Mutations in the Active Site of Penicillin-binding Protein PBP2x from *Streptococcus pneumoniae*

ROLE IN THE SPECIFICITY FOR β-LACTAM ANTIBIOTICS*

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Penicillin-binding protein 2x (PBP2x) isolated from clinical β-lactam-resistant strains of *Streptococcus pneumoniae* (R-PBP2x) have a reduced affinity for β-lactam antibiotics. Their transpeptidase domain carries numerous substitutions compared with homologous sequences from β-lactam-sensitive streptococci (S-PBP2x). Comparison of R-PBP2x sequences suggested that the mutation Gln552 → Glu is important for resistance development. Mutants selected in the laboratory with cephaparin frequently contain a mutation Thr550 → Ala. The high resolution structure of a complex between S-PBP2x and cefuroxime revealed that Gln552 and Thr550, which belong to strand β3, are in direct contact with the cephalosporin. We have studied the effect of alterations at positions 552 and 550 in soluble S-PBP2x (S-PBP2x*) expressed in *Escherichia coli*. Mutation Q552E lowered the acylation efficiency for both penicillin G and cefotaxime when compared with S-PBP2x*. We propose that the introduction of a negative charge in strand β3 conflicts with the negative charge of the β-lactam. Mutation T550A lowered the acylation efficiency of the protein for cefotaxime but not for penicillin G. The in vitro data presented here are in agreement with the distinct resistance profiles mediated by these mutations in vivo and underline their role as powerful resistance determinants.

The β-lactam antibiotics are powerful inhibitors of the transpeptidase (TP) activity of the bacterial penicillin-binding proteins (PBPs). These enzymes catalyze the formation of cross-linked peptidoglycan (1, 2). The sensitivity of PBPs to β-lactam antibiotics is related to the structural similarity between the β-lactam ring of penicillin and the carboxyl-terminal D-alanyl-D-alanine residues in the natural substrates of these enzymes.

*Streptococcus pneumoniae*, one of the major human pathogens of the upper respiratory tract, has developed resistance to β-lactam antibiotics via modification of the target enzymes, PBPs. Altered PBPs have a reduced affinity for the β-lactams, and increased drug concentrations are required for their in vivo inhibition. Low-affinity PBPs in resistant clinical strains of pneumococci (R-PBPs) are encoded by mosaic genes (3–9) and carry many amino acid substitutions when compared with PBPs from β-lactam-sensitive strains.

In resistant clinical strains of *S. pneumoniae*, up to five PBPs are phenotypically altered in β-lactam low-affinity variants: PBPs 1a, 1b, 2a, 2x, and 2b (4, 10–12). PBP2x and PBP2b are essential for cellular growth (13) and are primary targets for β-lactams (14). Therefore, a detailed understanding of the structural modifications conferring the β-lactam resistance properties of these two PBPs is a crucial step in designing new β-lactam antibiotics. A prime candidate for such an analysis is the PBP2x from *S. pneumoniae* strain R6 whose three-dimensional structure is available (15).

By comparing R-PBP2x sequences with sequences from sensitive strains of streptococci (S-PBP2x), we have established that the resistant character of PBP2x is often associated with substitution at position Thr550. This position is located immediately after the active site Ser177, but does not contact the antibiotic (15). Using site-directed mutagenesis we have confirmed the role of this position in modulating the reactivity of Ser177 toward β-lactams (16).

During the course of this work we have identified other amino acid positions that were preferentially altered in R-PBP2x. Herein, we extend our initial PBP2x sequence analysis and we present evidence linking the resistant phenotype to amino acid replacement at position 552 and 550. These positions are part of the active site groove strand β3 and defines one of the main contact region with the antibiotic as shown by the high resolution structure of a complex between PBP2x and the β-lactam cefuroxime (Fig. 1). Changes at both positions display different patterns of antibiotic acylation efficiency but a similar pattern of deacylation. These functional results highlight the role of the strand β3 residues of the active site groove in conferring the antibiotic-specific resistant phenotype.

MATERIALS AND METHODS

Source of PBP2x Sequences—Deduced peptide sequences of 28 pbp2x genes from penicillin-sensitive and -resistant *S. pneumoniae*, *Streptococcus orallis*, and *Streptococcus mitis*, were analyzed. In addition to published data (4, 9, 17, 18), pbp2x sequences were obtained from genetically distinct clones of penicillin-resistant isolates from different European countries and South Africa (9, 18) including (strain: accession number): J19, AJ238584; F1, AJ238585; F2, AJ238586; F5, AJ238587; Hu18, AJ238583; 2303, AJ238582; 2349, AJ238580; Sa9: AJ238581.

Construction of the Expression Plasmid—A DNA fragment encoding soluble PBP2x (PBP2x*), corresponding to Gly96 to Asp777, was ampli-
Site-directed Mutagenesis—The expression vector pGEX-S-PBP2x* was modified by introducing the replication origin of phage f1 as follows. First, a small DNA cassette resulting from the hybridization of oligonucleotides K7ori1 (5'-CAAGGTACCGCATGCAAGCTTCTA-9) and K7ori2 (5'-CGGTCTAGAAAGCTTGCATGCGG-9) was introduced into the unique XhoI restriction site of pGEX-4T1 (Pharmacia Biotech, Uppsala, Sweden) resulting in plasmid pGEX-S-PBP2x*. The complete nucleotide sequence encoding the glutathione S-transferase region fused to the NH₂ terminus of S-PBP2x* was determined by using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham France SA). No unexpected mutation was detected, and the resulting plasmid was digested simultaneously with RI and XhoI DNA fragments ligated into the expression vector pGEX-S-PBP2x* resulting from the hybridization of enzymes and the large DNA fragment was purified as described before (16). The resulting plasmid was digested simultaneously with EcoRI and XhoI restriction enzymes. The 2125-base pair polymerase 5'-deoxynucleotide 5'-transferase region fused to the NH₂ terminus of S-PBP2x* was determined using the T7 Sequenase version 2.0 DNA sequencing kit (New England Biolabs, Beverly, MA). The 2125-base pair polymerase 5'-deoxynucleotide 5'-transferase region fused to the NH₂ terminus of S-PBP2x* was ligated into the unique XhoI DNA fragment within the EcoRI-XhoI sites of pGEX-4T1 (Pharmacia Biotech, Uppsala, Sweden) resulting in plasmid pGEX-S-PBP2x*. The resulting plasmid was digested simultaneously with EcoRI and XhoI restriction enzymes. The 2114-base pair purified EcoRI-XhoI DNA fragment was ligated into the EcoRI-XhoI sites of plasmid pCG31 (Pharmacia Biotech, Uppsala, Sweden) resulting in plasmid pCG31-Pla hybridization of oligonucleotides K7ori1 (5'-CAAGGTACCGCATGCAAGCTTCTA-9) and K7ori2 (5'-CGGTCTAGAAAGCTTGCATGCGG-9) was introduced into the unique XhoI restriction site of pGEX-4T1 (Pharmacia Biotech, Uppsala, Sweden) resulting in plasmid pGEX-S-PBP2x*. The complete nucleotide sequence encoding the glutathione S-transferase region fused to the NH₂ terminus of S-PBP2x* was determined using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham France SA). No unexpected mutation was detected, and the resulting plasmid was digested simultaneously with RI and XhoI DNA fragments ligated into the expression vector pGEX-S-PBP2x* resulting from the hybridization of enzymes and the large DNA fragment was purified as described above. This DNA fragment was ligated with the 925-base pair KpnI/XhoI DNA fragment from pUC-11 (Pharmacia Biotech, Uppsala, Sweden) containing the replication origin of phage f1. The resulting expression vector pGEX-S-PBP2x*-f1 was used to transform CJ236 Escherichia coli (dur-1, ung-1, thi-1; relA-1; pCJ105 (CM')). The transformed strain was infected with the M13K07 helper phage (Bio-Rad). Site-directed mutagenesis was performed by using the single-stranded DNA produced from the resulting phagemid. Each mutant was controlled by DNA sequencing in the region of the expected mutation.

Identification of Residues Putatively Involved in Modulating the Affinity of β-Lactams for PBP2x—The sequences of 25 resistant PBP2x produced by S. pneumoniae clinical isolates were compared with those of penicillin-sensitive S. pneumoniae, S. mitis, and S. oralis. Overall, 73 sites within the 351-residue TP domain of PBP2x (residue 266 to 616) were changed in at least one of the isolates, and 29 positions were affected in at least 20% of the strains (Fig. 2). The number of amino acid differences between one R-PBP2x and the TP PBP2x from S. pneumoniae R6 strain ranged from 11 to 45. A substitution at Thr 338 occurred in 20 R-PBP2xss (80%). There was only one substitution Gin 552 → Glu common to the remaining five sequences. This mutation was found, furthermore, in combination with an Pro 358 in one, and with Ala 338 in another three cases. In summary, at least one of the two positions, 338 or 552, were changed in all 25 sequences examined. Most interestingly, both amino acid changes are known to confer a resistance phenotype in vivo and thus appear to be first candidates for a close in vitro investigation. The distance between the α carbon of the 15 remaining amino acid positions and the active site Ser 337 α carbon was measured. Two positions, 338 and 552, are within 10 Å of Ser 337 and are part of or near to conserved sequence motifs of the ASPRE family (active site serine penicillin recognizing enzymes) (Fig. 3) (22). Therefore, positions 338 and 552 are putative determinants for resistance to β-lactams. Indeed, position 338 was shown to be a key position in modulating the affinity to β-lactams, even thought this side chain is not in direct contact with either the substrate or the β-lactams (16).

Gln 552 in S-PBP2x from R6 is replaced by a Glu in 9 R-PBP2xss (36%). This position is located in the active site groove defined by strand β (Figs. 1 and 3). This element of secondary structure also includes position 550. A Thr 350 → Ala

2 J. Krauß and R. Hakenbeck, unpublished results.
mutation has been observed in five independently obtained laboratory mutants after selection with extended spectrum cephalosporins (cefotaxime or cefpodoxime) (Table I), and a mutation to Gly to Glu has also been reported (14, 23, 24). The mutation T550A occurs in one R-PBP2x, most likely also the result of selection with cephalosporins (17). The contribution of mutation T550A to Gly550 has also been reported (14, 23, 24). The cephalosporins (cefotaxime or cefpodoxime) (Table I), and a laboratory mutants after selection with extended spectrum mutation has been observed in five independently obtained (16). The molecular mass for all mutants measured by ESI-MS corresponds to the calculated mass deduced from the amino acid sequence (Table II). ESI-MS was also used to demonstrate that all mutants and S-PBP2x are fully acylated by cefotaxime when incubated with an excess of antibiotic (data not shown). These results show functional homogeneity of all mutants.

Accurate determination of enzyme kinetic parameters might be affected by variations in stability between mutants. This is especially true for $k_2$ measurement which requires long incubation periods at 37 °C. Therefore, we have measured the residual activity of the mutants following prolonged incubation at 37 °C which is the temperature used to measure all kinetic parameters ($k_{cat}/K_m$). Since the enzymatic activity remained constant over time, the kinetic parameters of all mutants can be directly compared.

**Characterization of Single S-PBP2x Mutants**—The effect on S-PBP2x of mutations most frequently found in $\beta$-lactam-resistant laboratory or clinical isolates, was determined. The acylation efficiency, represented by $k_{cat}/K_m$, of S-PBP2x mutants was measured by following the rapid accumulation of the acyl enzyme reported by the quenching of the protein intrinsic fluorescence (Fig. 4). The acylation efficiencies of S-PBP2x Q552E decrease by 72% for both Pen G and cefotaxime when compared with S-PBP2x. The pattern of acylation efficiency of the S-PBP2x T550A mutant is very different with a $k_{cat}/K_m$ reduction over 90% for cefotaxime whereas acylation by Pen G is not affected. To distinguish the influence of the hydroxyl group from the steric property of the side chain, the T550S S-PBP2x mutant was constructed. This mutation reduces the acylation efficiency of enzyme for both antibiotics by about two-thirds.

The reduction of $\beta$-lactam inhibition efficiency on laboratory and clinical resistant PBPs results mostly from a large decrease of the $k_{cat}/K_m$, the influence of the slow deacylation rate being negligible (16, 25). The deacylation rate of the three single mutants was measured by fluorography (Table III). This rate ranges from $1.3 \times 10^{-5}$ (s$^{-1}$) to $3.8 \times 10^{-5}$ (s$^{-1}$). These values remain very low, thus the effect of the mutations on the efficiency of acylation by $\beta$-lactams dominates.

To monitor the effect of the mutations on the enzymatic properties of S-PBP2x, we have determined the hydrolytic activity of the mutants for the S2d substrate analogue of the cell wall stem peptide (Table III). The hydrolytic activities of the S-PBP2x Q552E and T550A mutants were reduced by 32

**Expression, Purification, and Stability Measurement of S-PBP2x Mutants**—Six S-PBP2x mutants were expressed in E. coli as glutathione S-transferase fusion proteins. The recombinant proteins were purified to homogeneity by affinity chromatography following cleavage by thrombin. The yield of purified S-PBP2x mutants is comparable to the one reported for S-PBP2x and ranges from 20 to 50 mg/liter of bacterial culture (16). The molecular mass for all mutants measured by ESI-MS corresponds to the calculated mass deduced from the amino acid sequence (Table II). ESI-MS was also used to demonstrate that all mutants and S-PBP2x are fully acylated by cefotaxime when incubated with an excess of antibiotic (data not shown). These results show functional homogeneity of all mutants.

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and 46%, respectively, when compared with the S-PBP2x* activity. A marked decrease of $k_{cat}/K_m$ for the S2d thiol ester is observed for the S-PBP2x* T550S mutant, with less than 10% remaining activity of the wild type soluble enzyme. Thus, the S-PBP2x* T550S mutant, which is absent in the set of clinical or laboratory isolates, has a much reduced level of hydrolytic activity than the Q552E and T550A mutants.

Combining Mutations at Gln$^{552}$, Thr$^{338}$, and Ser$^{571}$ Mimic Clinical Resistant PBP2x—In previous work (16) we have shown that positions 338 and 571 were critical determinants for resistance to $\beta$-lactam antibiotics. In our set of 25 clinical isolate PBP2x sequences, three of them contain both the T338A and Q552E mutations and one displays the combined substitutions T338A, Q552E, and S571P. These two combinations of mutations were introduced in the context of S-PBP2x* as a mean to evaluate their effect in the enzymatic properties (Table III).

The acylation efficiencies of S-PBP2x* T338A,Q552E double mutant decrease by over 90 and 80% for cefotaxime and Pen G, respectively, when compared with S-PBP2x*. The deacylation rate of the mutant-Pen G complex decreases by 30% over the wild type enzyme. This slow rate remains negligible over the decrease of acylation efficiency. The $k_{cat}/K_m$ for the hydrolysis of S2d for the double mutant is reduced 8-fold when compared with S-PBP2x*.

The S-PBP2x* T338A,Q552E,S571P mutant is active since it can be fully acylated when incubated with a five times excess of cefotaxime as determined by ESI-MS (data not shown). However, the accurate kinetic parameters of this triple mutant cannot be determined because their values are too low. Based upon previous experiments (16), we have estimated the minimal measurable kinetic parameters as below 0.1 and 3.8% of the wild type value for $k_{2}/K$ for cefotaxime and Pen G, respectively. Similarly, the minimal measurable $k_{cat}/K_m$ for S2d is 0.2% of the wild type value.

### Table I

| Name            | Structure |
|-----------------|-----------|
| Penicillin G    | ![Penicillin G Structure](image) |
| Cefotaxime      | ![Cefotaxime Structure](image) |
| Cefuroxime      | ![Cefuroxime Structure](image) |
| Cefpodoxime     | ![Cefpodoxime Structure](image) |
| Cephalothin     | ![Cephalothin Structure](image) |
| S2d             | ![S2d Structure](image) |

**TABLE II**

Calculated and observed molecular masses (Da) of S-PBP2x* and variants, free and acylated by cefotaxime. These values were obtained by ESI-MS.

| Mutation(s) in S-PBP2x* | Free enzymes | Acyl enzymes |
|-------------------------|--------------|--------------|
|                         | Calculated   | Observed     | Calculated   | Observed     |
| S-PBP2x*                | 77,254       | 77,255       | 77,650       | 77,648       |
| Q552E                   | 77,255       | 77,263       | 77,651       | 77,660       |
| T550A                   | 77,224       | 77,230       | 77,620       | 77,621       |
| T550S                   | 77,240       | 77,247       | 77,636       | 77,637       |
| T338A                   | 77,224       | 77,230       | 77,620       | 77,621       |
| T338A-Q552E             | 77,225       | 77,226       | 77,621       | 77,644       |
| T338A-Q552E-S571P       | 77,235       | 77,238       | 77,631       | 77,633       |

FIG. 4. Comparison of acylation efficiencies of various PBP2x* mutants with cefotaxime and Pen G. Mean $k_{2}/K$ values relative to S-PBP2x* are given with standard deviation calculated from data obtained from three independent experiments.
TABLE III
Kinetic parameters of S-PBP2x* and its variants with cefotaxime, Pen G, and thiol ester substrate S2d

| Mutation(s) in S-PBP2x* | Cefotaxime | Pen G | Pen G | S2d |
|------------------------|------------|-------|-------|-----|
|                        | $k_{d}/K \times 10^{-9}$ (s$^{-1}$) | $k_{d} \times 10^{-9}$ (s$^{-1}$) | $k_{d}/K_{d}$ (s$^{-1}$) |
| S-PBP2x*               | 209.0 ± 18.0 | 99.0 ± 12.0 | 2.7 ± 0.6 | 2,500 ± 200 |
| Q552E                  | 59.4 ± 3.1 | 30.6 ± 0.2 | 3.8 ± 0.4 | 1,690 ± 270 |
| T550A                  | 11.9 ± 0.9 | 112.0 ± 10.0 | 1.3 ± 0.3 | 1,360 ± 140 |
| T550S                  | 62.0 ± 5.5 | 32.0 ± 2.8 | 2.1 ± 0.2 | 210 ± 20 |
| T338A                  | 114.0 ± 2.4 | 36.7 ± 3.9 | 4.1 ± 0.4 | 510 ± 40 |
| T338A-Q552E            | 18.3 ± 3.3 | 19.5 ± 1.6 | 1.9 ± 0.1 | 300 ± 20 |
| T338A-Q552E-S571P      | <0.2 | <3.8 | ND$^a$ | <5 |

$^a$ ND, not determined.

DISCUSSION

PBP2x is one of the main determinants for β-lactam resistance in the Gram-positive pathogen S. pneumoniae. The 351-residue long TP domain from S. pneumoniae R-PBP2x contains between 11 and 45 amino acid changes when compared with homologous genes from sensitive streptococci. Only a few of these changes are likely to be linked to the resistance phenotype. Two positions, located within 10 Å of the active site Ser$^{357}$, display a restricted pattern of substitutions in R-PBP2x but are strictly conserved in PBP2x from sensitive strains. These amino acid changes in PBP2x are likely to be relevant to the acquisition of the resistance phenotype.

The first position, Thr$^{338}$ in S-PBP2x from R6 strain was shown to be a major determinant for resistance to β-lactam (16). The second position, Gln$^{552}$ in S-PBP2x from R6 strain, follows the Lys-Ser-Gly conserved motif (Fig. 3) in the ASPRE family and is part of strand β3 which borders the enzyme active site (15). We have replaced this position by a Glu, a side chain frequently found in R-PBP2xs. This mutation reduced the efficiency of acylation by over two-thirds for both cefotaxime and Pen G but had only a marginal effect on the deacetylation step considering that this value remains very low. This result unambiguously identifies position 552 as another key position for the modulation of PBP2x sensitivity to β-lactams. The PBP2x active site displays a global positive charge favoring binding of β-lactams which presents a global negative charge. Introduction of a negative charge in active site via Gln$^{552}$ conflicts with β-lactams and consequently leads to the observed reduction in acylation efficiency. Electrostatic steering effects are important from 11 to 45 amino acid changes when compared with S. pneumoniae R-PBP2x contains.

The distance of 6.6 Å from active site Ser$^{357}$, is often altered to Ala in PBP2x isolated from β-lactam-resistant S. pneumoniae selected in the laboratory using a single cephalosporin antibiotic. The T550A mutation in S-PBP2x* is neutral for Pen G but reduces the acylation efficiency for cefotaxime almost 20-fold. The same mutation was found in one PBP2x from a clinical isolate. The mutation increases the resistance of S. pneumoniae to cephalosporin but reduces its resistance to penicillin (17). Taken together, these results show that, contrary to mutation Q552E, substitution T550A in S-PBP2x* affects the acylation efficiency for cefotaxime and Pen G differently. Our experimental data are in agreement with the observed differences between clinical and laboratory PBP2x mutants. Multiplete selective pressure leads to PBP2x with a reduced acylation efficiency toward a larger spectrum of β-lactams than when a single antibiotic is used under laboratory conditions. Interestingly, some natural variants of TEM-1 β-lactamases have been found to contain an A237T substitution in a position equivalent to residue 550 in PBP2xs (28, 29). These class A β-lactamases, generally penicillinases, have the ability to hydrolyze third-generation cephalosporins such as cefotaxime.

Preserving the hydroxyl group at position 550 while altering the steric property of the side chain (T550S mutation) leads to a PBP2x* mutant unable to discriminate between the Pen G and cefotaxime. This mutant displays acylation efficiency values very close to the Q552E mutant (Fig. 4). T550S mutation is not found in our collection of R-PBP2x, and this could be explained by the marked reduction in hydrolytic activity of the mutant. Thus, it is likely that the TP activity of S-PBP2x* T550S mutant is affected to a greater extent than it is for the Q552E mutant, which might be detrimental to the host bacteria. These differences might explain the selection of Glu at position 552 over Ser at position 550 in clinical isolates.

All R-PBP2x sequences of the considered set contain substitutions at position 338 or 552; in four cases, the R-PBP2x sequences contain both substitutions. Combining T338A with Q552E further reduces the acylation efficiency over the single mutation Q552E, substitution T550A in S-PBP2x* affects the acylation efficiency for cefotaxime and Pen G differently. Our experimental data are in agreement with the observed differences between clinical and laboratory PBP2x mutants. Multiple selective pressure leads to PBP2x with a reduced acylation efficiency toward a larger spectrum of β-lactams than when a single antibiotic is used under laboratory conditions. Interestingly, some natural variants of TEM-1 β-lactamases have been found to contain an A237T substitution in a position equivalent to residue 550 in PBP2xs (28, 29). These class A β-lactamases, generally penicillinases, have the ability to hydrolyze third-generation cephalosporins such as cefotaxime.

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