A PBP2x from a Clinical Isolate of *Streptococcus pneumoniae* Exhibits an Alternative Mechanism for Reduction of Susceptibility to β-Lactam Antibiotics*

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The human pathogen *Streptococcus pneumoniae* is one of the main causative agents of respiratory tract infections. At present, clinical isolates of *S. pneumoniae* often exhibit decreased susceptibility toward β-lactams, a phenomenon linked to multiple mutations within the penicillin-binding proteins (PBPs). PBP2x, one of the six PBPs of *S. pneumoniae*, is the first target to be modified under antibiotic pressure. By comparing 89 *S. pneumoniae* PBP2x sequences from clinical and public databases, we have identified one major group of sequences from drug-sensitive strains as well as two distinct groups from drug-resistant strains. The first group includes proteins that display high similarity to PBP2x from the well-characterized resistant strain Sp328. The second group includes sequences in which a signature mutation, Q552E, is found adjacent to the third catalytic motif. In this work, a PBP2x from a representative strain from the latter group (*S. pneumoniae* 5259) was biochemically and structurally characterized. Pheno- 
typical analyses of transformed pneumococci show that the Q552E substitution is responsible for most of the reduction of strain susceptibility toward β-lactams. The crystal structure of 5259-PBP2x reveals a change in polarity and charge distribution around the active site cavity, as well as rearrangement of strand β3, emulating structural changes observed for other PBPs that confer drug resistance to Gram-positive pathogens. Interestingly, the active site of 5259-PBP2x is in closed conformation, whereas that of Sp328-PBP2x is open. Consequently, *S. pneumoniae* has evolved to employ the same protein in two distinct mechanisms of antibiotic resistance.

*Streptococcus pneumoniae* is a commensal, Gram-positive bacterium that colonizes the upper respiratory tract in humans and is responsible for over one million deaths per year, mostly among the elderly and young children in developing countries. *S. pneumoniae* is a leading cause of community-acquired respiratory infections including pneumonia, acute otitis, and sinusitis as well as invasive infections such as bacteremia and meningitis (1). β-Lactam antibiotics are presently the most widely used molecules employed in the fight against pneumococcal infection. However, since the introduction of penicillin in the late 40s, β-lactam-resistant strains of *S. pneumoniae* have emerged in various locations and have now spread worldwide (2).

The *S. pneumoniae* bacterium is surrounded by a thick cell wall of which the main component is the peptidoglycan, a highly cross-linked mesh that is essential for bacterial cell division and protection from osmotic shock and lysis (3). Peptidoglycan synthesis occurs through a biosynthetic pathway that is initiated in the bacterial cytoplasm; the last steps occur on the periplasmic side of the bacterial membrane. The cytoplasmic steps of the pathway are catalyzed by Mur enzymes and result in the synthesis of lipid II molecules, which harbor the peptidoglycan building units and are subsequently translocated across the cytoplasmic membrane (4). The final steps of synthesis, namely the polymerization of the glycan chains and their reticulation, are catalyzed by the glycosyltransferase and transpeptidase activities of penicillin-binding proteins (PBPs)1 (5, 6).

In *S. pneumoniae*, six PBPs have been identified and classified on the basis of their sequence similarities (7, 8). High molecular mass PBPs are bifunctional (class A) enzymes harboring glycosyltransferase and transpeptidase (TP) activities or monofunctional enzymes (class B) with only the TP activity. Finally, a low molecular mass PBP displays a D,D-carboxypeptidase activity.

The TP activity of PBPs is responsible for cross-linking of peptidoglycan glycan chains through the formation of peptidoglycan bridges (5). β-Lactam antibiotics specifically inhibit the TP activity by acylating the active site serine (9). These molecules are specific toward the TP domain because of the structural analogy between the β-lactam ring and the d-alanyl-d-alanine peptidoglycan moiety, the natural substrate of the PBPs. The inhibition of the TP activity causes a disruption in cell wall synthesis that often leads to cell death (10).

1 The abbreviations used are: PBP, penicillin-binding protein; MIC, minimal inhibitory concentration; MIC<sub>CTX</sub>, MIC for cefotaxime; MIC<sub>PDC</sub>, MIC for penicillin G; r.m.s.d., root mean square deviation; TP, transpeptidase.
β-Lactam resistance in S. pneumoniae is a consequence of the generation of mosaic pbp genes, which result from intra- and interspecies recombination with sequences from related streptococci (11). Such genes encode PBPs harboring tens of substitutions spread throughout the entire protein (12, 13), leading to a loss of affinity for the antibiotics. Three of the streptococcal PBPs, namely PBP2x, PBP2b, and PBP1a, have been shown to be modified in penicillin-resistant strains isolated in a clinical setting (12, 14). PBP2x, one of the two monofunctional high molecular mass PBPs in S. pneumoniae, is described as the primary PBP target in β-lactam-resistant strains (15, 16). The appearance of a mosaic PBP2x is thus the first event occurring in the development of antibiotic resistance in S. pneumoniae (17).

We performed an analysis of 89 PBP2x sequences isolated from well characterized pneumococcal clinical isolates and found that they can be classified, based on cluster analysis, into three groups. The first group contains sequences closely related to the one isolated from the susceptible strain R6 (18). The two other groups contain sequences isolated from drug-resistant strains. The larger of these groups (53 sequences) contains mostly sequences related to PBP2x from β-lactam-resistant strain Sp328 (19). PBP2x from strains R6 and Sp328 have been well characterized, both biochemically (18-21) and structurally (22-24). The second group of mutants, however, which is distinguished by the Q552E mutation within all of the PBP2x sequences, remains uncharacterized both on the biochemical and structural level. This category of PBP2x variants appears to present a molecular mechanism selected to reduce the reactivity toward β-lactams that is different from that of the Sp328-like PBP2x proteins. One representative PBP2x, from strain 5259, was selected from this group for detailed study. In this work, we report the structural and functional analysis of 5259-PBP2x and demonstrate the role of the Q552E substitution in the β-lactam resistance process.

MATERIALS AND METHODS
Isolation of Drug-resistant Clones and Determination of Minimal Inhibitory Concentration (MIC) Values for β-Lactam Antibiotics—The S. pneumoniae clinical strain 5259 of serotype 15 was isolated from a tracheal noninvasive sample at the University Hospital in Grenoble, France. S. pneumoniae 5259 was grown at 37 °C in an atmosphere of 95% air, 5% CO₂ on Columbia blood agar plates (Biomérieux). Clones were isolated, and overnight liquid cultures were stored at −80 °C.

The MIC values for penicillin G and cefotaxime were determined using the E-test method on Muller-Hinton agar plates supplemented with a 20 μg/ml (penicillin) or 10 μg/ml (cefotaxime) plate (Biosciences) plated as well with 4% horse serum and cefotaxime at concentrations ranging from 0 to 0.3 μg/ml. Isolated clones were picked after 24 h of incubation at 37 °C in an atmosphere of 95% air, 5% CO₂ and then grown in glucose-buffered broth (Diagnostic Pasteur). Overnight cultures were then stored at −80 °C until the MIC determination was carried out. The MIC values were measured using the E-test method as described above for three independent isolates. All transformed colonies were confirmed as S. pneumoniae by agglutination and optochin susceptibility assays. Finally, genomic DNA of each transformant was extracted, and the pbp2x genes were PCR-amplified and sequenced.

Biochemical Characterization of the Purified Proteins—Acylation efficiencies were measured for R6-PBP2x*, R6-PBP2x*Q552E, 5259-PBP2x*, and 5259-PBP2x*-E552Q for both penicillin G and cefotaxime. The kₐ/K parameter was determined by following the intrinsic fluorescence of the proteins during antibiotic binding (9). Measurements were made at 37 °C using a spectrofluorimeter coupled to an SFM-400 stopped-flow apparatus (Bio-Logic). Proteins were used at a concentration of 0.6 μM and antibiotics at excess concentrations ranging from 25 μM to 2.5 mM in 10 mM sodium phosphate, pH 7.0. Excitation was measured at 280 nm, and emission was recorded above 305 nm. At a given concentration of antibiotic, the pseudo first-order rate constant, kₐp was determined by nonlinear least squares fitting to the equation Fluo = Fluo, exp(−kₐp t). The kₐ/K parameter, which accounts for the absence of efficiency, was obtained by linear square fitting to the equation kₐp = kₐ/K(antibiotic).

The deacylation rate was determined for all four proteins using [³H]penicillin G as previously described (28). The deacylation reaction obeys the following equation: kₐ = ln[Et]/Et,₀, where [Et,₀] is the initial concentration of acyl enzyme and [Et] is its concentration at time t. To initiate acylation, 2 μM protein was incubated with 1 μM radiolabeled penicillin G in a buffer composed of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA. After 10 or 30 min of incubation for R6-PBP2x* and 5259-PBP2x*, respectively, 15 μM unlabeled penicillin G was added, defining the beginning of the kinetic assay. Protein samples were withdrawn at various times and immediately boiled with 2% SDS prior to analysis by SDS-polyacrylamide gel electrophoresis. The decrease of radioactivity was measured in the protein bands by liquid scintillation counting. The rate constant kₐ was determined by nonlinear least squares fitting to the equation cpn = cpn,₀exp(−kₐ t).

Cryocrystallography—Commercial Hampton crystallization screens kits and employing the sitting drop vapor diffusion method (Hampton Research) were composed of 1 drop vapor diffusion method (Hampton Research). Crystallization drops were composed of 1 vol reservoir solution, which was 200 mM sodium citrate pH 6.0, 12% PEG 3350, 1 mM spermidine, and 1 mM MgCl₂. The precipitant was added to the crystallization drop and equilibrated overnight at 4 °C before being transferred to the mother liquor (27). Crystals grew as thin plates of dimensions 0.12 mm after 2 weeks. The crystals were flash-cooled by being plunged into liquid nitrogen. X-ray diffraction data were collected on the BM30 beamline at the European
Synchrotron Radiation Facility (Grenoble, France) operating at a wavelength of 0.999 Å. Angular increments between diffraction images of 1° and a distance crystal-to-detector of 210 mm were used. Raw diffraction images were indexed and integrated with MOSFLM, version 6.2.2 (29). Data scaling, merging, and reduction was carried out with programs of the CCP4 suite (30). The self-rotation function displays peaks showing a 2-fold axis perpendicular to the c axis.

Structure Solution and Refinement—The crystal structure of 5259-PBP2x* was determined by molecular replacement using AmoRe (31) with data between 30 and 3.0 Å and the R6-PBP2x* structure as a search model (22). (Protein Data Bank accession code 1QME). Residues differing from the 5259-PBP2x* sequence were replaced by alanines prior to the molecular replacement procedure. The rotation and the translation functions gave two clear single solutions, with an R-factor of 42.8% and a correlation coefficient of 68.1%, which corresponded to two molecules in the asymmetric unit related by a 2-fold noncrystallographic axis. These two solutions were employed in the refinement procedure performed with CNS 1.1 (32). The first cycle of the refinement procedure consisted of 20 cycles of rigid-body refinement with data between 31 and 4 Å. At this stage the N-terminal domain of the model was completely built in the electron density map for both molecules in the asymmetric unit. Subsequently, several cycles of simulated annealing, energy minimization, and grouped temperature factor refinement were performed. Cycles of refinement were interchanged with manual rebuilding sessions using the molecular graphics program O (33). Water molecules were positioned progressively if they were observed to be in an environment susceptible to providing hydrogen bonds. In the final stages of the refinement, individual restrained B-factor refinement was performed. These iterative cycles of refinement and map interpretation led to a model with R-factor = 23.5% and R-free = 25.6% (34). The stereochemistry of the model was analyzed with PROCHECK (35); a total of 83.1% of the non-glycine residues were R-free (MICPenG domain were sequenced. Among the 26 drug-sensitive strains for all of strains, and the selected from a large collection of well characterized clinical 53 remaining drug-resistant strains (MIC > 1) dis-

RESULTS

Characterization of pbp2x Sequences—Eighty-nine strains displaying a wide range of β-lactam resistance levels were selected from a large collection of well characterized clinical strains kept at the University Hospital in Grenoble, France. The MIC values for penicillin G and cefotaxime were measured for all of strains, and the pbp2x fragments coding for each TP domain were sequenced. Among the 26 drug-sensitive strains (MIC PenG < 0.1 μg/ml−1), only 10 PBP2x sequences displayed differences from those of PBP2x from strain R6. Similarly, the 53 remaining drug-resistant strains (MIC > 0.1 μg/ml−1) displayed 12 distinct PBP2x sequences. When the 22 different sequences (10 from sensitive strains, 12 from resistant strains) were submitted to a clustering algorithm (www.genebee. msu.su), they were found to fall into three groups: one contained 10 PBP2x sequences with major similarities to the R6-PBP2x sequence and originated from the susceptible strains; a second group displayed 11 PBP2x sequences that were similar to the previously characterized PBP2x from drug-resistant strain Sp328 (19, 23); and a third differing PBP2x sequence displayed similarities to PBP2x from strain F2. The existence of two distinct groups of sequences from resistant strains was further confirmed by analysis of public data bases. Because our laboratories had already biochemically and structurally characterized a PBP2x molecule from strain Sp328 (23, 26), we initiated the characterization of a representative PBP2x from the second group of strains. Two strains from our hospital-derived collection displayed this particular PBP2x sequence, and one of them, strain 5259, was further characterized in this work.

S. pneumoniae strain 5259 has MICs of 0.19 and 0.094 μg/ml−1 for penicillin G and cefotaxime, respectively. The 5259-PBP2x* sequence displays 28 mutations when compared with the R6-PBP2x* sequence. These mutations are as follows: three in the N-terminal domain (A172T, R254Q, and M256V), three in the C-terminal domain (L710F, Q721E, and T745K) and 22 in the TP domain. The latter 22 substitutions are within the C-terminal part of the TP domain, from amino acids 447 to 616. Among these substitutions, only 9 (I462L, T490S, A491V, D506E, N514H, L565S, D567V, A572V, and D616E) are common to both Sp328-PBP2x* and 5259-PBP2x* sequences. Among the 13 other substitutions in the TP domain, 9 are unique to 5259-PBP2x* (Q447M, S449A, I483L, L517M, P535A, T538N, Q552E, V563T, and Y568N), and 4 occur in both sequences, but the substituted amino acid residue is different (L510V, T513D, L532T, and N576H). The 5259-PBP2x sequence does not display the T398A substitution, which was shown to be an important factor in the development of drug resistance in strains such as Sp328 (26) and is located adjacent to the active site serine; but it does contain a Q552E mutation adjacent to the third catalytic motif of the active site.

Notably, public data bases contain 21 PBP2x* sequences harboring the Q552E substitution in the TP domain. When submitted to a cluster analysis, these sequences were found to differ markedly from those related to that from strain Sp328. Among these sequences, only four also harbor the T398A mutation, whereas one displays a T338P modification. Among the 16 remaining sequences, the ones from strains F2, G54, and Sp1465 are 100% identical from amino acids 266 to 616 to PBP2x from strain 5259.

In Vitro Analysis of 5259-PBP2x*, 5259-PBP2x*E552Q, and R6-PBP2x*Q552E—To gain insight into the molecular mechanism of resistance generated by 5259-PBP2x and to assess the role of the Q552E substitution in PBP2x catalysis and structure, different PBP2x* proteins were produced for structural analysis and in vitro characterization assays. In addition to the production of R6-PBP2x* and 5259-PBP2x*, site-directed mutagenesis was performed at position 552 of both proteins to generate the R6-PBP2x*-Q552E and the 5259-PBP2x*-E552Q mutants. All four proteins were expressed and purified analogously.

The kinetics of the interaction between PBPs and β-lactams can be described by a three-step reaction represented by the following equation (36).

\[
E + I \rightleftharpoons E^* \rightleftharpoons E + P
\]

The first step represents the formation of a noncovalent complex EI with the dissociation constant \( K = k_{-1}/k_{1} \), followed by the acylation of the active site serine by the β-lactam molecule with the rate constant \( k_{4} \). Those two steps are termed the “acylation efficiency” and are characterized by the second order rate constant \( k_{4}/K \). In this work, the acylation efficiency was measured at pH 7.0 by monitoring the decrease of the intrinsic fluorescence of each protein upon antibiotic binding. The acylation step is a very fast reaction and was thus analyzed with a stopped-flow apparatus coupled to a spectrofluorimeter.

The results are presented in Table I and Fig. 1. The introduction of the substitution Q552E into R6-PBP2x* is responsible for a 4.2- and 5.6-fold decrease of the acylation efficiency for cefotaxime and penicillin G, respectively. These values obtained for R6-PBP2x* and for R6-PBP2x*-Q552E are in agreement with the previously reported data (25). The acylation efficiency of 5259-PBP2x* is reduced 15-fold for cefotaxime and 23-fold for penicillin G when compared with R6-PBP2x*. The reversion of the substitution in position 552 within 5259-PBP2x* from Glu to Gln increases the acylation efficiency of the modified protein 2.7-fold for cefotaxime and 4.3-fold for penicillin G. The retained reactivities of R6-PBP2x*-Q552E and 5259-PBP2x*-E552Q toward both of the antibiotics tested are about 20% of the reactivity of the wild type R6-PBP2x* (as shown in Fig. 1).

The final step of the reaction between PBPs and β-lactams
Table II and Fig. 2. The introduction of any of the mutant genes always led to a higher resistance for cefotaxime—determined using the E-test method; results are presented in Table III. The MICs for penicillin G and cefotaxime were calculated on the 687 C–N-terminal domain was clear and interpretable and allowed the reconstruction of the structural model from residue Lys64 onward. Overall Structure of 5259-PBP2x*—To gain insight into a possible novel mechanism of β-lactam resistance displayed by 5259-PBP2x* in atomic detail, we solved its crystal structure by molecular replacement at a resolution of 3.0 Å. The relevant statistics of data collection and model refinement are given in Table III. The overall architecture of 5259-PBP2x* is very similar to that of R6-PBP2x* (22, 24) in that it consists of three domains: an elongated “sugar tong”-like N-terminal domain (residues 50–265) and a central TP domain (residues 266–616) that is connected to a C-terminal region (residues 635–750) by a linker segment (residues 617–634). For both monomers in the asymmetric unit of the crystal, the electron density map in the N-terminal domain was clear and interpretable and allowed the construction of the structural model from residue Lys64 onward. The two monomers in the 5259-PBP2x asymmetric unit are related by a 2-fold noncrystallographic axis that is perpendicular to the c axis and makes an angle of 12° with the a axis. Superposition of the two monomers (using LSQKAB from the CCP4 suite of programs) resulted in an r.m.s.d. of 0.9 Å. The monomers are related by a 2-fold noncrystallographic axis that is perpendicular to the c axis and makes an angle of 12° with the a axis. Superposition of the two monomers (using LSQKAB from the CCP4 suite of programs) resulted in an r.m.s.d. of 0.9 Å.

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The two monomers in the 5259-PBP2x asymmetric unit are related by a 2-fold noncrystallographic axis that is perpendicular to the c axis and makes an angle of 12° with the a axis. Superposition of the two monomers (using LSQKAB from the CCP4 suite of programs) resulted in an r.m.s.d. of 1.2 Å calculated on the 687 Cα positions of residues 64–750. The secondary structure elements are similar, but some differences are observed in the coil regions. The superposition of

Table I

| Variants            | Kinetic parameters of the reaction of the PBP2x* variants with cefotaxime and penicillin G | Penicillin G | Penicillin G |
|---------------------|------------------------------------------------------------------------------------------------|--------------|--------------|
|                     | $k_2/K$<sup>a</sup> | $k_3$<sup>b</sup> | $k_2/K$<sup>a</sup> | $k_3$<sup>b</sup> |
|---------------------|---------------------|---------------------|---------------------|---------------------|
| 5259-PBP2x<sup>a</sup> | 15,200 ± 900        | 3,670 ± 300         | 5.7 ± 0.9           |
| 5259-PBP2x<sup>a</sup>-E552Q | 40,400 ± 2,300 | 15,900 ± 900 | 3.8 ± 0.3 |
| R6-PBP2x<sup>*</sup> | 223,600 ± 20,700       | 84,900 ± 2,900     | 6.4 ± 0.4           |
| R6-PBP2x<sup>*</sup>-Q552E | 54,300 ± 5,800    | 15,100 ± 700        | 3.9 ± 0.4           |

<sup>a</sup> The error is the standard error obtained from the fit of the $k_{app}$ versus [antibiotic] data to the equation $k_{app} = (k_2/K_a)_{[antibiotic]}$.

<sup>b</sup> The error is the standard error obtained from the fit of the $[E^+]_{kin}$ data to the equation $[E^+]_{kin} = [E^+]_{kin}^{0} \exp(-k_3 t)$.

Fig. 1. Comparison of acylation efficiencies of the different PBP2x<sup>a</sup> variants with cefotaxime and penicillin G. The acylation rates were determined by measuring the decrease of intrinsic fluorescence of the proteins. Mean $k_2/K$ values relative to R6-PBP2x* are given with standard deviations calculated from data obtained from three independent experiments.

is deacylation. This step regenerates an active PBP, releasing an inactivated compound, and is characterized by the rate constant $k_3$. The deacylation rate was measured at pH 8.0 by fluorography as described previously (28); results are given in Table I. A slight modification of the deacylation rate was observed in 5259-PBP2x<sup>*</sup> as well as in the other point mutants.

In Vivo Analysis of 5259-PBP2x<sup>*</sup>—To assess the role of the 5259-pbp2x gene in the β-lactam resistance process in S. pneumoniae and to demonstrate the effect of the Q552E substitution, the various modified pbp2x genes were introduced into the recipient genome of the susceptible strain R6 by homologous recombination. The MICs for penicillin G and cefotaxime were determined using the E-test method; results are presented in Table II and Fig. 2. The introduction of any of the mutant pbp2x genes always led to a higher resistance for cefotaxime than for penicillin G. Note that the MIC<sub>CTX</sub> of the 5259-pbp2x2 transformant is thus the same as the MIC<sub>CTX</sub> of the originating strain 5259.

Overall Structure of 5259-PBP2x<sup>*</sup>—To gain insight into a possible novel mechanism of β-lactam resistance displayed by 5259-PBP2x<sup>*</sup> in atomic detail, we solved its crystal structure by molecular replacement at a resolution of 3.0 Å. The relevant statistics of data collection and model refinement are given in Table III. The overall architecture of 5259-PBP2x<sup>*</sup> is very similar to that of R6-PBP2x<sup>*</sup> (22, 24) in that it consists of three domains: an elongated “sugar tong”-like N-terminal domain (residues 50–265) and a central TP domain (residues 266–616) that is connected to a C-terminal region (residues 635–750) by a linker segment (residues 617–634). For both monomers in the asymmetric unit of the crystal, the electron density map in the N-terminal domain was clear and interpretable and allowed the construction of the structural model from residue Lys64 onward.

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Fig. 2. Comparison of the MIC for cefotaxime and penicillin G of S. pneumoniae strains R6 transformed with various pbp2x genes. The MIC<sub>CTX</sub> and MIC<sub>G</sub> of three isolated clones of each transformant were measured by the E-test method. wt, wild type.
the TP domains from the two monomers generates an r.m.s.d. of 0.31 Å (calculated on the 351 Cα of residues 266–616). Larger deviations are observed for residues in the N-terminal domain, which confirms the somewhat greater flexibility that has been observed for this domain in other PBP2x structures. Each N-terminal domain is stabilized in its position by close contacts with the N-terminal domain of the other monomer, involving helices α1, α2, and α3 and strands β2 and β5 (the nomenclature of secondary structural elements follows that of Parès et al. (24)) (Fig. 3). The monomer-monomer interface involves 17 residues from each monomer, of which a majority have hydrophilic side chains. The interaction is stabilized by eight hydrogen bonds and two salt bridges (Arg138 from one monomer to Asp313 from a neighboring monomer). We suggest that these intermolecular interactions stabilize the N-terminal domains, which may explain why they are less disordered than in other crystal structures of PBP2x. During model building, the electron density map corresponding to the side chains of residues that had been mutated into alanines was clearly interpretable.

A least squares fit of the main-chain residues of the TP domain between R6-PBP2x* and 5259-PBP2x* structures generates an r.m.s.d. of 0.6 Å for both monomers. The largest differences in backbone positions are observed in the loops 313–325, 354–361, 374–391, 517–535, and 555–568, which are known to be flexible. Similarly, a least-squares fit with the main-chain residues of the TP domain of Sp328-PBP2x* results in an r.m.s.d. of 0.9 Å. Again, the main differences are observed in the loop regions 353–360, 516–536, and 554–568.

The 5259-PBP2x* Active Site—In the active site of 5259-PBP2x*, the three conserved motifs involved in PBP catalytic activity were clearly traceable (Fig. 4). The first motif, Ser337-X-X-Lys, is located at the bottom of the cleft on the first turn of helix α2. The second element, Ser395−X-Asn, is on a short loop between the α4 and α5 helices. The side chains of Ser395 and Asn396 point into the active site cavity, forming one wall of the active site cavity. The third motif, Lys547−Ser-Gly, is situated on the innermost strand (β3) of the central sheet. These three latter residues form the opposite wall of the active site cavity.

The majority of the mutations (17 of 22) that are observed in 5259-PBP2x* are concentrated in the active site area of the TP domain (Fig. 3). Only a few point mutations occur in the N- and C-terminal domains. A similar observation was made in the crystal structure of PBP2x from drug-resistant strain Sp328. The lack of mutations within the sugar translocating region in both mutant structures thus reinforces the hypothesis that residues located within this domain may play important roles in protein-protein recognition (23). In the TP domain, most of the mutations are exposed to the surface and surround the active site cavity. Other groups of mutations are located above the active site cleft in helix α9 and in the loop connecting helix α9 to strand β3, as well as on strand β4 and in the preceding loop (E552–N568), which borders the right side of the active site. Except for strand β4, these segments are flexible and display different conformations in the various PBP2x crystal structures reported.

Among these substitutions, Q552E, Q447M, S449A, and Y568N are located within a distance of 10 to 12 Å from Ser537 and are positioned strategically to play important roles in the drug resistance phenomenon. Q552E is located after the third catalytic motif Lys547−Ser-Gly at the end of strand β3 and borders the active site cavity. This mutation replaces a polar residue and introduces a negative net charge at the entry of the active site (Fig. 5), an event that clearly disfavors the binding of β-lactams, which bear a global negative charge. The side chain of Glu552 in 5259-PBP2x* adopts the same orientation as Gln552 in R6-PBP2x* and points toward the outside of the active site.

Another active site mutation, Y568N, is located at the N terminus of β4; the Asn side chain thus lies across from that of Glu552. Interestingly, in the recently reported crystal structure of PBP2a from a methicillin-resistant Staphylococcus aureus strain, two identical residues (Glu602 and Asn613) are found in equivalent structural positions (the end of strand β3/beginning of strand β4) (37). The third and fourth mutations, Q447M and S449A, are located at the entry of the active site and buried in the vicinity of the active site serine, respectively. These two substitutions are absent from Sp328-PBP2x*. Whereas the Q552E substitution is found in 21 publicly available sequences and can be considered as a signature mutation, substitutions at positions 447, 449, and 568 are observed only in a subset of sequences.

**Table III**

Data collection and refinement statistics

| Data collection | No. of measured reflections | No. of unique reflections | Multiplicity | Completeness (%) | Rsym (%) |
|-----------------|-----------------------------|---------------------------|--------------|-----------------|----------|
| Resolution limits (Å) | 42–3.0 | 657,234 | 57,788 | 11.4 (5.1) | 96.5 (77.2) |
| [I/σ(I)] | 29.7 (3.0) | 9.2 (42.6) |

Refinement

| No. of reflections | 52,897 | 4,725 | 687 | 45 |
|-------------------|---------|--------|-----|----|
| No. of omitted reflections | | | | |
| No. of water molecules | | | | |
| R-factor (%)/R-free(%) | 23.1/25.6 |
| Mean on B-factors (Å²) | 60.3 |
| Protein | 60.3 |
| Solvent | 42.7 |
| r.m.s.d. from ideal geometry | 0.007 |
| Bond lengths (Å) | 1.3 |

* The numbers in parentheses represent the values for the highest resolution shell 3.16–3.0 Å.
The most noticeable difference between the 5259-PBP2x* active site and that of PBP2x* from the drug-sensitive R6 strain lies on the positioning of $\beta_3$, especially at the level of residues Ser548 to Thr550 (r.m.s.d. = 0.5 Å; Fig. 5, the overlaid yellow strand). It is interesting that, in PBP2a from S. aureus (37), $\beta_3$ also displays a noticeable bend (away from the central region of the sheet); these authors have suggested that such positioning plays an important role in the development of drug resistance by strains harboring PBP2a.

Interestingly, the 5259-PBP2x* active site is very different from the one observed for PBP2x* from pneumococcal strain Sp328. Although 5259-PBP2x* displays a closed active site that is reminiscent of the PBP2x* from the drug-sensitive strain R6, the active site of Sp328-PBP2x* is in an open configuration due to the presence of clashing mutations (23). The fact that the same protein may, by homologous recombination, acquire completely different active sites suggests that S. pneumoniae employs PBP2x in the development of drug resistance via two very different mechanisms.

**DISCUSSION**

$\beta$-Lactam resistance in S. pneumoniae is achieved by the acquisition of mosaic PBPs with decreased affinities for $\beta$-lactam antibiotics through intra- and interspecies homologous recombination (38, 39), which generate tens of substitutions within distinct sequences (12). PBP2x, the primary resistance determinant in S. pneumoniae (20), exists under different forms depending on the phenotypic background from which it comes (14). In this work, we have classified PBP2x proteins from clinical isolates into three main groups depending on their primary structures. Sequences coming from susceptible strains are closely related to the sequence of the drug-sensitive strain R6. Mosaic sequences isolated from resistant strains can be divided in two groups, those that harbor the T338A substitution and are related to PBP2x from the highly resistant strain Sp328 and those that can be defined by the landmark presence of the Q552E substitution. In this work, we have performed the biochemical and structural characterization of a PBP2x from drug-resistant strain 5259, which belongs to the latter group of sequences.

In previous work (25), the introduction of the Q552E mutation in R6-PBP2x was reported to play an important role in the modulation of the sensitivity of PBP2x to $\beta$-lactams by reducing the acylation efficiency. The role of this substitution is reinforced by the fact that the reversion from Glu to Gln in position 552 of the 5259-PBP2x* led to an increase of the acylation efficiency by a factor 2.7 for cefotaxime and 4.3 for penicillin G. The kinetic parameters of the reverted 5259-PBP2x* in position 552 are close to the ones determined for R6-PBP2x*-Q552E. The MICs of the transformant cells harboring either the 5259-pbp2x*-E552Q or the R6-pbp2x*-Q552E genes are identical and are 0.047 and 0.032 g.ml$^{-1}$ for cefotaxime and penicillin G, respectively. Thus, the effect of the single Q552E substitution is equivalent to the global effect of the 27 other substitutions spread throughout the 5259-PBP2x*. The Q552E mutation, the key determinant substitution for this group of PBP2x proteins, is thus responsible for a 4.1- and 5.6-fold decrease of the acylation efficiency for cefotaxime and penicillin G, respectively. Thus, the effect of the single Q552E substitution is equivalent to the global effect of the 27 other substitutions spread throughout the 5259-PBP2x*. The Q552E mutation, the key determinant substitution for this group of PBP2x proteins, is thus responsible for a 4.1- and 5.6-fold decrease of the acylation efficiency for cefotaxime and penicillin G, respectively. These values are in good agreement with the previously published data (25). The kinetic effect of this substitution in the molecular background of the R6-PBP2x* is 2-fold higher than that of the T338A substitution, which is the land-
mark mutation for PBP2x molecules from Sp328-like strains (26).

The crystal structure of 5259-PBP2x displays high similarity in the overall fold presented by the structure of PBP2x* from drug-sensitive strain R6 except within the active site region. In this area of the molecule, strand β3, notably within the segment Ser446–Thr550, is displaced by 0.5 Å (with a maximum of 0.7 Å for Thr550) from the position occupied by the same strand in R6-PBP2x. It should be noted that strand β3 is not particularly disordered (residues 544–552 display average temperature factors of ~ 60 Å² in both monomers). Moreover, the other strands, β4 and β5, superimpose very well with the corresponding strand in the R6-PBP2x structure. Consequently, we suggest that the movement of β3 could be related to the development of β-lactam resistance in pneumococci. Interestingly, the poor acylation rate of PBP2x from another Gram-positive pathogen, S. aureus, is known to be correlated to a distorted active site (37). The active site cavity of PBP2x is in a closed conformation, thus restricting the accessibility of the positive pathogen, pneumococcal strains is still marked. Consequently, it is the case for PBP2x* molecules from Sp328-like strains. This closed conformation, thus restricting the accessibility of the active site cavity of PBP2x is in a distorted active site (37). The active site cavity of PBP2a is in a closed conformation, thus restricting the accessibility of the active site cavity. Consequently, it is the case for PBP2x molecules from Sp328-like strains. This closed conformation, thus restricting the accessibility of the active site cavity of PBP2x is in a distorted active site (37). The active site cavity of PBP2x is in a closed conformation, thus restricting the accessibility of the active site cavity. Consequently, it is the case for PBP2x* molecules from Sp328-like strains.

Most mutations in the 5259-PBP2x structure are located within the active site region, with four mutations, namely Q447M, Y568N, S449A, and T552E, located within a 12-Å distance within the active site region, with four mutations, namely Q447M, Y568N, S449A, and T552E, located within a 12-Å distance. In the structure of PBP2a, strand β3 adopts a twisted conformation to accommodate the N terminus of the α2 helix. Furthermore, for acylation to occur, the N terminus of helix α2 undergoes a conformational change to position Ser403 for nucleophilic attack. Concomitantly, this conformational rearrangement engenders a twisting movement on β3 and a consequent displacement of the central (β-sheet) (37). It is thus conceivable that the mechanism of resistance displayed by pneumococcal strains that carry PBP2x molecules with similarities to 5259-PBP2x involves local movement of strand β3, as is the case for PBP2a.

In the structure of the acyl enzyme intermediate of R6-PBP2x* with cefuroxime, it was shown that, upon binding of the antibiotic, the side chains of Glu452 and Glu552 adopt new rotameric conformations and point toward the outside of the active site, thus opening the ground of the active site cavity (22). In particular, a hydrogen bond with the Glu447 side chain stabilizes the new conformation adopted by the Glu552 side chain. The Q447M mutation in 5259-PBP2x* abrogates this interaction and therefore disfavors the opening of the active site, and it may also affect the β-lactam resistance mechanism.

The crystal structure of PBP2x from the highly drug-resistant strain Sp328 showed that mutation of threonine 338 into an alanine led to the abrogation of a crucial hydrogen bond between the hydroxy group of the threonine residue and a buried water molecule. In addition, two other key mutations, S389L and N514H, led to an “open” active site due to the positioning of the His514 side chain within the region where α4 should be located in order to close the catalytic cleft. This clash could thus be responsible for the loss of affinity of the protein toward β-lactams. In the case of 5259-PBP2x, although the active site remains closed, the drug resistance profile of transformed and clinical strains is still marked. Consequently, it is conceivable that PBP2x proteins can participate in drug resistance phenomena by employing two distinct mechanisms, which are evident through sequence analyses and structural characterizations. In the first mechanism, an open active site causes a deficiency in ester bond formation and substrate acylation (Sp328 (23)). In the second, a closed active site undergoes a local modification of β3 and introduces a negative charge at its entrance, thus providing a less optimal cleft for recognition of antibiotic molecules.

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