Pneumococcal β-Lactam Resistance Due to a Conformational Change in Penicillin-binding Protein 2x

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Streptococcus pneumoniae is a life-threatening human pathogen that is increasingly resistant to a wide array of drugs. Resistance to β-lactams, the most widely used antibiotics, is correlated with tens of amino acid substitutions in their targets; that is, the penicillin-binding proteins (PBPs), resulting from multiple events of recombination. To discriminate relevant substitutions from those that are incidental to the recombination process, we report the exhaustive characterization of all the mutations in the transpeptidase domain of PBP2x from the highly resistant strain 5204. A semi-automated method combining biochemical and microbiological approaches singled out 6 mutations of 41 (15%) that are essential for high level resistance. The hitherto uncharacterized I371T, R384G, M400T, and N605T together with the previously studied T338M and M339F account for nearly all the loss of affinity of PBP2x for β-lactams. Most interestingly, I371T and R384G cause the conformational change of a loop that borders the entrance of the active site cavity, hampering antibiotic binding. For the first time all the mutations of a PBP relevant to β-lactam resistance have been identified, providing new mechanistic insights. Most notable is the relationship between the decreased susceptibility to β-lactams and the dynamic behavior of a loop.

The discovery of penicillin more than 60 years ago launched the antibiotic era, and β-lactams are still the most widely used drugs to combat bacterial infections today. β-Lactam antibiotics inhibit an essential step in bacterial cell wall synthesis, namely the cross-linking of the peptidoglycan by transpeptidases (TPs).2 The antibiotics hamper the TP activity by acylating the active site serine of the penicillin-binding proteins (PBPs) enzymes (1). The active site of PBPs is bordered by three defining conserved motifs, SXXK with the catalytic serine, SAX, and KSG.

However, several mechanisms have arisen that greatly threaten the clinical efficacy of β-lactams. Depending on the organisms, the strategies vary and can be combined. β-Lactamases can be expressed that degrade the antibiotics. Altered endogenous PBPs or an additional non-susceptible PBP can be expressed. In Gram-negative bacteria, the outer membrane permeability to the drugs can be decreased and/or the drugs can be actively expelled from the periplasm (2, 3).

Streptococcus pneumoniae is a major human pathogen of the upper respiratory tract that causes a number of serious life-threatening diseases. It is responsible for non-invasive infections such as otitis media, sinusitis, or pneumonia. Invasive S. pneumoniae infections lead to more severe afflictions like bacteremia or meningitis. In resistant strains altered PBPs are less susceptible to inhibition by antibiotics while remaining physiologically functional (4). The low susceptibility of PBPs from resistant strains to β-lactam results mainly from a decreased rate of acylation (5–7), although PBPs with both slower acylation and faster deacylation rates are known (8).

S. pneumoniae contains five high molecular weight PBPs. Mainly three of them are involved in the mechanism of β-lactam resistance: PBP2x, -1a, and -2b. PBP2x and 2b are essential. PBP2x is a primary determinant of β-lactam resistance, because it is the first one modified under selective pressure by some β-lactams (9, 10).

Because of its natural competence, S. pneumoniae is able to recombine its genomic DNA with that from other strains or from related species such as Streptococcus mitis or Streptococcus oralis to form mosaic pbp genes (11, 12). Because of this mechanism and additional point mutations, a wide variety of amino acid substitutions (13) is observed in PBP2x, -1a, and -2b, of which only a restricted set is likely relevant for resistance.

Pbp2x sequences of clinical strains can be classified in three groups. Sequences of β-lactam-susceptible strains are similar to that of the susceptible reference laboratory strain R6. The two other groups harbor generally more than 30 mutations in the TP domain and originate from strains with reduced susceptibility to β-lactams (14). One group is characterized by the well studied T338A substitution (5). The other group is composed of sequences containing the Q552E substitution (6).

The two groups of proteins display reduced reactivity toward β-lactam because of two different mechanisms (5, 6). These mechanisms may sometimes be combined, as a few sequences can harbor mutations at both positions 338 and 552.

Four PBP2x mutations that play a role in resistance have been studied in detail. The earlier cited substitution T338A, immediately adjacent to the catalytic Ser-337, and the additional modification at position 339, which further decreases the susceptibility to β-lactams, likely affect the reactivity of the active site serine (5); so does T550A, although its effect is restricted to cephalosporins (15). Q552E likely influences binding of the antibiotic (6). In each case these individual mutations explain only part of the decrease in reactivity toward β-lactams measured for PBP2x from clinical strains and the corresponding resistance. However, there is no clear structure- or alignment-based clue to identify other mutations involved. Identification of all the mutations participating in the resistance process is a crucial step in the development of sequence-based diagnostic tools, in understanding the molecular mechanisms of resistance, and in the future design of better β-lactam antibiotics or adjuvants that might restore susceptibility.

We present here an exhaustive study of all the 41 amino acid substitutions located in the TP domain of PBP2x from the highly resistant...
Resistance-relevant Substitutions in PBP2x

clinical strain 5204 (penicillin G minimal inhibitory concentration (MIC) = 6.0 μg/ml−1; cefotaxime MIC = 12.0 μg/ml−1; serotype 14) (16). The effect of each mutation on the acylation efficiency was investigated. We report five new mutations (I371T, R384G, M400T, D567N, and N605T) affecting the kinetics of reaction with β-lactams in a way that is correlated with resistance phenotypes. Substitutions at positions 371 and 384 affect the resistance by modulating the dynamic behavior of a loop that borders the entrance of the active site.

EXPERIMENTAL PROCEDURES

Transformations and MICs—The non-encapsulated S. pneumoniae R6 was used as a recipient strain for genetic transformation. To prevent correction of the introduced mutations by the Hex mismatch repair system of the bacteria, an altered R6 strain (hexA:spc) (17) was used in some cases. The modified strain was obtained by transforming R6 S. pneumoniae with genomic DNA of the R320 strain (generously provided by B. Martin, CNRS, Toulouse) and selection on spectinomycin. Genomic DNA of the R320 strain was extracted by resuspension in 1 ml of CelLytic B-II (Sigma) containing 100 μg/ml of sodium phosphate, pH 7, 200 mM KCl in a 96-well filter plate (Pall) on the MicroLab STAR robot (Hamilton). Proteins were eluted with 800 μl of 5 mM reduced glutathione (Sigma). For proteins used in the proteolysis assay, the resin was washed with 3 ml of 50 mM Tris–HCl, pH 8, 100 mM NaCl, 1 mM EDTA and eluted in 300 μl of the same buffer after cleavage with 10 units of thrombin (Sigma).

Kinetcs—The k_{app} acylation parameter was determined directly on the eluates by monitoring the decrease of the intrinsic fluorescence of the protein after the addition of the antibiotic to the proteins in a 96-well black flattrac-200 plate (Greiner Bio-One) with a Fluostar Optima micro-plate reader (BMG Labtech). The protein concentration was about 0.5 μM, and that of cefotaxime (Sigma) was varied between 5 and 320 μM. The excitation wavelength was 280 nm, and the emission was recorded at 320 nm. At a given concentration of antibiotic, the apparent pseudo-first order rate constant k_{app} was determined by non-linear least-squares fitting to the equation Fluo_0 = Fluo_∞ exp(\(-k_{app} t\)). The least-squares fitting to the equation k_{app} = (k_{p}/K) [antibiotic].

Monitoring of Loop 365–394 Dynamics by Proteolysis—Purified PBP2x* variants were subjected to trypsin digestion as follows. About 40 μg of protein were incubated with 80 ng of trypsin (Sigma) in 56 μl at room temperature. At various times aliquots were withdrawn and heated for 10 min at 95 °C in SDS-PAGE sample buffer. Aliquots containing about 5 μg of protein were analyzed by SDS-PAGE (15% acrylamide) and Coomassie staining.

RESULTS

PBP2x Sequences Comparison—5204-PBP2x has 80 amino acid changes compared with R6-PBP2x, including 41 in the TP domain. There are the two well characterized T338A and M339F substitutions and 39 other previously uncharacterized modifications (between residues 281 and 616, Fig. 1). The TP domain of 5204-PBP2x differs only at 7 positions from that of PBP2x from strain Sp328, the structure of which is known (20); they are F339M, M343T, F364L, A378E, T400M, V572A, and F595Y, where the first amino acid is that from 5204-PBP2x, and the second is from Sp328-PBP2x. The TP domain of 5204-PBP2x differs from that of PBP2x from strain 4790 at positions 339, 343, 378, 400, and 595. 4790-PBP2x has been well characterized previously together with 5204-PBP2x (14). The results presented below concern both 5204-specific mutations (positions 339 and 400) and substitutions common to the two related Sp328- and 4790-PBP2x sequences. Thus, some of the described phenomena and conclusions certainly apply to a wide class of PBP2x from clinical strains, including strains Sp328 and 4790.

Mutagenesis and Protein Production—A large set of mutated PBP2x* was produced to test the influence of substitutions in different contexts. In a first screen, each modified residue of the PBP2x TP domain of strain 5204 was reverted to the original R6-PBP2x amino acid. This strategy was chosen to detect substitutions that may be involved in cooperative effects. With the converse strategy, cooperativity between substitutions would require the introduction of multiple mutations in R6-PBP2x to produce an effect. In a second time, a limited set of pertinent R6-PBP2x point mutants was created to assess the importance of some substitutions in the context of a sequence from a susceptible strain. For in vitro characterization, the modified-PBP2x*, R6-PBP2x* and 5204-PBP2x*, were expressed in E. coli and purified by glutathione affinity chromatography. The fusion proteins were eluted by competition with reduced glutathione. The homogeneity was assessed by Coomassie-stained SDS-PAGE. Yields were about 35–40 mg/liter of culture.
Resistance-relevant Substitutions in PBP2x

The efficiency of acylation by cefotaxime at 37 °C of 5204-PBP2x* and R6-PBP2x* was determined. To avoid masking the effect of mutations in PBP2x by the presence of glutathione or to the retention of the fusion with glutathione S-transferase, the substitutions A338T, M339F, T371I, G384R, T400M, N567D, and N605T R6-PBP2x* mutants were measured. Because of the rapid kinetics of R6-PBP2x* and its variants and the long dead time of the measurements in the microplate reader (2 s), the experiments were performed at 28 °C. For direct comparison, the acylation efficiencies of the corresponding A338T, F339M, T371I, G384R, T400M, N567D, and T605N mutants of 5204-PBP2x* were also determined at 28 °C. Moreover, the kinetic analysis was also performed on a set of multiple mutants of R6-PBP2x* and 5204-PBP2x*. Results are presented in Table 1. Among the four new positions (371, 384, 400, and 605) important for the lower reactivity of 5204-PBP2x*, only substitutions at residues 384 and 605 showed a corresponding inverse effect on the acylation of R6-PBP2x*. Both R384G and N605T induced a 27% decrease of the k2/K value in R6-PBP2x*. Because I371T had no influence on the kinetics of R6-PBP2x*, the threonine 371 of strain 5204 is most likely involved in a cooperative action with one or more other substituted amino acids. Surprisingly, M400T changes the efficiency of acylation of R6-PBP2x* in a way opposite to that expected from the effect of the reverse mutant in 5204-PBP2x*; M400T in R6-PBP2x* increases the k2/K value 2.3-fold, whereas the T400M in 5204-PBP2x* also increases the reactivity 3.4-fold.

Mutations at position 567 show the same inverse effect, i.e. N567D in 5204-PBP2x* and D567N in R6-PBP2x* decreases and increases, respectively, the acylation rate. These effects are observed for the substitution alone or combined with the six other mutations in R6-PBP2x* or the 5204-PBP2x*.

Combination of the six substitutions T338A, F339M, T371I, R384G, M400T, and N605T in R6-PBP2x* decreases the acylation efficiency more than 100-fold so that it remains only 10-fold higher than that of 5204-PBP2x*. Reversion of the six residues at the same positions (A338T, M339F, T371I, G384R, T400M, and T605N) in 5204-PBP2x* causes a near 1000-fold increase of the k2/K value. This hexa-mutant of 5204-PBP2x* is acetylated by cefotaxime with an efficiency close to that of R6-PBP2x* (78%).

Phenotypes—To assess the phenotypic impact of the newly identified substitutions, the modified 5204- and R6-pbp2x* were transformed into S. pneumoniae R6, and the resistance levels to cefotaxime (MIC) were determined. To avoid masking the effect of mutations in PBP2x by the inhibition of PBP1a (14), all the modified pbp2x* was co-transformed with pbp1a* of strain 5204. The results are presented in Table 2. Inversion of the desired mutations in the R6 genome was checked in every case.

In accordance with the k2/K values, the single A338T, F339M, T371I, G384R, T400M, and T605N 5204-PBP2x* reversion mutants showed a decreased level of resistance from 1 to 0.5–0.75 μg/ml−1. The N567D mutation in 5204-PBP2x* had no effect on the MIC. The combinations of F339M/T400M, A338T/F339M, and T371I/G384R reversion induced greater decreases of resistance level with MICs of 0.5, 0.38–0.5, and 0.5 μg/ml−1, respectively. After transformation with the 5204-pbp2x* hexa-mutant A338T/M339F/T371I/G384R/T400M/T605N, sequencing of the gene showed that recombination had occurred.
Resistance-relevant Substitutions in PBP2x

![Image](50x557 to 564x732)

**TABLE 1**

**Acylation efficiencies of PBP2x variants for cefotaxime at 28 °C**

The reactions were done in 100 mM sodium phosphate buffer, pH 7, 200 mM KCl. Acylation rates are given with S.E. calculated from three independent experiments.

| PBP2x*                              | $k_a/K$ (m$^{-1}$s$^{-1}$) |
|--------------------------------------|---------------------------|
| 5204-PBP2x*                         |                           |
| Wild type                            | 85 ± 14                   |
| A338T                               | 630 ± 60                  |
| F339M                               | 1,680 ± 240               |
| T371I                               | 1,380 ± 180               |
| G384R                               | 930 ± 170                 |
| T400M                               | 290 ± 40                  |
| T605N                               | 380 ± 35                  |
| N567D                               | 48 ± 4                    |
| A338T/F339M                         | 3,020 ± 500               |
| F339M/T400M                         | 1,435 ± 90                |
| T371I/G384R                         | 1,280 ± 160               |
| A338T/F339M/T371I/G384R/T400M/T605N | 78,100 ± 12,800           |
| A338T/F339M/T371I/G384R/T400M/N567D/T605N | 60,000 ± 6,500 |
| R6-PBP2x*                           |                           |
| Wild type                            | 100,000 ± 3800             |
| T338A                               | 51,000 ± 3200             |
| F339M                               | 30,100 ± 950              |
| I371T                               | 101,000 ± 21,000          |
| R384G                               | 73,000 ± 4500             |
| M400T                               | 229,000 ± 19,000          |
| N605T                               | 73,000 ± 3700             |
| D567N                               | 189,000 ± 14,500          |
| T338A/M339F                         | 3,660 ± 635               |
| M339F/M400T                         | 41,000 ± 1700             |
| I371T/R384G                         | 47,000 ± 1700             |
| T338A/M339F/I371T/R384G/M400T/N605T | 830 ± 70                  |
| T338A/M339F/I371T/R384G/M400T/D567N/N605T | 2,500 ± 190 |

TABLE 2

**Acylation efficiencies of 5204-PBP2x* variants for cefotaxime at 37 °C.** The reactions were performed in 100 mM sodium phosphate buffer, pH 7, 200 mM KCl using a micro-plate reader equipped with an injector. Mean $k_a/K$ values are given with S.E. calculated from three independent experiments.

| PBP2x*                              | $k_a/K$ (m$^{-1}$s$^{-1}$) |
|--------------------------------------|---------------------------|
| 5204-PBP2x*                         |                           |
| Wild type                            | 85 ± 14                   |
| A338T                               | 630 ± 60                  |
| F339M                               | 1,680 ± 240               |
| T371I                               | 1,380 ± 180               |
| G384R                               | 930 ± 170                 |
| T400M                               | 290 ± 40                  |
| T605N                               | 380 ± 35                  |
| N567D                               | 48 ± 4                    |
| A338T/F339M                         | 3,020 ± 500               |
| F339M/T400M                         | 1,435 ± 90                |
| T371I/G384R                         | 1,280 ± 160               |
| A338T/F339M/T371I/G384R/T400M/T605N | 78,100 ± 12,800           |
| A338T/F339M/T371I/G384R/T400M/N567D/T605N | 60,000 ± 6,500 |
| R6-PBP2x*                           |                           |
| Wild type                            | 100,000 ± 3800             |
| T338A                               | 51,000 ± 3200             |
| F339M                               | 30,100 ± 950              |
| I371T                               | 101,000 ± 21,000          |
| R384G                               | 73,000 ± 4500             |
| M400T                               | 229,000 ± 19,000          |
| N605T                               | 73,000 ± 3700             |
| D567N                               | 189,000 ± 14,500          |
| T338A/M339F                         | 3,660 ± 635               |
| M339F/M400T                         | 41,000 ± 1700             |
| I371T/R384G                         | 47,000 ± 1700             |
| T338A/M339F/I371T/R384G/M400T/N605T | 830 ± 70                  |
| T338A/M339F/I371T/R384G/M400T/D567N/N605T | 2,500 ± 190 |

The reactions were done in 100 mM sodium phosphate buffer, pH 7, 200 mM KCl using a micro-plate reader equipped with an injector. Mean $k_a/K$ values are given with S.E. calculated from three independent experiments.

**FIGURE 2.** Acylation efficiencies of 5204-PBP2x* variants for cefotaxime at 37 °C. The reactions were performed in 100 mM sodium phosphate buffer, pH 7, 200 mM KCl using a micro-plate reader equipped with an injector. Mean $k_a/K$ values are given with S.E. calculated from three independent experiments.

DISCUSSION

**Monitoring of Loop 365–394 Dynamics by Proteolysis**—Two of the four newly found resistance-related amino acid positions (371 and 384) are located in a region that is flexible in the related Sp328-PBP2x* (4). It consists of a loop region between residues 365 and 394, which is not traceable on the crystal structure of Sp328-PBP2x* because of a very weak electron density (20). This loop is not mobile in R6-PBP2x (21, 22). Previous work had shown that the conformational change of the 365–394 loop could be assayed by trypsic digestion. Under the same conditions, R6-PBP2x* is resistant to digestion by trypsin, whereas the very similar Sp328-PBP2x* and PBP2x* are cleaved in two fragments of 35 and 40 kDa. N-terminal sequencing of both fragments positioned the cleavage site after Arg-372. We decided to use this assay to investigate the relationship between mutations in position 371 and 384 and the conformational change of the region.

As shown in Fig. 3, R6-PBP2x* is resistant to trypsin digestion and shows an apparent molecular mass of 75 kDa, whereas 5204-PBP2x* is cleaved. By inserting R384G and I371T individually or together in R6-PBP2x*, the protein becomes susceptible to digestion, indicating a destabilization of the loop. Conversely, reversion of the substitutions at the same positions in 5204-PBP2x* prevents digestion by trypsin, indicating a stabilization of the loop. Substitutions in position 389 and 514, which were proposed previously to destabilize the region 365–394 (20), were investigated in the same manner and showed no influence on the susceptibility to trypsin.

**DISCUSSION**

PBP2x is a primary determinant of the resistance to β-lactams in S. pneumoniae (9, 10) and shows the greatest sequence variability (13). Previous biochemical and structural studies focused on conserved sub-
Institutions within the active site (T338A, M339F, Q552E, and T550A) have illuminated crucial mechanistic aspects (5, 6, 14, 15, 23). These results were, however, frustrating in that the effect of the individual mutations accounted for less than half of the reduction in acylation efficiency measured for proteins from clinical resistant strains. To identify additional mutations that contribute to the low acylation efficiency of PBP2x, we undertook an exhaustive mutagenesis of the protein from the highly resistant isolate 5204. With the exception of the substitution T550A (5, 6, 14, 15, 23), which is present in some PBPs that have a decreased susceptibility to cephalosporins but near normal reactivity with penicillins, the effect of the previously studied substitutions is similar on the reaction with both penicillins and cephalosporins (5, 6, 14, 15, 23). Because 5204-PBP2x* is equally poorly acylated by penicillins and cephalosporins (5, 6, 14, 15, 23), it is most likely that the findings presented here with cefotaxime apply qualitatively to penicillins as well.

Of 41 mutations present in the TP domain, our study has uncovered the role of four new substitutions in addition to the previously characterized T338A and M339F mutations. The localization of the four new positions on the crystal structure of R6-PBP2x (16, 17) is shown in Fig. 4.

Dynamics of a Loop in PBP2x Is an Important Resistance Determinant—Most interesting is the role of residues 371 and 384 in the acylation kinetics and the conformation of the loop spanning residues 364–395. In 5204-PBP2x*, the T371I and G384R reversions to the amino acids found in R6-PBP2x, individually and together, increase the efficiency of acylation about 10-fold at 28 °C. In R6-PBP2x*, only the R384G mutation has a modest effect, decreasing the acylation efficiency, with the I371T substitution having no influence. In contrast, the double mutations I371T/R384G cause a major 2-fold drop of the efficiency of acylation. Taken together, these results indicate cooperativity between both substitutions, with the I371T substitution having no influence. Of 83 different publicly available PBP2x sequences, a mutation in position 371 is always associated with the R384G substitution (44 times), whereas the latter appears 9 times in the absence of the former.

### Table 2

| 5204-pbp2x<sup>a</sup> | Cefotaxime MIC | Actual reverted substitutions |
|------------------------|----------------|-------------------------------|
| Wild type              | 1              | NS<sup>a</sup>                |
| A338T                  | 0.75           | A338T                         |
| F339M                  | 0.5            | F339M                         |
| T371I                  | 0.5            | T371I                         |
| G384R                  | 0.5            | G384R                         |
| T400M                  | 0.75           | T400M                         |
| T605N                  | 0.75           | T605N                         |
| N567D                  | 1              | N567D                         |
| F339M/T400M            | 0.5            | F339M/T400M                   |
| A338T/F339M            | 0.38–0.5       | A338T/F339M                   |
| T371I/G384R            | 0.38           | T371I/G384R                   |
| A338T/F339M/T371I/G384R/T400M/T605N | 0.064 | G384R/T400M/T605N          |
| A338T/F339M/T371I/G384R/T400M/N567D/T605N | 0.064 | A338T/F339M/T371I/G384R/T400M/N567D/T605N |

| 5204-pbp2x<sup>a</sup> | Cefotaxime MIC | Actual inserted substitutions |
|------------------------|----------------|-------------------------------|
| T338A                  | NT<sup>a</sup> | NS                            |
| M339F                  | 0.064          | M339F                         |
| I371T                  | NT             | NS                            |
| R384G                  | NT             | NS                            |
| M400T                  | NT             | NS                            |
| N605T                  | NT             | NS                            |
| D567N                  | NT             | NS                            |
| M339F/M400T            | NT             | NS                            |
| T338A/M339F            | 0.125          | T338A/M339F                   |
| I371T/R384G            | 0.064          | I371T/R384G                   |
| T338A/M339F/I371T/R384G/M400T/N605T | 0.19 | T338A/M339F/I371T/R384G/N605T |
| T338A/M339F/I371T/R384G/M400T/N605T | 0.19 | T338A/M339F/I371T/R384G/N605T |
| T338A/M339F/I371T/R384G/M400T/N605T | 0.5 | T338A/M339F/I371T/R384G/N605T |
| T338A/M339F/I371T/R384G/M400T/D567N/N605T | 0.19 | T338A/M339F/I371T/R384G/N605T |

<sup>a</sup> NS, no substitution was inverted or inserted.
<sup>b</sup> NT, no transformants were found.

### Figure 3

Digestion of PBP2x<sup>a</sup> variants with trypsin. A, digestions of 5204-PBP2x<sup>a</sup> and point-mutants. B, digestions of R6-PBP2x<sup>a</sup> and point mutants. M stands for molecular mass markers, and t<sub>0</sub>, t<sub>10</sub>, t<sub>30</sub>, and t<sub>60</sub> correspond to incubation times with trypsin (1/500 w/w) at room temperature for 0, 10, 30, and 60 min, respectively, before analysis by SDS-PAGE and Coomassie staining. wt, wild type.
The crystal structure of Sp328-PBP2x*, which is nearly identical in sequence to 5204-PBP2x*, had revealed that residues 364–395 are in a mobile loop, as no electron densities were observed, with deviation from the structure of R6-PBP2x* starting at residue 354 (20). The peptide bond after Arg-372 is sensitive to tryptic digestion in several related PBP2x’s from clinical strains (Sp328, 4790, and 5204) but not in R6- or 5259-PBP2x*, in which the region 354–395 is structurally well defined (20, 22). Using this differential sensitivity to trypsin, we probed the effect of substitution in positions 371 and 384 on the dynamic of the loop.

Both positions 371 and 384 were found to be important conformational determinants of the region, both in R6- and 5204-PBP2x*, although substitutions at position 384 seem to have a greater effect (Fig. 3). The destabilization caused by the I371T mutation in R6-PBP2x* is weaker (with most of the protein remaining undigested after 60 min) than that caused by the R384G substitution. This difference might be correlated with the different effects on the acylation kinetics. Conversely, the T371I mutation in 5204-PBP2x* does not completely abolish tryptic digestion after 60 min, whereas the G384R substitution does.

The influence of substitutions in position 384 and 371 on the acylation by β-lactams and the loop conformation suggests a causal relationship between the two phenomena. The destabilization of the loop may weaken the non-covalent interactions with the antibiotic (increase the K).

Note that the amino acids at positions 371 and 384 are identical in R6-PBP2x*-I371T and 5204-PBP2x*-G384R and conversely in R6-PBP2x*-R384G and 5204-PBP2x*-T371I. However, the two R6-PBP2x* variants are more trypsin-sensitive than the two 5204-PBP2x* variants (Fig. 3). The 5204-PBP2x* sequence context tolerates better Thr-371 and Gly-384, and both are necessary to destabilize the loop. The reasons for this difference might be found in two other substitutions in the loop itself. R6-PBP2x* holds two glycine residues at positions 355 and 382, which are expected to favor flexibility, whereas 5204-PBP2x* has a serine and a threonine, respectively, which offer fewer degrees of freedom. Thus, with the same amino acids in positions 371 and 384, the loop of 5204-PBP2x* is less flexible than that of the R6-PBP2x* variant.

The importance for resistance of substitutions at position 371 and 384 and the resulting mobility of loop 365–394 is confirmed in vivo. Reversions T371I and G384R in 5204-pbp2x induced an important 50% decrease of the resistance level. Both substitutions together further decreased the MICs to 38% of the level conferred by the wild type 5204-PBP2x. This is somewhat surprising as the double mutant 5204-PBP2x* has roughly the same acylation efficiency as the two simple mutants. The double mutant may be physiologically less efficient. The double substitution I371T/R384G in R6-ppb2x induced a MIC of 0.064 μg/ml−1, whereas each substitution alone did not result in a resistance level sufficient for selection.

**Substitution M400T, a Context-specific Resistance Determinant**—In the available sequences of PBP2x, the substitution M400T is always associated with the major resistance determinant M339F (16). In the context of 5204-PBP2x*, the presence of a threonine in position 400 is important for the low acylation efficiency. However, this role may be restricted to potentiating the effect of the M339F mutation, as reversion of the latter has the same consequences with and without the concomitant reversion T400M. Structurally, the importance of the residue in position 400 can be rationalized by its core localization and its immediate proximity to the long side chain of Lys-340 of the first catalytic motif. In vivo the effects on resistance of the substitution in position 400 in 5204-pbp2x parallel the in vitro kinetic results. The T400M reversion decreases resistance alone. However, if the F339M reversion is also present, T400M mutation causes no further decrease of resistance.

It is paradoxical that the presence of a threonine in position 400 in the context of R6-PBP2x* in the absence or the presence of the M339F substitution has the opposite effect of increasing the acylation efficiency. The reason for this behavior is not known but may be related to the orientation of the distal methyl of Met-400, which points in the opposite direction in the structure of R6-PBP2x* and the 5204-related Sp328-PBP2x* (20).

The paradoxical effect of the threonine in position 400 in the context of R6-PBP2x was also observed in vivo, because no transformants could be selected with the double mutation M339F/M400T, whereas the single M339F substitution could be obtained. Also, when transformed with DNA encoding the hexa-mutant R6-PBP2x/T338A/F339M/I371T/R384G/M400T/N605T, no clones were obtained that had incorporated the M400T substitution despite using a hex β-lactam strain as recipient. It is possible that by picking among the most resistant clones, we have screened against the introduction of the M400T if this one decreases the resistance.

**The Individual Role of Mutation N605T**—Substitutions in position 605 have an individual and consistent impact on the efficiency of acylation in both R6- and 5204-PBP2x*. Examination of the available structures (14, 15, 20, 22, 24) reveals that Asn-605 in R6-PBP2x* forms 2 hydrogen bonds to the backbone nitrogen of residue 538 and carbonyl oxygen of residue 536. This bonding pattern is disrupted in the mutant with the hydroxyl of Thr-605, forming a single weaker hydrogen bond with the main chain nitrogen in position 538, as observed in Sp328-PBP2x*. It is possible that these modifications participate to the destabilization of the adjacent loop 524–535, which borders the active site and is mobile and invisible in the structure of Sp328-PBP2x*, like the facing 365–394 region discussed above.

The reversion T605N in 5204-PBP2x reduces the MIC as expected. Conversely, in vivo incorporation of the five R6-PBP2x substitutions T338A/M339F/I371T/R384G/N605T into the R6-strain led to transformants with greater resistance than integration of the four substitutions T338A/M339F/I371T/R384G. Thus, position 605 has most likely an individual additive effect on resistance.

**Six Mutations Account for Nearly all the Resistance Conferred by** 5204-PBP2x*—Reversion of the six substitutions in positions 338, 339, 371, 384, 400, and 605 in 5204-PBP2x* results in an efficiency of acylation (increased 1000-fold) of the same order of magnitude as that of R6-PBP2x*, indicating that they are the substitutions most relevant for the resistance. However, the six converse substitutions in R6-PBP2x* generate a species with an acylation efficiency reduced 100-fold, with a difference of an order of magnitude remaining with that of 5204-PBP2x*. This discrepancy indicates that some cooperativity in lowering...
the acylation efficiency operates between the six identified substitutions and the remaining 35 mutations. The cooperativity effect is probably collective, as no other single substitution in 5204-PBP2x* had a significant impact on the k$_{a}$/K.

When transforming S. pneumoniae with hexa- or hepta-reverse 5204-PBP2x variants, two types of transformants were obtained. In the first, recombination occurred just before codon 382 so that the resulting gene encodes a mosaic PBP2x, with the first third of the TP domain of R6 origin and the last two-thirds from strain 5204 but with some reverted substitutions. The second have the entire TP domain of strain 5204 without the seven substitutions at positions 338, 339, 371, 384, 400, 567, and 605. Because the two types of clones have the same MICs, the 7 mutations preceding residue 382 (i.e. 281, 311, 346, 347, 355, 358, 364, and 378) are most likely unnecessary to develop resistance. Moreover, the 28 mutations encoded by the region downstream of codon 378, not including those in positions 384, 400, 567, and 605, are together responsible for the very low observed resistance level (MIC 0.064 μg/ml$^{-1}$).

This level would classify the transformants into the susceptible group of S. pneumoniae clinical strains. Thus, all the key mutations responsible for the high level of resistance acting in the 5204-PBP2x context have been identified.

The combination of all the resistance-related substitutions of strain 5204 (T338A, F339M, I371T, R384G, M400T, and N605T) in R6-PBP2x could not be obtained in vivo. Among the 16 analyzed transformants resulting from transformations with R6-pbp2x* hexa- and hepta-mutants, R384G and M400T were never observed together, suggesting that a combination of these two mutations in an R6-context is lethal. The most resistant transformants harbor mutations in positions 338, 339, 371, 384, and 605 as expected from the in vitro acylation kinetics. However, the sampling may be too small to conclude definitely, and a bias against the incorporation of the M400T substitution may have arisen from picking the most resistant clones, as mentioned above.

The Highly Conserved Substitution (N567D) Modifies Acylation Efficiency in the Unexpected Direction—The Asp to Asn substitution at position 567 is the most conserved mutation in PBP2x from resistant strains. Surprisingly, the reversion or integration of the mutation in 5204- or R6-PBP2x*, respectively, influences the acylation in a direction that is contrary to the expectation. The D567N substitution further sensitizes R6-PBP2x* to cefotaxime, whereas the reversion in 5204-PBP2x* further diminishes the acylation efficiency. These effects are also observed in the context of both hexa-mutants. Why the N567D mutation is so conserved in PBP2x proteins with a reduced affinity for β-lactams is a matter of speculations. The fact that it is found in all sorts of unrelated sequences does not support a stabilizing compensatory function. However, a compensatory role in terms of physiological function is possible. Indeed, substitutions that decrease the reactivity of PBP2x toward β-lactams are unlikely to impact favorably on the physiological transpeptidation reaction. The increased reactivity caused by the N567D mutations with β-lactams may be much greater toward the natural substrates than toward the antibiotics. Another possibility is that Asn-567 is involved in interactions with other proteins involved in resistance.

Structurally, an effect of residue 567 is difficult to rationalize as it is surface-exposed and is not particularly near the active site cavity. The amino acid in position 567 is in the loop connecting strands β3 and β4. It is conceivable that it, thus, influences the conformation of strand β3, which bears the third catalytic motif. Note that the loop connecting β3 and β4 was found to adopt different conformations in the apo and acylated form of S. pneumoniae PBP1b. These modifications, which extend to the strand β3, were proposed to have a role in the activation of the physiological enzymatic activity (25).

The substitution N567D in 5204-PBP2x* did not measurably decrease resistance in vivo. However transformants were obtained, indicating that the above speculation about a role of Asn-567 in maintaining the physiological function of PBP2x mutants must be subtle if it is correct.

Conclusions—This study demonstrates the role of four new PBP2x amino acid substitutions (I371T, R384G, M400T, and N605T) in the development of high level β-lactam resistance in a clinical isolate of S. pneumoniae. Two of these substitutions (I371T and R384G) reveal a new mechanism of resistance that involves a dynamic loop of PBP2x. Moreover, because of the permanent emergence of resistant strains with new mutations, the automated mutagenesis and analysis methods used for this study could be a useful tool for the rapid adaptation of sequence based diagnostic tools for predicting resistance. Finally, we hope that this extensive study will help designing new β-lactams or adjuvants that would restore the efficacy of older drugs by stabilizing a crucial mobile loop in PBP2x.

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REFERENCES

1. Waxman, D. J., and Strominger, J. L. (1983) Annu. Rev. Biochem. 52, 825–869
2. Nikaido, H. (1994) Science 264, 382–388
3. Wilke, M. S., Lovering, A. L., and Strynadka, N. C. (2005) Curr. Opin. Microbiol. 8, 525–533
4. Hakenbeck, R., Kaminski, K., Konig, A., van der Linden, M., Paik, J., Reichmann, P., and Zahner, D. (1999) Microb. Drug Resist. 5, 91–99
5. Mouz, N., Gordon, E., Di Guilmi, A. M., Petit, I., Petillot, Y., Dupont, Y., Hakenbeck, R., Vernet, T., and Dideberg, O. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13403–13406
6. Mouz, N., Di Guilmi, A. M., Gordon, E., Hakenbeck, R., Dideberg, O., and Vernet, T. (1999) J. Biol. Chem. 274, 19175–19180
7. Zannin, M., Hakenbeck, R., and Freer, J. M. (1993) FEBS Lett. 331, 101–104
8. Lu, W. P., Kincad, E., Sun, Y., and Bauer, M. D. (2001) J. Biol. Chem. 276, 31494–31501
9. Grebe, T., and Hakenbeck, R. (1996) Antimicrob. Agents Chemother. 40, 829–834
10. Krauss, J., van der Linden, M., Grebe, T., and Hakenbeck, R. (1996) Microb. Drug Resist. 2, 183–186
11. Dowson, C. G., Coffey, T. J., and Spratt, B. G. (1994) Trends Microbiol. 2, 361–366
12. Laible, G., Spratt, B. G., and Hakenbeck, R. (1993) Mol. Microbiol. 5, 193–202
13. Asahi, Y., Takeuchi, Y., and Uchibata, K. (1999) Antimicrob. Agents Chemother. 43, 1252–1255
14. Chesnel, L., Pernot, L., Lemaire, D., Champelovier, D., Croize, J., Dideberg, O., Vernet, T., and Zapun, A. (2003) J. Biol. Chem. 278, 44448–44456
15. Pernot, L., Chesnel, L., Le Gouellec, A., Croize, J., Vernet, T., Dideberg, O., and Dessen, A. (2004) J. Biol. Chem. 279, 16463–16470
16. Chesnel, L., Carapito, R., Croize, J., Dideberg, O., Vernet, T., and Zapun, A. (2005) Antimicrob. Agents Chemother. 49, 2895–2902
17. Claverys, J. P., and Lacks, S. A. (1986) Microbiol. Rev. 50, 133–165
18. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) Methods Enzymol. 204, 125–139
19. Studier, F. W. (2005) Protein Expression Purif. 41, 207–234
20. Dessen, A., Mouz, N., Gordon, E., Hopkins, J., and Dideberg, O. (2001) J. Biol. Chem. 276, 45106–45112
21. Freer, J. M., Ghayesb, I. M., and Beatson, M. (1975) Eur. J. Biochem. 57, 343–351
22. Gordon, E., Mouz, N., Duee, E., and Dideberg, O. (2000) J. Mol. Biol. 299, 477–485
23. Chesnel, L., Zapun, A., Mouz, N., Dideberg, O., and Vernet, T. (2002) Eur. J. Biochem. 269, 1678–1683
24. Parex, S., Mouz, N., Petillot, Y., Hakenbeck, R., and Dideberg, O. (1996) Nat. Struct. Biol. 3, 264–269
25. Macbeochef, P., Di Guilmi, A. M., Job, V., Vernet, T., Dideberg, O., and Dessen, A. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 577–582