylene glycol into the crystallization drop. After a 5-min equilibration period, crystals were transferred into a larger volume of the above solution. The amount of ethylene glycol in the cryoprotection buffer was slowly increased to 15% in a total period of 24 h, after which crystals were flash-cooled by rapid introduction into liquid nitrogen. All attempts to perform cryoprotection in less than 24 h or with a protectant other than ethylene glycol yielded poor quality, highly mosaic diffraction patterns. Data were collected at the ID14-EH4 beamline (European Synchrotron Radiation Facility, Grenoble, France) at 0.94 \AA using a 250-mm crystal-to-detector distance and 1° oscillations. Crystals presented unit cell dimensions $a = b = 146.56$ and $c = 132.61 \text{\AA}$ in space group $P3_2$, with four monomers per asymmetric unit. Integration, scaling, and merging of data were performed with the programs Denzo and Scalepack (16).

Molecular Replacement and Refinement—Phases were obtained by molecular replacement using the 2.5- \AA structure of the penicillin-sensitive form of R6 PBP2x* (Ref. 15; 1QME.pdb) with all different amino acids (83 in total within the soluble region, starting from residue 49) mutated to alanines (Fig. 1). The program AMoRE (17) was employed with data from 10 to 4 \AA . No noncrystallographic symmetry was used during refinement of the four monomers in the asymmetric unit. Atomic refinement of Sp328 PBP2x* was performed with CNS (18) employing all measured reflections of the data set using positional and B -factor refinement interspersed with rounds of manual rebuilding in O (19). Statistics for data collection and refinement are included in Table I. The final structure includes 2237 residues (67–99, 181–236, 242–364, 395–523, and 536–750 (monomer 1); 1066–1100, 1150–1235, 1246–1360, 1397–1520, and 1535–1749 (monomer 2); 2067–2099, 2181–2236, 2242–2360, 2397–2523, and 2536–2750 (monomer 3); 3066–3100, 3150–3235, 3246–3360, 3397–3520, 3535–3612, and 3632–3749 (monomer 4)), and 21 water molecules, and has an R -factor of 23.4% for all reflections. An R_{free} of 31.1% was obtained from a subset of 9% of the data, which were excluded from the refinement. The model has good stereochemistry and structural quality, with an average bond length and bond angle deviation of 0.009 \AA and 1.6° , respectively. A total of 96.8% of all residues are in most favorable and additionally allowed regions of a Ramachandran plot.

RESULTS

Overview of Structure and Mapping of Mutations—The Sp328 PBP2x* monomer consists of three domains: an N-terminal unit, which folds into an elongated, sugar tong shape; a central transpeptidase domain (residues 266–616), which harbors the peptidoglycan cross-linking catalytic machinery; and a C-terminal unit (residues 635–750). Fig. 1 relates positions of all mutations in Sp328 PBP2x* (as compared with the penicillin-sensitive R6) and secondary structural elements in the present structure. Limits between domains are shown by *red arrows*. Most mutations are nonconservative (*orange*) and are located in loop regions. During refinement, side chain density was clearly visible for all mutated residues (which during mono-

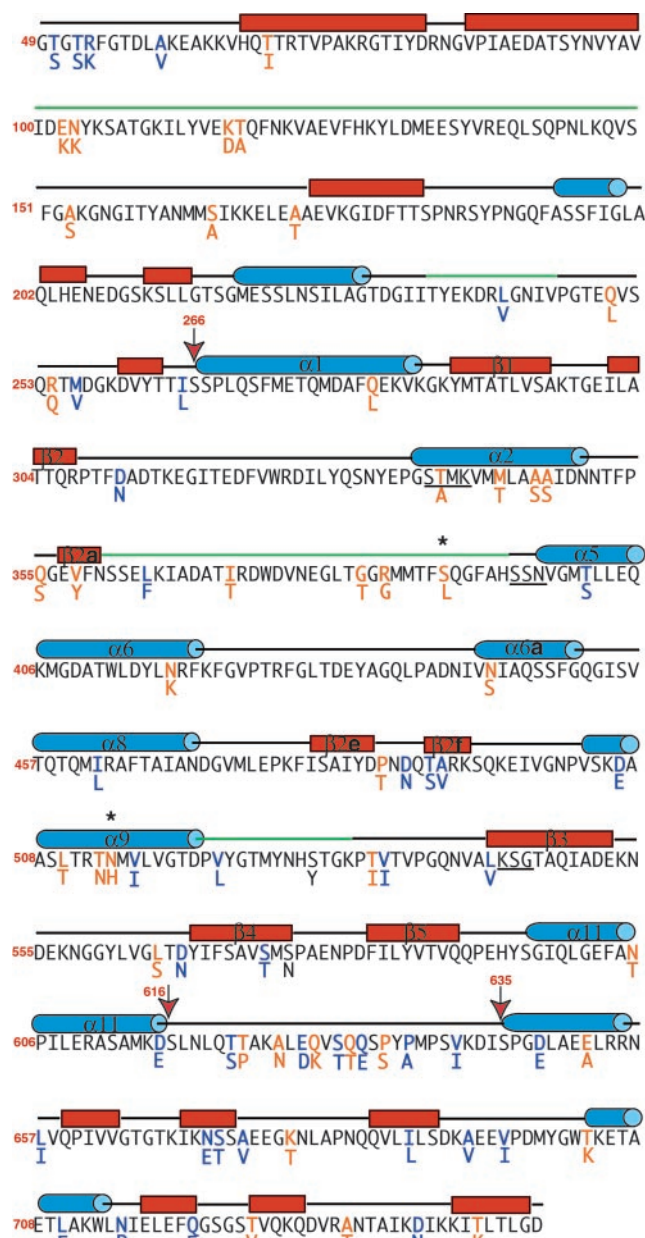


FIG. 1. Sequence alignment and secondary structure assignment. Figure shows sequence of PBP2x* from strain R6 (top) and mutations present in the Sp328 that was clinically resistant (bottom line). Secondary structural elements referring to the Sp328 PBP2x* structure are shown as blue cylinders (α -helices), red rectangles (β -sheets), or black bars (loops). Green bars represent regions that are not traceable in the electron density map. Conserved mutations are represented in blue, whereas nonconserved changes are shown in orange. Red arrows pinpoint the limits between different domains in the molecule. Sequence motifs relevant for catalysis (SXXX, SXN, KSG) are underlined. Secondary structure nomenclature for the transpeptidase domain is as described by Parès *et al.* (12). Two important mutations, Ser³⁹⁸ → Leu and Asn⁵¹⁴ → His, are marked with a star.

lecular replacement had been included as alanines) with very few exceptions.

Mutations present in Sp328 PBP2x* are mapped as red marks onto a α representation in Fig. 2. Although it is clear that punctual modifications have occurred throughout the entire structure, it is evident that there are two main regions where they are concentrated: the active site area and the C-terminal domain, including the C-terminal/transpeptidase domain linker region. Interestingly, an analysis of 26 available PBP2x sequences from β -lactam-resistant *S. pneumoniae* iso-

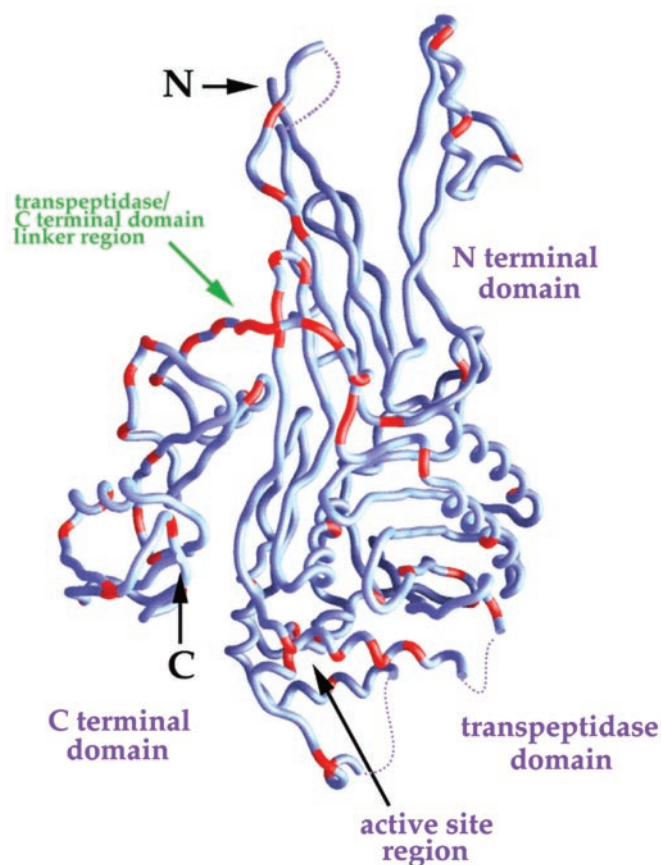


FIG. 2. Worm representation of the Sp328 PBP2x* fold. Figure shows α trace of PBP2x* from the highly penicillin-resistant Sp328; amino acids that diverge from the R6 sequence are shown in red. Loop regions that are not visible in the electron density map are shown as dotted lines.

lates available in GenBank[®] revealed that, although there is only a 16% sequence variation within the N-terminal domain, the transpeptidase and the C-terminal regions display 26 and 31% variability, respectively. The sequence of the C-terminal/transpeptidase domain linker region (residues 617–634) displays 64% variability among the 26 strains. The relevance of the localization of these mutations will be discussed below.

Resistant and Sensitive Forms of PBP2x*—Sp328 PBP2x* crystallizes in space group $P3_2$ with four molecules (2237 traceable residues) and 43% solvent per asymmetric unit. Interestingly, this new, distinct arrangement from the tetragonal ($P4_12_12$) and hexagonal ($P6_122$) forms reported previously for the β -lactam-sensitive molecule (12, 15) displays an N-terminal domain that is clearly more stable than in either form (green in Fig. 3). This is especially relevant upon comparison with the monomer crystallized in tetragonal form, which diffracted to 1-Å higher resolution than the hexagonal samples but contained a cleavage site at peptide bond 182–183 and had a largely disordered N-terminal region. The Sp328 PBP2x* structure reveals traceable density that had not been detected before for N-terminal domain residues 66–70, 93–99, 151–182, and 245–253 (and corresponding residues in monomers 2, 3, and 4). Interestingly, the structure of Sp328 PBP2x* clearly displays the linker between the transpeptidase domain and the C-terminal unit for three of the four monomers in the asymmetric unit; this region was also absent in the tetragonal crystals of the β -lactam-sensitive molecule.

Notably, in Sp328 PBP2x*, the loop region between residues 365 and 394 has very weak electron density for monomer 1 and

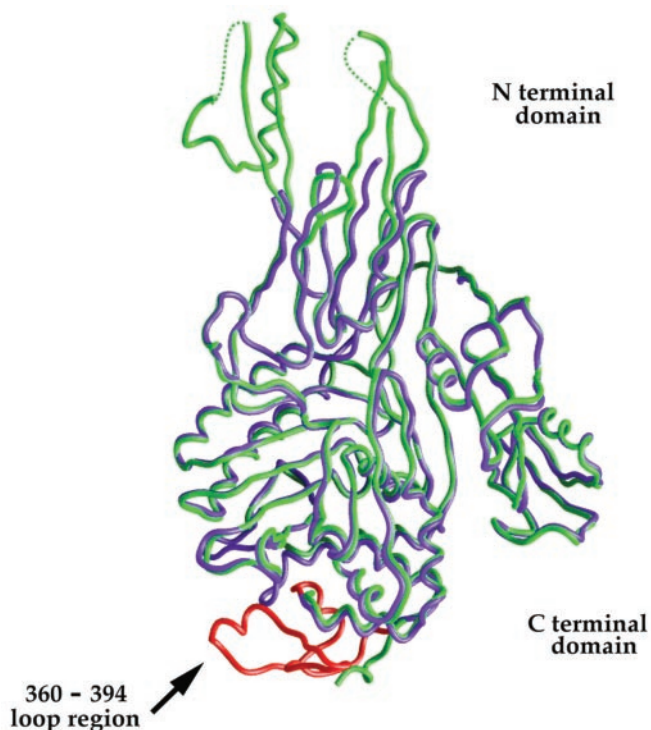


FIG. 3. **Structural comparison between Sp328 and R6 PBP2x*.** Figure shows superposition of PBP2x* from Sp328 (green) and from the penicillin-sensitive R6 strain (blue). Most α chain divergences occur at the level of the N-terminal domain, which is much more stable and thus provided a higher quality trace in the Sp328 form, and the 360–394 loop region (red), flexible in the penicillin-resistant molecule. View is 180° away from the one presented in Fig. 2.

is untraceable for the other three molecules (red in Fig. 3). Because a SDS-PAGE analysis of dissolved crystals of Sp328 PBP2x* proved the protein to be stable (a unique band at the expected molecular weight was observed), we interpret this observation as being a result of inherent flexibility of the molecule in this region.

The PBP2x* Active Site: Penicillin-sensitive and Resistant Forms—The active sites of both sensitive and resistant forms of PBP2x* are depicted in Fig. 4 (a and b), respectively, showing the three highly conserved sequence motifs for the transpeptidase domain (S³³⁷XXK; S³⁹⁵XN; K⁵⁴⁷SG). The nucleophilic serine (Ser³³⁷) is found at the N-terminal end of $\alpha 2$ (secondary structure nomenclature as in Ref. 12) and, in both resistant and sensitive forms, is in close proximity to the Ne atoms of Lys³⁴⁰ (not shown for clarity) and Lys⁵⁴⁷. In the sensitive form (Fig. 4a), the loop between $\alpha 4$ and $\alpha 5$ is stably positioned, allowing Ser³⁹⁵ to point into the active site and make close contact with Ser³³⁷. O γ . Asn⁵¹⁴, present in $\alpha 9$, points away from the active site region and does not interfere with the position of Ser³⁸⁹ in $\alpha 4$. Thus, the conserved active site residues form a rigid network of hydrogen bonds; these involve Ser³³⁷, Lys³⁴⁰, Ser³⁹⁵, Asn³⁹⁷, and Lys⁵⁴⁷. In the structure of R6 PBP2x* complexed to cefotaxime, Ser³³⁷ and Ser³⁹⁵ are critical for stabilization of the antibiotic in the active site cavity (15).

The active site of Sp328 PBP2x* contains a number of discrepancies from the enzymatic cavity of the protein from the antibiotic-sensitive strain (Fig. 4b). A slight modification in the polarity of the site is evident because of the presence of Ser³⁴⁷ (Ala in the sensitive protein), which forms a hydrogen bond with the backbone carbonyl of Thr³⁵², a residue present in the loop that follows $\alpha 2$ (not shown for clarity). It is precisely at this point that the tracing of the sensitive and resistant proteins start to differ significantly, an observation that culminates

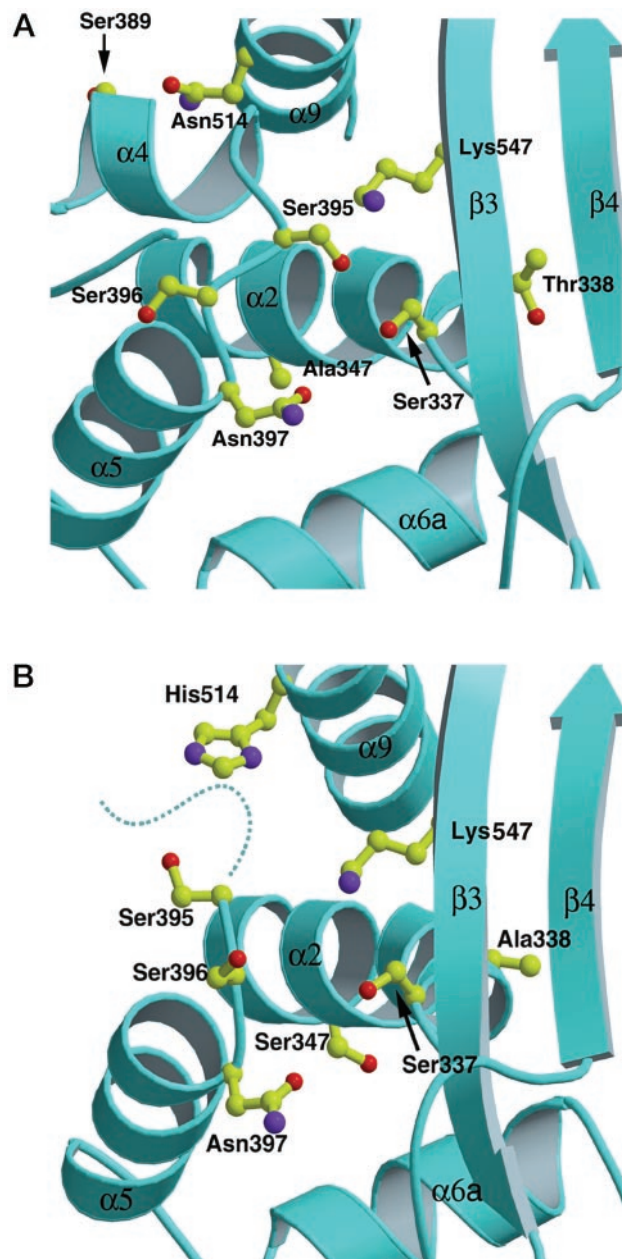


FIG. 4. **Drug-sensitive and -resistant active sites.** A, active site of PBP2x* from penicillin-sensitive strain R6 (1QME.pdb). The three crucial motifs for enzymatic activity are represented by Ser³³⁷ (SXXK), Ser³⁹⁵ (SXN), and Lys⁵⁴⁷ (K(S/T)G). Thr³³⁸ is at the N-terminal end of $\alpha 2$, and Asn⁵¹⁴ points away from $\alpha 4$, which harbors Ser³⁸⁹. B, active site of PBP2x* from penicillin-resistant strain Sp328. Although all three motifs are represented, the SXN loop is clearly displaced, probably the result of a steric clash between Leu³⁸⁹ and His⁵¹⁴, which points into where $\alpha 4$ should be located. Ser³⁴⁷ not only adds a polar character to the region but also makes contact with Thr³⁵², a residue present in the loop that follows $\alpha 2$ (not shown for clarity).

with the non-traceability of residues 365–394. This region includes $\alpha 4$, whose stability is affected by the presence of the Asn⁵¹⁴ \rightarrow His mutation. The His⁵¹⁴ side chain thus lies within the region where $\alpha 4$ is located in R6 PBP2x*. In the resistant form of PBP2x*, the lack of stability of this region engenders the flexibility of the $\alpha 4$ - $\alpha 5$ loop, which in turn causes Ser³⁹⁵ (from the SXN motif) to point away from the active site, thus not being readily available for participation in the enzymatic reaction. Chain stability is regained at the level of Asn³⁹⁷, which is positioned analogously in both active sites.

The active site of Sp328 PBP2x* also harbors the Thr³³⁸ → Ala mutation, which has been shown to be a key structural determinant for β -lactam resistance in *S. pneumoniae* (13). In the sensitive R6 PBP2x* structure, Thr³³⁸ is at the N-terminal end of $\alpha 2$ but does not point into the active site cavity. Instead, it is involved in a constellation of hydrogen bonds which include Ser⁵⁷¹, Tyr⁵⁸⁶, and a buried, tightly bound H₂O molecule. In the active site of Sp328 PBP2x*, the loss of the Thr O γ group occurs concomitantly with the loss of the stable water molecule; none of the four molecules in the asymmetric unit display such a molecule in the vicinity of the hydroxyl groups of Ser⁵⁷¹ and Tyr⁵⁸⁶. Although it was not surprising that the loss of a key hydrogen bond donor (Thr³³⁸ O γ) would cause instability of a tightly bound H₂O molecule, the proposed importance of the constellation of interactions in this area of the protein for generation of antibiotic resistance (13) led us to test our data. Thus, we compared positions of H₂O molecules present in the resistant and in the β -lactam-sensitive structure (15). Molecules analyzed in the sensitive structure had *B*-factors < 30 Å² and made at least three hydrogen bonds within the structure (and were therefore considered to be buried). Of the 41 molecules with *B*-factors < 30 Å², 21 made at least three contacts within the structure; 2 molecules from this group interacted with residues which lost hydrogen bonding capabilities in the resistant PBP2x* structure. Thus, from the 19 molecules searched for in Sp328 PBP2x*, only 5 were not identified in *F_o* - *F_c* maps contoured at 2.8 σ in any of the four monomers, including the Thr³³⁸ ligand. Further testing was performed by introducing water molecules at the expected sites in all four monomers within the Thr³³⁸-Ser⁵⁷¹-Tyr⁵⁸⁶ vicinity, performing several rounds of individual *B*-factor refinement and energy minimization with CNS (18), and generating 2*F_o* - *F_c* maps. *B*-factors for all artificially introduced water molecules were above 50 Å², and none of the molecules had any identifiable density in the maps. The average *B*-factor value for all water molecules present in the Sp328 PBP2x* structure is 25.5 Å².

DISCUSSION

Gene Recombination and Antibiotic Resistance—Unlike classical drug resistance mechanisms in which the bacterial target is mutated by a few residues in “hot spot” regions (20, 21), the molecular mechanism which underlies the development of β -lactam resistance in streptococci results from the acquisition of mosaic genes, sometimes with 100 mutations throughout the target protein. Such mosaic structures are believed to have arisen by intra- and interspecies homologous recombination involving PBPs, penicillin resistance determinants of the highly transformable streptococcal spp. (10). Closely related DNA sequences of PBP genes occur in penicillin-resistant *S. pneumoniae*, *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguis*, all of which may generate low affinity PBP forms by acquiring mosaic PBP-encoding genes in which sequence blocks are replaced by homologous regions that may diverge as much as 20% from the sequence of the drug-sensitive strain (9).

Protein Structure and Antibiotic Resistance—Of the six PBPs present in *S. pneumoniae*, PBP2x is the first to be altered in cefotaxime-resistant laboratory mutants (22) and also in clinical isolates (23). In this work, we have solved the crystal structure of a soluble form of PBP2x from a highly penicillin-resistant clinical strain of *S. pneumoniae*, Sp328. The fold of PBP2x* had been described previously for the β -lactam-sensitive structure from strain R6 (12, 15), but the present structure displays a number of topological changes. This mutant contains 83 amino acids that are different from PBP2x from the penicillin-sensitive R6 strain (in the crystallized region). Although mutations exist throughout the entire protein structure, two

heavily mutated hot spots are of interest: the active site and the C-terminal region, including the loop that links the transpeptidase and C-terminal domains.

PBPs have been proposed as being essential members of a multi-enzymatic cell division network of central importance in peptidoglycan biosynthesis (the divisome (24)). It has been suggested that the sugar tong format of the N-terminal domain of PBP2x may be useful in promoting protein-protein contacts with other members of the divisome (12, 15). Interestingly, Fig. 2 clearly shows that the N-terminal domain of Sp328 PBP2x* harbors very few mutations, with no mutated amino acids seen in the interior part of the sugar tong hole. In addition, the few mutations that are present in the N-terminal domain are mostly solvent-exposed, and several of them face the C-terminal domain hot spot area. It is of note that a sequence comparison between other PBP2x penicillin-resistant pneumococcal mutants reveals that the N-terminal domain is also the one that presents the least amount of variation, with the highest variability seen for the linker region. The lack of variation within the N-terminal region for a variety of PBP2x molecules may be related to the function of the protein within the divisome. A domain that provides several key amino acids for complex formation will tend to be more conserved, even in strains with different antibiotic susceptibilities. Mutations in an important protein contact region within a macromolecular network cannot be tolerated because they would affect peptidoglycan biosynthesis, cell division, and daughter cell formation in highly penicillin-resistant strains.

Conversely, the linker region contains a high number of mutations. In addition, the loop encompassing residues 484–489 within the transpeptidase domain contains four mutations and is closely positioned to the linker region (Figs. 1 and 2). These observations suggest that these regions, which are both localized on the same face of the molecule, may not be crucial for contacting other proteins within the peptidoglycan synthesis macromolecular assembly and can thus tolerate a variety of mutations.

The most dramatic differences that can be observed between resistant and sensitive forms of PBP2x*, however, are located in the vicinity of and within the active site region of the transpeptidase domain. The Thr³³⁸ → Ala mutation within the SXXK motif weakens the hydrogen bonding pattern in the region involving $\beta 4$ and $\alpha 2$, thus abrogating the contribution of an essential water molecule. The biochemical consequences of mutations at this site were verified by the construction of a collection of single point mutants whose kinetic parameters showed decreased β -lactam acylation efficiency (13). Interestingly, the mutation of Ala³³⁸ in Sp328 PBP2x* back to Thr increased the acylation efficiency of the enzyme by the penicillin analog cefotaxime by a factor of 6, underlining the importance of the Thr³³⁸ O γ group. It is of note that PBP2x mutants that harbor Thr³³⁸ → Pro or Thr³³⁸ → Gly mutations, isolated in hospital settings, have been reported to display unusually high level of β -lactam resistance (25).

The second motif to be structurally affected in PBP2x* from Sp328 is SXN (SSN), although it displays no internal mutation. In the crystal structure of R6 PBP2x*, $\alpha 9$, which harbors Asn⁵¹⁴, is closely positioned to $\alpha 4$, the helix that immediately precedes the loop harboring the SXN motif (Fig. 4a). It is clear from the structure of Sp328 PBP2x* that the Asn⁵¹⁴ → His mutation places the imidazole ring of His⁵¹⁴ in the direction of $\alpha 4$ (Fig. 4b). Interestingly, Sp328 PBP2x* harbors a Ser³⁸⁹ → Leu mutation on $\alpha 4$; the presence of both His⁵¹⁴ and Leu³⁸⁹ within the $\alpha 4/\alpha 9$ interface could generate a steric clash whose consequence is the instability of $\alpha 4$, the least buried of the two

helices. In only one of the four molecules present in the asymmetric unit of the Sp328 PBP2x* structure was there any density visible for $\alpha 4$ (although at a low, untraceable level). The possibility of a steric clash at the $\alpha 4/\alpha 9$ region was tested by mutating Sp328 PBP2x* residues onto the R6 PBP2x* structure (which contains a stable $\alpha 4$ -loop- $\alpha 5$ region; Ref. 12) using the Swiss-model server (26). The calculations revealed that if the $\alpha 4$ -loop- $\alpha 5$ region were to have the same conformation in the resistant molecule as it does in the sensitive one, the side chain of His⁵¹⁴ ($\alpha 9$) would clash with that of Leu³⁸⁹ ($\alpha 4$), at a high energetic cost. Thus $\alpha 4$ must occupy a different position in the β -lactam-resistant PBP2x to avoid direct contact with the His⁵¹⁴ side chain (and hence $\alpha 9$). Interestingly, in all 26 PBP2x sequences from β -lactam-resistant *S. pneumoniae* clinical isolates available, 15 have either the Ser³⁸⁹ \rightarrow Leu or the Asn⁵¹⁴ \rightarrow His mutation, whereas 11 have both.

An "Open" Active Site: A New Structural Feature Linked to Antibiotic Resistance—The flexibility of a critical region containing an active site residue in a β -lactam target enzyme is not unprecedented. β -Lactamases hydrolyze β -lactam antibiotics with high efficiency by employing a catalytic machinery that is reminiscent of that used by PBPs (a nucleophilic serine, as well as the SXXK and K(S/TG) motifs). In the recent crystal structure of a class D β -lactamase from *Pseudomonas aeruginosa*, OXA10, of the four residues directly involved in catalysis (Ser⁶⁷, Lys⁷⁰, Ser¹¹⁵, Lys²⁰⁵), Ser¹¹⁵ is located in a flexible loop (Ser¹¹⁵-Ala¹¹⁶-Val¹¹⁷; Ref. 27). Although the flexibility of this region is not of the same magnitude as the $\alpha 4$ -loop- $\alpha 5$ movement seen in Sp328 PBP2x*, the inclusion of a catalytic residue in a flexible region nonetheless reflects the possibility of movement within the active site, possibly for differential or optimal substrate binding.

In Sp328 PBP2x*, the movement of $\alpha 4$ caused by the Leu³⁸⁹-His⁵¹⁴ steric clash places Ser³⁹⁵, a catalytic residue, in an unfavorable position for enzymatic function, with its O γ atom pointing away from the catalytic cleft. Importantly, Ser³⁹⁵ O γ is responsible for two of the six stabilizing interactions made by cefotaxime within the active site of β -lactam-sensitive R6 PBP2x* (Ref. 15; 1QMF.pdb). The displacement of $\alpha 4$, and consequently Ser³⁹⁵, thus eliminates one third of the necessary contacts for antibiotic binding within the active site. Notably, when compared with R6 PBP2x*, Sp328 PBP2x* exhibits a 10-fold lower affinity for *N*-benzoyl-D-alanylmercaptoacetic thioester, a thioester analog of stem peptides, as well as a 30-fold lower activity (V_{\max}) (28). Interestingly, the microscopic kinetic parameters for acylation of a PBP2x from a closely related β -lactam-resistant *S. pneumoniae* strain, CS109, have been recently determined (29). For this molecule, the K_d values for penicillin and cefotaxime binding increase only 5- and 3-fold, respectively, when compared with the already high millimolar K_d values for the β -lactam-sensitive strain. However, ester bond formation, reflected in a 300-fold (penicillin) and 60-fold (cefotaxime) decrease in k_2 , is highly compromised. It is thus apparent that the low binding affinity of β -lactams for resistant forms of PBP2x is mostly caused by a lower efficiency in ester bond formation and, to a lesser extent, poor recognition of the active site cleft (29). These results are in agreement with our observations for Sp328 PBP2x*, where mutations in the structure generate a steric clash that displaces an active site residue from the expected site. A distortion in the position of the constellation of catalytic residues may, to some extent, play a role in poor antibiotic binding, but is a key factor in the deficiency in ester bond formation and substrate acylation. These observations are reflected in the fact that Sp328 is a high level penicillin-resistant clinical isolate (28).

A second consequence of the flexibility of the SXN loop is that the Sp328 PBP2x* active site is "open." A variety of studies have shown that the cell wall peptidoglycan composition of penicillin-resistant clinical isolates of *S. pneumoniae* may differ significantly from that of sensitive strains. Resistant forms have been reported to contain a large amount of abnormal, branched peptides, whereas sensitive strains display linear forms of stem peptides. In penicillin-resistant strains isolated from South Africa, Hungary, and the Czech Republic, an increased proportion of branched peptides was detected, whereas sensitive strains isolated from different genetic backgrounds had branched peptides in only a small proportion of their peptidoglycan (30, 31). Although genetic transformation experiments by the Tomasz group questioned the linkage between abnormality of cell wall composition of *S. pneumoniae* strains and development of resistance (32), the same team has recently suggested that cell wall precursors with branched structures may be superior competitors against β -lactam antibiotics for sites on PBPs from drug-resistant strains (33). Consequently, it is possible that enzymes from penicillin-resistant and -sensitive *S. pneumoniae* strains utilize two different physiological substrates *in vivo* (28). The flexible loop of Sp328 PBP2x*, which generates an "open" active site, may facilitate recognition of branched substrates. The presence of the mutations that cause loop flexibility (389/514) in different combinations in all PBP2x sequences of β -lactam-resistant *S. pneumoniae* strains suggests that active site elasticity, affecting both substrate recognition and turnover, may be an important mechanism in pneumococcal drug resistance profiles.

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