The structural modifications induced by the M339F substitution in PBP2x from *Streptococcus pneumoniae*, further decreases the susceptibility to β-lactams of resistant strains.

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SUMMARY

PBP2x is a primary determinant of β-lactams resistance in *Streptococcus pneumoniae*. Altered PBP2x with multiple mutations have a reduced “affinity” for the antibiotics. An important polymorphism is found in PBP2x sequences from clinical resistant strains. To understand the mechanism of resistance, it is necessary to identify and characterize the relevant substitutions. Many similar PBP2x sequences from resistant isolates have the previously studied T338A mutation, adjacent to the active site S337. We report here the structural and functional analysis of the M339F substitution which is found in a subset of these sequences, originating from highly resistant strains. The M339F mutation causes a 4- to 10-fold reduction of the reaction rate with β-lactams, depending on the molecular context. In addition, release of the inactivated antibiotic from the active site is up to 3-fold faster as a result from the M339F mutation. These effects measured *in vitro* are correlated with the level of β-lactam resistance *in vivo* conferred by several PBP2x variants. Thus, a single amino acid difference between similar PBP2x from clinical isolates can strongly modulate the degree of β-lactam resistance. The crystal structure of the double mutant T338A/M339F solved to a resolution of 2.4 Å, shows a distortion of the active site and a reorientation of the hydroxyl group of the active site S337, which can explain the kinetic effects of the mutations.
INTRODUCTION

*Streptococcus pneumoniae* is one of the major human pathogens of the upper respiratory tract. The emergence and increase of resistance to β-lactams, the most widely used antibiotics, is of great concern, as it limits the available options for the treatment of serious pneumococcal infections (1).

β-lactam antibiotics inhibit the transpeptidase activity of the penicillin-binding proteins (PBPs), causing a deficit of cell wall synthesis (2). The peptidoglycan sacculus, the main component of the cell wall, consists of chains of disaccharides reticulated by peptidic cross-bridges. Peptides linked to adjacent glycan chains are joined by transpeptidation, a reaction catalyzed by the transpeptidase domain of the PBPs (3). β-lactams acylate the active site serine of the transpeptidase domain to form a stable covalent adduct. The deacylation, which is slow and insignificant on a bacterial time scale, regenerates the active enzyme and releases an inactivated drug. The resistance of *S. pneumoniae* to β-lactams results from altered PBPs with decreased susceptibilities to these antibiotics (4).

*S. pneumoniae* contains five high molecular mass PBPs. PBP2x is the best studied (8,9) and has the highest affinity for cephalosporins, a class of β-lactam (10,11). The low susceptibility to β-lactam of PBP2x from resistant strains results mainly from a decreased rate of acylation (12-14), although strain CS109 harbors a PBP2x with both decreased acylation and faster deacylation rates (15).

Altered PBP2x from resistant *S. pneumoniae* are encoded by mosaic genes, which are the result of inter- and intra-species recombinational events (6,16). Point mutations may provide another layer of diversity. These two processes lead to great variety of amino acid substitutions (17), of which only a restricted set is likely relevant for the resistance. The observed polymorphism is an important hurdle for the development of sequence-based diagnostic tools for predicting resistance. Moreover, understanding the effect of the
substitutions conferring β-lactam resistance should help the design of better and new β-lactams. It is thus necessary to unambiguously identify and characterize the relevant mutations.

The sequences of \( \text{pbp2x} \) can be divided in three families. Sequences originating from β-lactam susceptible strains are similar to that from the reference strain R6. The two other families of sequences are from resistant strains and exhibit numerous amino acid substitutions, typically more than 30 in the transpeptidase domain. One type is characterized by the T338A substitution. The other type harbors the Q552E substitution, which may sometimes be associated with the mutation in position 338. Within one sequence family, numerous variations are observed. These differences are probably mostly irrelevant to the resistance, but sometimes not. Unique substitutions can modulate the spectrum of sensitivity to β-lactams of resistant strains. For example the T550A mutation increases the susceptibility to penicillins while it decreases the reactivity to cephalosporins (17,18).

We present here a functional and structural study of the M339F substitution, found in several PBP2x sequences harboring the T338A mutation. M339 is located in the active site of the transpeptidase domain, being part of the first conserved motif defining the PBPs: S337XXK. The M339F mutation has been found in the highly resistant clinical strain 5204 isolated in 1999 in Grenoble, France. This substitution has also been described in two strains isolated in 1991 in the USA (strain CS109 and CS111 (18)), and in several strains from Japan (isolated from 1993 to 1997) (17,19). The kinetic parameters of PBP2x from strain CS109 have been measured in details and are characterized by both a decreased acylation and a faster deacylation rate (15).

For comparison, we have chosen a PBP2x with a similar sequence harboring the T338A mutation but retaining M339. This PBP2x is from strain 4790 isolated in 1999 in Grenoble. The sequence of PBP2x from strain 4790 is very similar to that from strain Sp328
of known structure (20). In addition, PBP2x from the laboratory strain R6 was used as reference as it exhibits a maximal susceptibility to β-lactams. Various point mutants of these proteins were made and characterized to assess the importance of the M339F substitution in different sequence background. The *pbp2x* gene encoding these proteins were introduced in *S. pneumoniae* to test the correlation between their kinetic parameters and the resistance that they confer. To gain mechanistic insight, the crystal structure of PBP2x harboring the double substitution T338A/M339F has been solved to a resolution of 2.4 Å.

Many PBP2x sequences confer a moderate level of resistance without the M339F substitution. The high level of resistance due to the additional M339F point mutation demonstrates that the level of β-lactam resistance will be predicted with greater accuracy by a number of well characterized substitutions than from the overall sequence profile of the various PBPs.
EXPERIMENTAL PROCEDURES

Bacterial strains, growth conditions and MICs determination—S. pneumoniae strains 5204 and 4790 were isolated at the University Hospital in Grenoble, France, from sputum in 1999 and blood culture in 1996, respectively. S. pneumoniae was grown at 37 °C in an atmosphere of 95 % air-5 % CO₂ on Columbia blood agar plates (bioMérieux). Clones were isolated and overnight cultures were stored at -80 °C.

The minimal inhibitory concentration (MIC) for penicillin-G and cefotaxime for each strain was determined using the agar dilution method with Mueller-Hinton agar supplemented with 5% sheep blood. The concentrations of antibiotics ranged from 0.027 to 0.625 µg.mL⁻¹ and 0.035 to 8 µg.mL⁻¹ for penicillin-G and cefotaxime respectively in 1.25-fold dilution series.

Inocula containing 10⁵ colony forming units per 10 µL spot were prepared by resuspension of overnight agar plate cultures in Brain-Heart Infusion broth (BHI) to a turbidity of 0.5 McFarland standard. After 18 h, the lowest concentration of antibiotic for which the bacteria showed no growth was taken as the MIC. S. pneumoniae control strains ATCC 49619 and R6 were included in each run.

Expression plasmids—S. pneumoniae strains 4790 and 5204 were used as a source of chromosomal DNA for PCR amplification of the pbp2x and pbp1a genes. Genomic DNA was extracted using the High Pure PCR Preparation Kit (Roche). The pbp2x* gene fragment (encoding the extra-cellular domain, residues 49-750) from strain 5204 was amplified as a BamHI-SmaI fragment using the pair of primers: 5’-
TCCCCCGGGTTAGTCTCCTAAAGTTAATTTAAT-3’ and 5’-
CGGGATCCGGGACAGGCCTCGC-3’. The pbp2x* gene fragments from strain 4790 was amplified as a BamHI-SalI fragment using the following primers: 5’-
GTGCGACGGTAGTCTCCTAAAGTTAATTTAATTTTATGTTTTTG-3’ and 5’-
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GGATCCGGGACAGGCACTCGC-3’. The complete pbp1a gene from strain 5204 was amplified as a BamHI-XhoI fragment using the primer pair: 5’-
CCGGGATCCGGATGAACAAACCAACGATTCTGCACC-3’ and 5’-
CCGCTCGAGCGGTCTTTGGGAGGTTGAGG-3’.

The resulting PCR products were cloned in the pGEX-4T1 vector to create the pGEX-4790-pbp2x*, pGEX-5204-pbp2x*, and pGEX-5204-pbp1a plasmids. Inserts were sequenced. The previously described pGEX-R6-pbp2x* plasmid was used to express PBP2x* from strain R6 (12).

Site-directed mutagenesis—Site-directed mutagenesis was performed using the Quick-Change kit (Stratagene). The following primers and their reverse complements were used with pGEX-R6-pbp2x* to introduce the T338A, M339F and T338A/M339F substitution, respectively: 5’-
CCAAAGTAACATGAGCCAGGATGCGCTATGAAAGTGATGTTGTCG-3’, 5’-
GTAACTATGAGCCAGGTTCTTTGACTTAAGTGATGTTGTCG-3’, and 5’-
GTAACTATGAGCCAGGATGCTTTTAAAGTGATGTTGTCG-3’. These primers also introduce the Eco47III, DraI and the double DraI/BamHI restriction sites, respectively, to allow screening for successful mutagenesis.

The pGEX-4790-pbp2x* plasmid was mutated with the following primers and their reverse complements, which introduce the A338T and M339F, respectively, and the respective diagnostic restriction sites Scal and DraI: 5’-
CTATGAAACCAGAAATGACTATGAAAGTTATGACGTTAGCTTTCTTC-3’, 5’-
CTATGAAACCAGATCAGCCTTTAAAGTTATGACGTTAGCTTTCTTC-3’.

The primer 5’-
CAAAAGTAACATGAAACCAGGATCCGCCATGAAGGTCTGATGTTGAGC-3’ and its
reverse complement were used with the pGEX-5204-pbp2x* plasmid to introduce the F339M substitution and the diagnostic BamHI restriction site. The region of the expected mutations was sequenced for each mutant.

Transformation—The non-encapsulated S. pneumoniae R6 strain was used as recipient for genetic transformation. Bacteria were grown in C-medium supplemented with 0.18% albumin (8%, boiled) to an optical density at 620 nm of 0.15, taken as the onset of the exponential phase. Aliquots withdrawn immediately or after further incubation for 30 min were stored in 20% glycerol at −80°C until used. About 50 ng of plasmid DNA was added to 100 µL of competent cells (thawed on ice and diluted 10-fold in C-medium with 0.18% albumin added (8%, boiled)). After incubation for 30 min at 30 °C and 120 min at 37 °C, cells were plated on Columbia blood agar Base EH (Becton-Dickinson) plates enriched with 6% horse blood containing concentrations of cefotaxime ranging from 0 to 0.3 µg.mL⁻¹. After overnight incubation at 37 °C, isolated colonies were picked and grown overnight in glucose buffered broth (Diagnostics Pasteur) to be stored at −80 °C in 20% glycerol. The isolated transformants were verified to be S. pneumoniae by optochine susceptibility and agglutination test. The MIC of the transformed cells for penicillin-G and cefotaxime was determined by the agar dilution method. Finally, genomic DNA was extracted and the php2x and php1a genes were PCR amplified and sequenced.

Protein production and purification—Recombinant R6-PBP2x*, 4790-PBP2x*, 5204-PBP2x* and all the point-mutants described in this work were expressed in E. coli as fusion with glutathione-S-transferase and purified by affinity chromatography and thrombin cleavage as described previously (13). The purity was assessed by SDS-polyacrylamide gel electrophoresis and Coomassie-staining, and their molecular masses were measured by
electro-spray ionization mass spectrometry (ESI-MS). For crystallization purpose, R6-PBP2x*-T338A/M339F was further purified by ion exchange chromatography on a 3 mL Source 15Q resin (Amersham) equilibrated in 50 mM Tris pH 8.0, 10 mM NaCl and 1 mM EDTA. The protein was eluted with a gradient of NaCl concentration (10-120 mM, 30 ml) in the same buffer. Finally, pooled fractions were loaded onto a 120 mL Superdex-200 gel filtration column (Amersham) equilibrated with 50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA. The protein was eluted as a single symmetrical peak.

**Crystallisation, data collection and processing**—Purified R6-PBP2x*-T338A/M339F in 50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA was concentrated to 12 mg/mL. Crystals were obtained using the vapor diffusion and hanging-drop method. The drops were prepared by addition of glycyl-glycyl-glycine to the protein solution to a final concentration of 100 mM, 2 µL of this solution were then mixed with 1µL of the reservoir solution containing 14% polyethylene glycol (PEG) 6000, 100 mM sodium acetate pH 4.6 and 200 mM ammonium sulfate and finally, 0.4 µL of 24.4 mM n-octanoylsucrose (detergent screen n°1 Hampton Research, Laguna Niguel, CA) was added. The drops were equilibrated against 500 µL of the reservoir solution and kept at 8 °C. Under these conditions, crystals appeared within one day and reached their maximum size after five days.

R6-PBP2x*-T338A/M339F crystals belong to the monoclinic system and have the unit-cell parameters \(a = 128.22 \, \text{Å}, \ b = 64.75 \, \text{Å}, \ c = 144.97 \, \text{Å} \) and \(\beta = 118.89^\circ\) with the space-group \(C2\). The presence of one molecule in the asymmetric unit gives a crystal packing parameter (\(V_M\)) of 3.5 Å\(^3\)/Da and a solvent content of 64.4%.

Prior to data collection, crystals were flash-cooled by immersion into liquid nitrogen, following successive soaking for 2 min in solutions having the same composition as the mother liquor but with 16% PEG, 5% PEG 400 and 5% 2-methyl-2,4-pentanediol (MPD),
increased to 10% MPD and 15% MPD. X-ray diffraction data were collected on beamline BM14 at the European Synchrotron Radiation Facility (Grenoble, France) equipped with a MAR CCD detector and tuned to the wavelength of 0.999 Å. The dataset was obtained from a single crystal with 1° angular increments between images and a crystal-to-detector distance of 140 mm. Raw diffraction images were indexed and integrated with MOSFLM version 6.2.2 (21). Data scaling, merging and reduction was carried out with programs of the CCP4 suite (22). Relevant statistics are given in Table III.

Structure determination—The structure was determined by molecular replacement using the program MOLREP (23) with data from 30 Å to 3.5 Å resolution. The structure of PBP2x from *S. pneumoniae* R6 at 2.4 Å (24) (PDB accession code 1QME) was used as a search model with residues T338 and M339 changed to glycines. The rotation and translation functions gave a single solution with a correlation coefficient of 59.5% and a crystallographic $R$-factor of 39.1%. The coordinates were transformed with the rotation and translation function solution and submitted to refinement with CNS 1.1 (25). A total of 5% of the reflections were randomly selected and excluded from the refinement to constitute a test set for $R_{\text{free}}$ calculations (26) and monitor the refinement progress.

The first cycle of the procedure consisted of 20 cycles of rigid-body refinement with data between 31 and 4 Å. Then, several cycles composed of a simulated annealing with data from 31 to 2.4 Å using a slow cooling protocol from 2000 to 100 °K, an energy minimization, and a grouped-temperature factor refinement (one temperature factor per residue) were applied. The molecular graphic program O (27) was used for map inspection and model building at each cycle. Electron density maps with sigma-A weighted coefficients of type $(3F_{\text{obs}} - 2F_{\text{calc}})$ and $(F_{\text{obs}} - F_{\text{calc}})$ and with phases calculated from the current model were inspected simultaneously. Water molecules, a detergent molecule and a sulfate ion, were
positioned as they were observed as strong peaks in the \((F_{\text{obs}} - F_{\text{calc}})\) electron density map contoured at 3\(\sigma\). The maps showed a significant electron density attributed to one molecule of n-octanoylsucrose. In the final stages, the individual restrained \(B\)-factor refinement was performed.

The stereochemistry of the model was analysed with PROCHECK (28): all non-glycine residues are in the ‘most favored’ (87.4\%) or the ‘additionally allowed’ \(f/y\) regions of the Ramachandran plot. The final model contains 607 of the 702 residues of the recombinant protein. Relevant statistics of the refinement are presented in Table III.

**Determination of the efficiency of acylation**—The \(k_2/K\) parameter was measured by following the decrease of the intrinsic fluorescence of the protein, at various concentrations of a large excess of antibiotic, using an SFM-400 stopped-flow apparatus (Biol-Logic) (13, 29). Measurements were performed at 37°C in a solution of 10 mM sodium phosphate pH7 with 0.6 \(\mu\)M of protein and 10 to 5000 \(\mu\)M of penicillin-G or cefotaxime. The excitation wavelength was 280 nm and the emission was measured above 305 nm using a cut-off filter. The apparent pseudo-first order rate constant \(k_{\text{app}}\) was determined by non-linear fitting of the fluorescence data to equation \(\text{Fluo}_t = \text{Fluo}_0 \cdot \exp(-k_{\text{app}} \cdot t)\) using the Bio-Kine software (Biol-Logic). The efficiency of acylation was determined by least squares linear fitting to the equation \(k_{\text{app}} = (k_2/K) \cdot [\text{antibiotic}]\) with the Kaleidagraph software.

**Determination of the deacylation rate**—PBP2x* proteins were dialysed against a solution of 50 mM Tris (pH 7), 100 mM NaCl and 1 mM EDTA or 100 mM ammonium acetate (pH 7). The proteins (15 to 35 \(\mu\)M) were acylated by incubation at 37°C with an excess of penicillin-G or cefotaxime (500 \(\mu\)M) for 30 min, prior to 5 minutes of incubation with 2 \(\mu\)M recombinant FEZ-1 metallo-\(\beta\)-lactamase from *Legionella gormanii* to remove the
unreacted antibiotic. Aliquots were then withdrawn at various time. The deacylation reaction was stopped by the addition of 0.05% trifluoroacetic acid. Samples were stored at −20°C until analyzed by ESI-MS (13,30).

The relative amount of acylated, EI*, and deacylated enzyme E was determined using either of two mass spectrometers. Using a PE Sciex (Toronto, Ontario, Canada) AP III+ triple quadrupole mass spectrometer equipped with an ionspray source, samples were loaded on a C4 Macrotrap cartridge (Michrom) desalted with H₂O 0.1% trifluoroacetic acid, and proteins were eluted with 60% of CH₃CN/H₂O 9/1 (v/v) 0.1% trifluoroacetic acid. Spectra were recorded in the 1100-1300 and 500-1500 range of mass-to-charge (m/z) ratios by steps of m/z 0.5, with a 8- and 2-ms dwell time, respectively. The signal was averaged over 4 scans. The electrospray probe tip was held at 5 kV, and the declustering voltage was set at 80 V. Data were processed using the MasSpec 3.3 software. Using a Q-TOF Micro spectrometer (Micromass, Manchester, UK) equipped with an electro-spray ion source and operated with a needle voltage of 2.7 kV and a sample cone voltage of 60 V, samples were diluted in CH₃CN/H₂O 1/1 (v/v) 0.2% formic acid and the flow rate was 5 µL.min⁻¹. Spectra were recorded in the 700-2000 range of mass-to-charge (m/z). The rate constant k₃ was determined by non-linear least-square fitting to the first order equation [EI*]ₜ = [EI*]₀exp(-k₃t) using the Kaleidagraph software.
RESULTS

Sequences—*S. pneumoniae* strains 5204 and 4790 have MICs of 6.0 mg L\(^{-1}\) and 1.5 mg L\(^{-1}\) for penicillin-G and 12.0 mg L\(^{-1}\) and 1.0 mg L\(^{-1}\) for cefotaxime, respectively, as measured by E-test on blood agar plates. The *php2x* gene from both strains has been cloned and sequenced.

5204-PBP2x possesses the M339F mutation in the catalytic motif close to the active site S337. 5204-PBP2x has 80 amino acid changes compared to R6-PBP2x, of which 41 are located within the transpeptidase domain (residues 266-616). 4790-PBP2x has 75 residue substitutions relative to R6-PBP2x, including 39 changes in the transpeptidase domain. Of the 41 amino acid changes in the transpeptidase domain of 5204-PBP2x, 37 are shared with 4790-PBP2x. The PBP2x transpeptidase domain from strain 4790 and 5204 are nearly identical as they differ only at 5 out of 350 positions: M339F, T343M, D378A, M400T and Y595F, where the first amino acid is that from 4790-PBP2x and the second from 5204-PBP2x. Both proteins harbor the T338A substitution, which has been studied previously (13) and defines one family of resistant PBP2x sequences.

Two substitutions are found between the transpeptidase domain of 4790-PBP2x and that from strain Sp328, which has been well characterized (31) and whose structure is known (20). Those changes are D378E and V572A, which are both conservative, are not part of the active site, and likely unimportant.

The transpeptidase domain of 5204-PBP2x differs only at four positions from that of strain CS109 which has been extensively characterized (18,19): A378E, L389S, N574T and F595Y. The PBP2x from strains 5204 and CS109 are thus nearly identical.

*Site-directed mutagenesis and protein production*—To assess the importance of the M339F mutation in various sequence background, different PBP2x proteins have been
produced for in vitro characterization. The soluble extracellular domain (residues 49 to 750, denoted by an asterisk) of PBP2x from strains 5204, 4790, and R6, termed 5204-PBP2x*, 4790-PBP2x*, and R6-PBP2x*, respectively, were expressed in E. coli as glutathione S-transferase fusion proteins. In addition, various point mutants were generated in positions 338 and 339. The recombinant fusion proteins were bound on a glutathione-sepharose matrix and PBP2x* proteins were released by thrombin cleavage at a specific site engineered between PBP2x and the glutathione S-transferase. The proteins were homogenous as judged by Coomassie-stained polyacrylamide gels and yields ranged from 10 to 30 mg per liter of culture. The molecular masses of all proteins were checked by ESI-MS. For crystallography, R6-PBP2x-T338A/M339F was further purified by anion-exchange and gel filtration chromatography.

Kinetics of acylation—The rate of acylation by β-lactam antibiotics is severely diminished in PBP2x from resistant strains. To assess the effect of the M339F substitution on the acylation process, we measured the kinetics of the reaction of various PBP2x* with two β-lactams. The interaction between PBPs and β-lactams is classically described by the following equation:

\[
E + I \xrightleftharpoons[K_{-1}]{k_2} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E + P
\]

where E is the enzyme, I is the β-lactam, EI is a non-covalent complex with the dissociation constant \(K\), the covalent acyl-enzyme EI* is formed with the rate constant \(k_2\), and finally the enzyme is deacylated with the rate constant \(k_3\), to regenerate an active enzyme and release an inactivated compound P. The acylation efficiency, characterized by the second order rate constant \(k_2/K\), was measured at pH 7 with cefotaxime and penicillin-G in a stopped-flow apparatus by monitoring the decrease of the intrinsic fluorescence of the protein upon
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binding of the antibiotic. The results are presented in Table I and Figure 1. The values obtained for R6-PBP2x* and R6-PBP2x*-T338A are in agreement with previously published data (12,15,29). Acylation efficiency is reduced by the M339F substitution for both cefotaxime and penicillin. For cefotaxime, the acylation efficiency is decreased 4- and 6-fold in the context of R6-PBP2x* and 4790-PBP2x*, respectively. The reverse substitution, i.e. F339M in 5204-PBP2x*, causes a 9.8-fold increase of the acylation efficiency for cefotaxime. For penicillin-G, the M339F substitution decreases the efficiency of acylation 6- and 3-fold in R6-PBP2x* and 4790-PBP2x*, respectively, whereas F339M in 5204-PBP2x* increases the reaction rate 3.6-fold. In either R6-PBP2x* or 4790-PBP2x* background sequences, the double substitution T338A/M339F causes a 20-fold reduction of the acylation efficiency for both cefotaxime and penicillin-G.

Kinetics of deacylation—The deacylation rates of PBP acyl-enzymes can be determined using either fluorography or ESI-MS (15,30). ESI-MS was used as it allowed the study of proteins with low acylation rates, which could not be significantly acylated with the low amounts of radio-labeled antibiotics available for fluorography. Using high concentration of unlabeled β-lactams, it was possible to acylate completely even the slowest reacting proteins. The deacylation rates were determined at pH 7 with cefotaxime and penicillin-G. Results are given in Table I and Figure 2. The values of $k_3$ obtained for R6-PBP2x* and R6-PBP2x-T338A are in agreement with previously published data (13,15,30). The values measured with 4790-PBP2x* are close to those reported for the nearly identical PBP2x from strain Sp328 (30). The deacylation of 5204-PBP2x* with penicillin-G is more than 30-fold faster than the deacylation of R6-PBP2x*. PBP2x from strain CS109, which also contains the M339F mutation, has been reported to deacetylate 70-fold faster than R6-PBP2x* (15).
The effect of the M339F mutation on the deacylation is readily apparent as its reversal in the context of 5204-PBP2x* slows the deacylation for penicillin-G by a factor of 3. Conversely, introduction of the M339F substitution in 4790-PBP2x accelerates the deacylation 1.5-fold. However, in the sequence context of R6-PBP2x*, the M339F substitution does not affect significantly the deacylation rate.

The deacylation rates of the PBP2x*’s from clinical strains and their derivatives could not be measured with cefotaxime. With the high concentration of antibiotic required to acylate these proteins, the deacylation kinetics were obscured by additional species observed by mass spectrometry. These extra-species resulted possibly from non-covalent binding of native and β-lactamase-hydrolyzed cefotaxime, as observed in the crystal structure of R6-PBP2x (24).

*Phenotypes*—The degree of resistance in clinical strains of *S. pneumoniae* depends on several modified PBPs. Altered *pbp2x, pbp2b* and *pbp1a* genes are always found in pneumococci that have MIC ≥0.1 µg.mL⁻¹ for penicillin-G (6). It is therefore difficult to assess the relative importance of individual mutations in *pbp* genes directly from the clinical strains. To measure the phenotypic importance of the M339F mutation, the R6 susceptible strain was transformed with plasmids encoding 5204-PBP2x*, 4790-PBP2x*, R6-PBP2x*, and their variants. PBP2x is the most sensitive target of cefotaxime and is therefore the primary determinant of first level resistance to this antibiotic in laboratory mutants (9). Strains growing in the presence of a cefotaxime concentration higher than the non-transformed strain were isolated and had their MIC determined. Results are presented in Table II and Figure 3. Transformation using an empty pGex vector or the plasmid encoding R6-PBP2x* did not confer resistance to penicillin-G or cefotaxime. The *pbp2x* gene fragment
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coding the transpeptidase domain was PCR amplified from the transformed strains and sequenced to insure that proper allelic replacement had occurred.

The point mutant R6-\textit{pbp2x*}-T338A did not allow the selection of transformed cells whereas R6-\textit{pbp2x*}-M339F was sufficient to confer a selectable resistance. Transformation with the double mutant R6-\textit{pbp2x*}-T338A/M339F produced strains with a higher level of resistance. Except for the 4790-\textit{pbp2x*}-A338T transformants, strains transformed with \textit{pbp2x} genes from clinical origin displayed identical resistance for cefotaxime. This threshold of susceptibility indicated that the MIC in these strains is probably not determined by the inhibition of PBP2x.

Pneumococci have six PBPs, yet alterations in PBP2x and PBP1a are sufficient to confer high resistance to cephalosporins (18,32), indicating that PBP1a is the second most sensitive PBP to this class of β-lactam. Consequently, in order to reveal the different levels of resistance due to the PBP2x variants, which might be obscured by the inhibition of R6-PBP1a, the \textit{pbp1a} mosaic gene from the resistant strain 5204 was co-transformed with the \textit{pbp2x} genes. Transformed cells were selected on cefotaxime and both \textit{pbp} genes of the isolated strains were sequenced.

No R6-\textit{pbp2x*} /5204-\textit{pbp1a} and R6-\textit{pbp2x*}-T338A /5204-\textit{pbp1a} co-transformants were selected on cefotaxime. Co-transformation of R6-\textit{pbp2x*}-M339F and R6-\textit{pbp2x*}-T338A/M339F with 5204-\textit{pbp1a} yielded strains with the same decreased sensitivity to cefotaxime as strains singly transformed with the corresponding \textit{pbp2x} genes. Thus, in those cases, the \textit{pbp1a} mosaic gene did not increase the resistance as the inhibition of PBP2x is likely preventing growth.

In contrast, co-transformation of 5204-\textit{pbp1a} with the \textit{pbp2x*} genes from or derived from the clinical strains 4790 and 5204 produced a greater resistance than single transformations. Thus, the presence of the 5204-\textit{pbp1a} gene reveals the phenotypic
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difference conferred by the M339F mutation. The M339F substitution in 4790-PBP2x causes an increase of the resistance, whereas the reverse F339M substitution in 5204-PBP2x causes the resistance level to drop.

Surprisingly, cotransformants of 4790-pbp2x*-A338T and 5204-pbp1a could be isolated and have a higher MIC_{CTX} than the single 4790-pbp2x*-A338T transformant, although the low resistance of the latter indicated that inhibition of PBP2x, but not of PBP1a, was limiting. This paradox may indicate that complex relationships exist between the various PBPs.

Overall architecture— The double mutant R6-PBP2x*-T338A/M339F is folded in three domains: an elongated ‘sugar tong’-like N-terminal domain (residues 50–265), a central transpeptidase domain (residues 266–616, Figure 4) followed by a linker segment (residues 617-634) and a C-terminal domain (residues 635–750). The N-terminal domain could not be constructed entirely because the electron density map was discontinuous over several segments. The segments that were visible and could be modeled are 73-98, 120-141, 149-164, 183-231 and 257-265. The linker segment connecting the transpeptidase domain to the C-terminal domain was clearly visible in the electron density map and could be traced, whereas this region was not defined in the map of the R6-PBP2x* crystal structure (24). The overall structure of double mutant protein is close to that of R6-PBP2x* with few exceptions. A least-squares fit of the main-chain residues of the transpeptidase domain between the two structures gives a r.m.s.d. (root mean square deviation) of 1.03 Å and of 0.52 Å if residues 359–386 are excluded. A detailed comparison reveals differences higher than 0.8 Å in five regions (Figure 4). The largest deviations are located in loop regions, which are known to be flexible (319-324, 359-386, 564-567). In the other PBP2x structures available (20,24) these regions have high temperature factors and/or display different conformations. The largest
differences in backbone positions are observed in the loop 359-386 (up to 9.2 Å). Interestingly this region to the left of the active-site cleft (Figure 4) was poorly resolved in the native and acyl-enzyme complex structures (24). Moreover, a longer region (364-394) was not traceable in PBP2x from the penicillin-resistant clinical isolate Sp328 (20). In the present structure the stabilization and conformational changes in this region (359-386) could be related to the binding of a detergent molecule, which is visible in the electron density map in the vicinity of the loop. Lastly, two differences are observed near the active site: the main-chain tracing deviates at the N-terminus of helix α2 between residues 336–340 (mean r.m.s.d. of 0.85 Å) and in strand β3 between residues 548–550 (mean r.m.s.d. of 0.71 Å).

Comparison of the active sites of R6-PBP2x* and R6-PBP2x*-T338A/M339F—A superposition of the active sites of double mutant and R6-PBP2x* is shown in Figure 5, where the three conserved sequence motifs (S337XXK; S395XN; K547SG) of the transpeptidase domain are represented. The first motif contains the active site serine S337 located at the N-terminus of the helix α2. The second motif is on a loop between helices α3 and α4. The third motif is located on strand β3 that is facing the active site serine S337. The electron density in these regions was well defined and alanine and phenylalanine side-chains could be attributed in positions 338 and 339, respectively.

A significant difference between the main-chain tracings in the two structures is found in the N-terminal extremity of the helix α2, especially for the residues 338 and 339 whose main-chain atoms deviate by ca. 1 Å. Another major difference is observed in the substrate-binding site where the side-chain of the nucleophilic serine S337 adopts a different orientation. In the R6-PBP2x* structure the side-chain of S337 is directed into the active site and is connected by a hydrogen bonding network to the extremity of the side-chain of K340 (2.9 Å) and S395 (3.2 Å). In sharp contrast, the double mutant features an Oγ atom of S337
that points towards the β3 strand, and is hydrogen bonded (3.0 Å) to the main-chain nitrogen atom of T550 and to a sulfate ion (3.0 Å). This orientation of the S337 side-chain in double mutant was confirmed by the calculation of omit-type electron density maps, where the atoms of S337 were excluded, thus minimizing model bias. Furthermore, the two alternative rotamer conformations of S337 were generated and these models were subjected to slow-cool simulated-annealing refinement with CNS 1.1. In each case, the refinement eventually placed the S337 side-chain in the orientation that is observed.
DISCUSSION

Antibiotic resistance has spread in recent years and the current medical practice, in case of severe infection, is to treat with a combination of wide spectrum antibiotics, while the pathogen is being identified and its susceptibility to various drugs established by classical microbiology techniques. It is likely that the advent of rapid sequencing and the various DNA chip technologies will speed diagnostic and resistance determination to the point that an adapted treatment can be prescribed immediately.

The challenge with the resistance to β-lactams in *S. pneumoniae* is that numerous similar sequences of PBP variants are associated with various levels of resistance. Thus, one finds in the public databases 26 closely related sequences of the PBP2x transpeptidase domain containing the T338A substitution. These sequences from amino acid 277 to 576 differ by at least 32 and at most 45 residues from that of the sensitive R6-PBP2x. For example, if PBP2x from strain Sp328 (31) is taken as the prototypical example of this family, 4790-PBP2x differs at two positions, while 5204-PBP2x and PBP2x from strain CS109 differ by 4 residues. Most of the minor modifications of sequence within a family are likely non-significant but some may be highly relevant as demonstrated with the M339F mutation in the present work.

The importance of the M339F substitution was suggested by the fact that it is reported only in 8 highly resistant strains (MIC$_{CTX}$ ≥ 2) (17,18,33,34). Comparing the reaction rates of 5204-PBP2x* (or PBP2x isolated from strain CS109 (15)) to that of 4790-PBP2x* (or the very similar PBP2x isolated from strain Sp328 (30)), suggests that the M339F mutation causes a 6- to 8-fold decrease of the efficiency of acylation by cefotaxime. Site-directed mutagenesis demonstrates that the M339F mutation decreases the acylation efficiency 3- to 10-fold, depending on the sequence context and the β-lactam. The reason for the discrepancy between the effect of the M339F mutation in the sequence background of 5204-PBP2x* and...
4790-PBP2x* has to be found in the few other residues that differ between these two proteins and are listed in the Results section. Of those positions, the M400T substitution is present in all PBP2x that have the M339F mutation and is therefore likely to modulate the effect of the latter.

The effect of the M339F substitution seems greater on the reaction with cefotaxime than with penicillin-G, when the T338A mutation is present. It is likely that the M339F substitution found in clinical strains was selected by treatment with cephalosporins. Indeed, when comparing the strains that harbor modified PBP1a, PBP2b and PBP2x, characterized by Asahi and coworkers, those with the additional M339F have a resistance increased by a larger factor to cephalosporins than to penicillin (17). However, the differential effect of the M339F substitution is not as marked as that of the T550A mutation, which results in a 17-fold decrease of the acylation efficiency for cefotaxime only (12).

In position 338 the mutation of the threonine into alanine has been shown to be a key structural determinant for β-lactam resistance in S. pneumoniae (13). In the R6-PBP2x* structure, the threonine T338 points out of the active site and is hydrogen-bonded to a buried water molecule (O2, B = 22.5 Å²) which is stabilized by a constellation of hydrogen bonds involving P335, S571, Y586 and another water molecule (O93, B = 36.9 Å²). In the structure of double mutant, both water molecules (labeled O7 and O18 respectively) are clearly seen in the electron density map and the same network of hydrogen bonds is present, except the one involving the side-chain of residue 338. The values of the temperature factors for O7 and O18 are B = 26.2 Å² and 28.1 Å², respectively. Interestingly, in the structure of PBP2x from strain Sp328 solved at 3.2 Å, which also contains the T338A mutation (20) the O7 water molecule was not observed.

In the structure of the double mutant the side-chain of the phenylalanine at position 339 points in the same direction as the corresponding methionine side-chain in R6-PBP2x*. The
phenylalanine side-chain of the residue is buried in a narrow hydrophobic cleft. Only one rotameric configuration is possible for this side-chain; the alternative rotamers would generate steric clashes, either with the side-chain of P335 or with the carbon backbone of F450-G451. Consequently, the replacement of a flexible methionine by a rigid phenylalanine induces a strain in the backbone protein near the active site serine. The strain induces a movement of up to 0.94 Å in the Cα position. Interestingly, the residue volume sum of the two side chains (Thr and Met) for the native or (Ala and Phe) for the double mutant have exactly the same value (279 Å³). The distortion of the active side allows the rotation of Oγ S337, from -69.9° to 57.4° and the formation of a hydrogen bond between Oγ and the main-chain nitrogen atom of T550, which is displaced by 0.83 Å. This interaction may contribute to stabilize a local distortion of strand β3. The high resolution structure of a complex between R6-PBP2x* and cefuroxime revealed that T550 is in direct contact with the cephalosporin (24). The substitution T550A was shown to decrease 17-fold the efficiency of acylation by cefotaxime, whereas the reaction rate with penicillin-G is not affected (13). Displacement of T550 due to the M339F substitution could similarly affect binding of cephalosporins to a greater extent than binding of penicillin-G, and explain the different effect of the M339F mutation on the reaction with cefotaxime and penicillin-G.

The observed position of the Oγ atom of S337 could be the result of different factors: distortion of the active site, presence of the sulfate ion and crystal packing. An alternative rotamer of S337 is observed in all the other available structures of PBP2x. We can speculate that in one of the two possible rotamers, the Oγ atom of S337 is correctly positioned for nucleophilic attack on the β-lactam ring, whereas the Oγ atom is inappropriately located in the other rotamer. While the various crystal structures may freeze one conformation or the other, both rotamers may be in dynamic equilibrium in solution. Mutations may change the rotameric equilibrium of S337 in favor of the reactive or non-reactive conformation. Thus,
the acylation rate $k_2$ would lowered if the proportion of enzyme with the reactive rotamer of S337 were reduced.

To summarize, the T338A mutation disrupts the stabilizing hydrogen bond to the conserved water molecule O7 and the M339F mutation introduces a bulkier side-chain which puts additional constraints on the conformation of the main-chain. These two mutations can thus explain the rearrangement of the N-terminus extremity of the helix $\alpha_2$ and the displacement of strand $\beta_3$ in the double mutant. These local changes favor an alternative conformation of the active site S337, a structural modification that is likely to affect the catalytic machinery of the protein and could explain the reduced acylation rate of R6-PBP2x*-T338A/M339F.

Besides the effect of the T338A/M339F double substitution, the other 39 mutations spread along the transpeptidase domain of 5204-PBP2x* are also responsible for a 20-fold decrease of the acylation efficiency. The combined 39 others mutations have thus an equivalent effect to that of the T338A/M339F double mutations. Indeed 4790-PBP2x-A338T, which is R6-like at the active site position 338 and 339, still has a decreased reactivity towards $\beta$-lactams and can confer resistance to $S. pneumoniiae$. Extensive mutagenesis studies will be required to identify, among the substitutions that are not localized in the active site, those that are relevant for the diminished reactivity. Moreover, substitutions that do not reduce the efficiency of acylation may be important for the resistance as they can compensate for a loss of stability due to kinetically relevant mutations (35).

The $php2x$ genes of this study, once transformed into the R6 recipient strain, were able to confer a selectable resistance to cefotaxime, with the exception of that encoding R6-PBP2x-T338A. Remarkably, the single amino acid substitution M339F increases the resistance towards cefotaxime of the host bacteria from \( \leq 0.016 \mu g.mL^{-1} \) to 0.055 $\mu g.mL^{-1}$, whereas the single T338A mutation is not sufficient to induce a selectable modification of
resistance. As these PBP2x variants have similar deacylation rates, it implies that a 6-fold reduction of the efficiency of acylation can impart a significant level of resistance whereas a diminution by a factor two cannot.

The double substitution T338A/M339F confer a level of resistance to β-lactams that is comparable to that of some clinical strains. This observation raises the question of why point mutations in these positions have never been obtained by artificial selection in laboratory experiments (9,10,36). One possibility is that picking the most resistant clones, as performed in laboratory experiments, necessarily picks the substitution with the greatest effect, such as T550A, but ignores substitutions with milder consequences such as M339F.

PBP2x proteins, even with very low reactivities towards cefotaxime, do not confer a high level of resistance in strain R6, because inhibition of PBP1a is then detrimental to the cells. When this limitation is suppressed by the presence of a modified PBP1a, the full effect of the PBP2x modifications is revealed. Indeed, there is a rough correlation between the MIC_{CTX} resulting from co-transformations of altered pbp2x and pbp1a genes and the reduced efficiency of acylation ($k_2/K$) of the corresponding PBP2x proteins. The proportion of acylated vs. total amount of enzyme at steady-state is given by the following equation when the concentration of antibiotic [I] is in large excess (37): $[\text{EI}^*]/[\text{E}]_{\text{total}} = (k_2/K)[I]/(k_2/K)[I]+k_3$, hence, the concentration of antibiotic for which half of the enzyme is free is given by: $c_{50} = k_3/(k_2/K)$. If the deacylation rate $k_3$ is important for the resistance phenomenon as hypothesized by Lu and coworkers (15), the MIC should show a better correlation with $c_{50}$ than with $k_2/K$. Unfortunately, we were not able to measure the deacylation rates of 4790-PBP2x*, 5204-PBP2x* and their derivatives for cefotaxime. Nevertheless, assuming that the $c_{50}$ for penicillin-G reflects that for β-lactams in general, the resistance conferred by the PBP2x’s with lowest susceptibilities to β-lactams appears better correlated with $c_{50}$ than with $k_2/K$, supporting the phenotypic relevance of the deacylation.
One curious observation is that co-transformants 4790-\textit{pbp2x}-A338T/5204-\textit{pbp1a} could be isolated that have a MIC\textsubscript{CTX} greater than the single 4790-\textit{pbp2x}-A338T transformant. This was unexpected as the resistance of the 4790-\textit{pbp2x}-A338T transformant is lower than the susceptibility threshold due to the inhibition of PBP1a. An explanation is that mosaic genes were selected not only for conferring a low susceptibility to β-lactams, but also for the optimal functional interaction of the two PBPs. In support of this hypothesis, it has been observed that successive round of transformation of R6, starting with a \textit{pbp2x} gene from \textit{S. oralis} conferring resistance to cefotaxime, restrict the mosaicity to the immediate vicinity of the important codon (8). This observation implies that a selective pressure exists that tends to fit the overall sequence of PBP2x to its cellular environment (8). The recent finding that PBP2x and PBP1a are both located to the septum during the division of \textit{S. pneumoniae} (Cécile Morlot, personal communication) also suggests that they might physically interact.

In conclusion, the present study demonstrates that a single mutation in PBP2x, in the context of a sequence that confers a moderate resistance to β-lactams, can significantly increase the level of resistance. This finding suggests that diagnostic tools based on fingerprinting of the \textit{pbp} genes might miss important mutations, and fail to accurately predict the level of resistance of a tested strain. The comprehensive identification of all the relevant mutations will therefore be a prerequisite to the development of sequence-based methods able to accurately predict the level and spectrum of β-lactam resistance of \textit{S. pneumoniae} clinical isolates.

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FOOTNOTES

The abbreviations used are: CTX, cefotaxime; ESI-MS, electro-spray ionization mass spectrometry; MIC, minimal inhibitory concentration; MPD, 2-methyl-2,4-pentanediol; PBP, penicillin-binding protein; PEG, polyethylene glycol; r.m.s.d., root mean square deviation.

The nucleotide sequences for the \textit{pbp2x} gene from strains 4790 and 5204 have been deposited in the GenBank database under GenBank accession numbers #AJ560761 and #AJ560762, respectively. The amino acid sequence of these proteins can be accessed through NCBI Protein Database under NCBI accession numbers #CAD90770 and #CAD90771. The atomic coordinates and structure factor amplitudes for the crystal structure of R6-PBP2x*-T338A/M339F are available in the Research Collaboratory for Structural Bioinformatics Protein Databank with the accession numbers 1PYY and 1pyysf.
FIGURE LEGENDS

FIG. 1. Comparison of the acylation efficiencies of PBP2x* variants with cefotaxime. A, values of $k_2/K$ for R6-PBP2x* and its derivatives. B, values for PBP2x* from clinical isolates and their derivatives. The acylation rates with various excess concentration of antibiotic were determined by measuring the decrease of intrinsic fluorescence of the proteins with a stopped-flow apparatus.

FIG. 2. Comparison of the deacylation rates of PBP2x* variants with penicillin-G. After complete acylation with an excess of antibiotic, unbound penicillin-G was degraded by addition of a β-lactamase. The relative amount of acylated protein was measured by ESI-MS after various time intervals.

FIG. 3. Comparison of the MIC for cefotaxime of S. pneumoniae strains transformed with mutant pbp2x and pbp1a genes. The R6 strain was transformed with variants of the pbp2x gene alone (-) or together with the pbp1a gene from strain 5204 (+). The MIC$_{CTX}$ of three isolated clones of each transformant was measured by the agar dilution method.

FIG. 4. Worm representation of the R6-PBP2x*-T338A/M339F transpeptidase domain. The flexible regions are colored in magenta. Figures 4 and 5 were prepared with MOLSCRIPT (38) and Raster3D (39).

FIG. 5. Superposition of the active sites of R6-PBP2x* and R6-PBP2x*-T338A/M339F. R6-PBP2x* is in magenta. For R6-PBP2x*-T338A/M339F, carbon atoms
are in green, oxygen atoms in red, nitrogen atoms in blue, sulfur atoms in yellow; water molecules in red and the side-chains of A338 and F339 are in cyan. Hydrogen bonds are drawn as orange dashed lines for the double mutant. The sulfate ion in the active site is labeled S1. In this view the helix α2 goes out of the plane. The average temperature factor for the residues of R6-PBP2x*-T338A/M339F represented in this figure is 32.9 Å².
TABLE I

| Kinetic parameters of the reaction of R6-PBP2x, 4790-PBP2x and their variants with penicillin-G and ceftazidime | Penicillin-G | Ceftazidime |
|---|---|---|
| | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x |
| | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x |
| | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x |
| | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x |
| | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x |
| | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x |
| | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x |
| | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x |
| | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x |
| | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x |
| | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x |
| | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x |
| | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x |
| | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x |
| | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x | 4790-PBP2x |
| | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x | 5204-PBP2x |
### TABLE II

β-lactam resistance of the R6 strain transformed with various *pbp2x* genes

| *pbp2x* used for transformation | Penicillin-G MIC (µg.mL⁻¹) | Cefotaxime MIC (µg.mL⁻¹) |
|---------------------------------|-----------------------------|---------------------------|
|                                 | R6 | R6 cotransformation 5204-*pbp1a* | R6 | R6 cotransformation 5204-*pbp1a* |
| R6-*pbp2x*                      | NT | NT | NT | NT |
| R6-*pbp2x*-T338A                | NT | NT | NT | NT |
| R6-*pbp2x*-M339F                | 0.027 | NI | 0.055 | NI |
| R6-*pbp2x*-T338A/M339F          | 0.034 | NI | 0.134 | NI |
| 4790-*pbp2x*                    | 0.043 | 0.125 | 0.262 | 0.64 |
| 4790-*pbp2x*-A338T              | 0.034 | 0.034 | 0.064 | 0.134 |
| 4790-*pbp2x*-M339F              | 0.043 | 0.064 | 0.262 | 1.0 |
| 5204-*pbp2x*                    | 0.043 | 0.125 | 0.262 | 2.0 |
| 5204-*pbp2x*-F339M              | 0.055 | 0.125 | 0.262 | 0.41 |

* NT, no transformant were obtained. MIC of strain R6 < 0.016 µg.mL⁻¹ for both penicillin-G and cefotaxime.

* NI, no clones were isolated as no clones with a sensitivity lower than that of the single transformants were obtained.
TABLE III
Crystallographic data collection and refinement statistics

| Crystal data                   |            |
|-------------------------------|------------|
| Space group                   | C2         |
| Unit cell parameters (Å)      | $a = 128.22$, $b = 64.75$, $c = 144.97$, $\beta = 118.89^\circ$ |
| Resolution (last shell) (Å)   | 31-2.42 (2.55-2.42) |
| No. unique/total reflections  | 40275/299369 |
| Multiplicity (last shell)     | 7.4 (6.0) |
| $R_{\text{merge}}$ (last shell) (%)$^a$ | 4.8 (19.8) |
| <$I/\sigma I>$ (last shell)   | 29.7 (3.5) |
| Completeness (last shell)     | 99.6 (98.2) |

| Refinement                    |            |
|-------------------------------|------------|
| No. of reflections            | 38261      |
| No. of omitted reflections    | 2014       |
| No. of residues               | 607        |
| No. of water/sulfate/detergent | 241/1/1   |
| $R_{\text{cryst}}/R_{\text{free}}$ (%) | 21.8/24.4 |
| Mean $B$-factor (Å$^2$) protein / transpeptidase domain / solvent / detergent | 53.8/43.3/44.9/49.2 |
| Root mean square bond/angle deviation (Å)/($) | 0.007/1.43 |

$^a$ $R_{\text{merge}} = \sum_{hkl} \sum_i |I(hkl,i) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i |I(hkl,i)|$ where $I(hkl,i)$ represents the $i^{th}$ measurement of the intensity of the $hkl$ reflection and its symmetry equivalent, and $\langle I(hkl) \rangle$ is the average intensity of the $hkl$ reflection.

$^b$ $R_{\text{cryst}}$ is defined as $\sum_{hkl} |F_{\text{obs}}| - |F_{\text{calc}}| / \sum_{hkl} |F_{\text{obs}}|$ where $F_{\text{obs}}$ and $F_{\text{calc}}$ are the observed and calculated structure factor. $R_{\text{free}} = \sum_{hkl \in T} |F_{\text{obs}}| - |F_{\text{calc}}| / \sum_{hkl \in T} |F_{\text{obs}}|$ where $hkl \in T$ represents the reflections belonging to the test set $T$ of unique reflections.
Role of PBP2x M339F substitution in β-lactam resistance

Fig. 1
Fig. 2

Role of PBP2x M339F substitution in β-lactam resistance
Role of PBP2x M339F substitution in β-lactam resistance

Fig. 3
Role of PBP2x M339F substitution in β-lactam resistance

Fig. 4
Role of PBP2x M339F substitution in β-lactam resistance

Fig. 5
The structural modifications induced by the M339F substitution in PBP2x from Streptococcus pneumoniae, further decreases the susceptibility to β-lactams of resistant strains

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