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# Penicillin-Binding Proteins 2b and 2x of *Streptococcus pneumoniae* Are Primary Resistance Determinants for Different Classes of β-Lactam Antibiotics

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**High-level resistance to β-lactam antibiotics in *Streptococcus pneumoniae* is mediated by successive alterations in essential penicillin-binding proteins (PBPs). In the present work, single amino acid changes in *S. pneumoniae* PBP 2x and PBP 2b that result in reduced affinity for the antibiotic and that confer first-level β-lactam resistance are defined. Point mutations in the PBP genes were generated by PCR-derived mutagenesis. Those conferring maximal resistance to either cefotaxime (*pbp2x*) or piperacillin (*pbp2b*) were obtained after transformation of the susceptible laboratory strain R6 with the PCR-amplified PBP genes and selection on agar with various concentrations of the antibiotic. In the case of PBP 2x, transformants for which the cefotaxime MIC was 0.16 μg/ml contained the substitution of a Thr for an Ala at position 550 (Thr550→Ala), close to the PBP homology box Lys547SerGly, a mutation frequently observed in laboratory mutants and in a high-level cefotaxime-resistant clinical isolate as well. After further selection, transformants resisting 0.3 μg of cefotaxime per ml were obtained; they contained the substitution Gly550 as the result of two mutations in the same codon. In PBP 2b, Thr446→Ala, adjacent to another homology box Ser443SerAsn, was the mutation selected with piperacillin. This substitution has been described in all clinical isolates with a low-affinity PBP 2b but was distinct from point mutations found in laboratory mutants. Both *pbp2b* with the single mutation and a mosaic *pbp2b* of a clinical isolate conferred a twofold increase in piperacillin resistance. Attempts to select PBP 2b variants at higher piperacillin concentrations were unsuccessful. The mutated PBP 2b also markedly reduced the lytic response to piperacillin, suggesting that such a mutation is an important step in resistance development in clinical isolates.**

Penicillin-binding proteins (PBPs) are the classical target enzymes for β-lactam antibiotics. A resistance mechanism that is independent of the production of β-lactamases has been described in several gram-positive bacterial species. It involves alterations of essential PBPs in such a way that interaction with β-lactams takes place at much higher antibiotic concentrations than with PBPs of susceptible strains, and hence, the biological activity of the drug is greatly reduced (for a review, see reference 28).

PBPs interact with β-lactams enzymatically by forming a covalent complex via the active-site serine. The affinity for a given β-lactam is different for different PBPs, and conversely, one PBP has distinct affinities for different β-lactams. Therefore, point mutations reducing the affinity for one β-lactam do not necessarily affect the affinity for another compound, and different β-lactams may interact with and inhibit different PBPs, as has been described for *Escherichia coli* (29). The primary target PBPs are essential enzymes, and therefore, mutations affecting the active site in such a way that the inhibitor molecule can no longer bind must not interfere with the actual in vivo function, i.e., binding to the actual substrate molecule.

*Streptococcus pneumoniae* has served as a model organism for development of PBP-mediated resistance for various reasons (9). The appearance and spread of clinical pneumococcal isolates resistant to penicillin is a frightful development, reinforcing the need for understanding the selective conditions and the evolutionary mechanism. However, the situation is complicated by the fact that up to four PBPs are altered in highly resistant clinical isolates (18). In those strains, low-affinity variants of the three PBPs 1a, 2x, and 2b contain multiple amino acid substitutions because of the mosaic structure of the respective genes (5, 18, 20), and the contribution of these changes to resistance is not clear. So far, only PBP 2x has been described as the primary PBP target in clinical isolates (18), since *pbp2x* genes encoding low-affinity variants transfer β-lactam resistance to susceptible strains. In contrast, low-affinity variants of PBP 2b were selected with benzylpenicillin in low-level-resistant recipient strains that already contained a low-affinity PBP 2x (6), and also, selection of a low-affinity PBP 1a required the presence of a low-affinity PBP 2x in the recipients (23).

One approach for analyzing structural prerequisites of an essential PBP for remodeling the active site is the identification and characterization of point mutations in the PBPs of laboratory mutants. Such strains have been obtained by using increasing concentrations of a β-lactam antibiotic belonging to either one of two classes (16): piperacillin, a lytic β-lactam that binds with a high affinity to all pneumococcal PBPs, and cefotaxime, which results in a tolerant response and that does not bind to PBP 2b (13). All mutants isolated after five selection steps on increasing antibiotic concentrations contained point mutations in their PBP genes. In addition, non-PBP genes play a role in resistance development in the laboratory mutants, and some of these non-PBP genes were even responsible for the first step in the increase in resistance (8, 12). The first PBP gene (but, as outlined above, not necessarily the primary gene)

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affected in cefotaxime-resistant mutants was PBP 2x (17), whereas it was PBP 2b in piperacillin-resistant mutants (12).

Comparison of the location of point mutations in several independently isolated mutants revealed that two regions were preferentially affected in both PBP 2x and PBP 2b, although different β-lactams were used for selection, suggesting that they are generally important for interaction with β-lactams: the KT/SG box (an amino acid sequence common to all PBPs and β-lactamases) and adjacent amino acids and the end of the (theoretically defined) penicillin-binding domain, approximately 50 amino acids C terminal of the KT/SG box (for a review, see reference 7).

We investigated whether we could obtain mutations not only in PBP 2x but also in PBP 2b that allowed for the direct selection of β-lactam resistance in a penicillin-susceptible strain. Here we report on the isolation and characterization of mutations in PBP genes generated via PCR amplification and show that a mutation in PBP 2b that elevates the MIC of piperacillin affects the rate of β-lactam-induced lysis as well.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The penicillin-susceptible laboratory strain *S. pneumoniae* R6 is an unencapsulated derivative of the Rockefeller University strain R36A (1). The piperacillin-resistant mutant P506 is a derivative of the R6 strain; it is a member of the mutant lineage 6 (last digit) and has been obtained by five successive selections (first digit) with increasing concentrations of piperacillin (16). The clinical isolate *S. pneumoniae* 2349 from Spain was obtained from J. Liñares (Barcelona, Spain) and is a representative of the highly penicillin-resistant and multiple-antibiotic-resistant serotype 23F clone (22, 27). *Streptococcus oralis* M3 is a penicillin-susceptible isolate from South Africa (26). *S. pneumoniae* and *S. oralis* were grown in C medium (15) supplemented with 0.2% yeast extract at 37°C without aeration, and cellular growth was monitored by nephelometry. Cultures were stored as glycerol stocks at −80°C. *E. coli* INVαF′ (Invitrogen, Leek, The Netherlands) was grown in SOC medium (24) and was used for propagation of the recombinant PCR II Vector (Invitrogen).

**MIC determination.** Pneumococci were tested on blood agar plates (3% sheep blood) containing narrow dilutions of the respective antibiotic.

**PCR.** The standard method of PCR amplification was optimized for short cycles by using pneumococcal cells instead of isolated DNA. Cells were taken from glycerol stock cultures (approximately 100 μl) and were pelleted in an Eppendorf centrifuge. A total of 1 μl of the pellet was used in a 100-μl sample containing 6 mM $MgCl_2$, 50 pmol of the oligonucleotide primers, 0.5 μl of *Taq* DNA polymerase (Gibco BRL, Berlin, Germany), and buffer according to the manufacturer. The PCR was carried out for 28 cycles in a Biomed thermocycler with denaturation for 5 s at 96°C, annealing for 5 s at 52°C, extension for 10 s at 72°C, and then a 2-min delay. The following oligonucleotide primers were used for amplification of the 3′ end of the PBP genes: for *pbp2x*, primers Pn2xUP and Pn2xDOWN (18), with the resulting DNA fragment including codons 75 to 750 of the structural *pbp2x* gene; for *pbp2b*, primers 5′-TAACAGCCATTCGATTC CGA from positions 244 to 263 and 5′-TTTCCTTTCTAGTTCATTGG from positions 2283 to 2264, corresponding to codons 5 to 680.

**Transformation.** Transformation of *S. pneumoniae* R6 was carried out essentially by the method of Tiraby and Fox (30), but with a phenotypic expression period of 90 to 120 min in liquid medium. β-Lactam-resistant transformants were isolated at the antibiotic concentrations stated below, and further details are given in the Results section. In a standard transformation assay, $10^6$ competent cells were plated under selective conditions. The number of transformants obtained with PCR-amplified PBP genes from susceptible strains (i.e., a randomly mutagenized population of PBP genes) decreased with an increasing concentration of the antibiotic used for selection. In the case of piperacillin, with which selection was only possible close to the MIC for the susceptible strain R6 (0.04 μg/ml), competent cells with no added DNA gave rise to approximately 50 colonies compared with the $2 \times 10^2$ to $5 \times 10^2$ colonies obtained with the transformed cells. In the case of cefotaxime, with which transformants could be selected up to a concentration of 0.15 μg/ml, which is well above the MIC for strain R6 (0.02 μg/ml), no colonies were seen when competent cells without DNA were plated. Colonies were picked under conditions at which not more than 500 transformants appeared on the selection plate. When PBP genes known to encode low-affinity PBP variants were tested in a transformation assay under the same selective conditions, $>10^4$ transformants were obtained.

Transformation of *E. coli* INVαF′ with recombinant plasmid DNA was performed as described by Invitrogen.

**DNA sequencing.** DNA sequencing of the cloned PBP gene fragments was performed by the dideoxy-nucleotide chain termination method (25) with the T7 sequencing kit (Pharmacia). Mutations were verified by sequencing the respective regions in independent PBP clones.

**Detection of PBPs.** *S. pneumoniae* cells were resuspended in 20 mM sodium phosphate buffer (pH 7.2). After the addition of 0.1% Triton X-100 (final concentration) and [$^3$H]propionylampicillin, the cells were lysed and the PBPs were labeled during an incubation period of 20 min at 37°C. The PBPs in the cell lysates were visualized after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography as described previously (11).

## RESULTS AND DISCUSSION

The approach taken here for the isolation of point mutations in PBP genes differed from the approaches taken in earlier attempts in several respects. First, isolated PBP gene fragments were used for selection, thereby avoiding the contribution of other non-PBP genes known to play a role in β-lactam resistance in laboratory mutants (8, 12). Second, the antibiotic concentrations used for selection were as high as possible in order to obtain the most effective mutation(s), whereas in a previous study (16), the lowest antibiotic concentration that allowed differentiation between the parental strain and a less susceptible mutant was used in order to obtain a wide variety of mutations. Third, the PBP genes used were randomly mutagenized via PCR by *Taq* polymerase-introduced errors during the polymerization reaction rather than by using specific in vitro mutagenesis. One should keep in mind that only those mutations that do not severely affect the essential in vivo function of the PBP will be found.

**Selection of PCR-derived mutated PBP 2x.** Mutations in PBP 2x that result in reduced penicillin affinity can readily be selected with cefotaxime in the laboratory (17). Such mutated *pbp2x* genes can be used as donor DNA in transformation assays and can easily confer to recipient cells of the susceptible strain R6 resistance to 0.06 to 0.08 μg of cefotaxime per ml. With PCR-amplified R6 *pbp2x* as the donor DNA, $>10^4$ transformants of R6 were obtained up to a selective concentration of 0.06 μg of cefotaxime per ml, and at between 0.13 and 0.15 μg/ml, the number of transformants decreased to approximately $1 \times 10^3$ to $0.5 \times 10^3$. This indicates that several different mutations in PBP 2x can confer resistance at the lower concentrations, whereas the number of mutations conferring resistance at higher concentrations is more restricted. Concentrations above 0.15 μg/ml gave only negative results. Transformants were picked from the plate with the highest concentration possible. In order to verify that a mutated PBP 2x was responsible for the resistance increase, *pbp2x* DNA from such transformants was amplified by PCR, cloned, and tested again in a transformation assay with the R6 strain as the recipient, and the high number of transformants ($>10^4$) generally achieved with altered *pbp2x* genes was obtained. The mutations in the *pbp2x* genes of the respective transformants were then identified after sequencing of the cloned *pbp2x* fragment, and the transformant was characterized further as described in the following paragraphs.

Three types of experiments were performed. (i) *pbp2x* from the susceptible laboratory strain R6 was used to select resistant transformants after one round of PCR amplification (transformants of class R1). Under these conditions, transformants resistant to 0.12 to 0.15 μg/ml were obtained, as outlined in the previous paragraph, and no colonies were obtained when competent R6 cells with no DNA added were plated under such conditions. (ii) The R6 *pbp2x* gene was subjected to 10 rounds of selection, e.g., after PCR amplification, transformation, and selection, the *pbp2x* gene of a resistant transformant was again amplified by PCR and was used as the donor in a second round of transformation and selection in order to determine the limit of selectability under these conditions (class R2 transformants), and only then did we obtain transformants that were resistant to at least 0.2 μg/ml. The extremely low frequency of this event (more than nine PCR of 28 cycles each) strongly

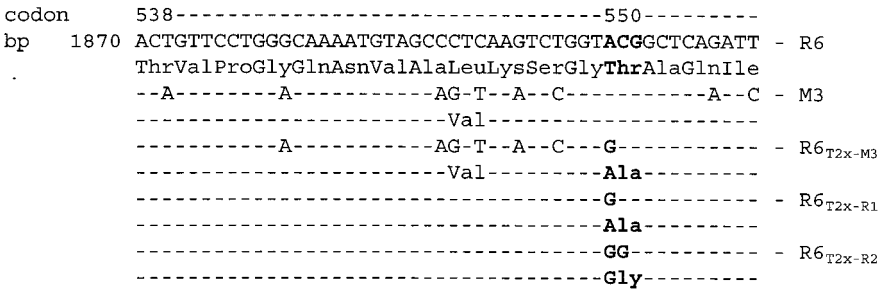


FIG. 1. Mutations obtained in *S. pneumoniae* R6 transformants obtained with PCR-amplified *pbp2x* as donor DNA and cefotaxime selection. The DNA and amino acid sequences of *S. pneumoniae* R6 *pbp2x* (R6) and *S. oralis* M3 *pbp2x* (M3) between codons 538 to 553 are aligned at the top. Alterations of R6 transformants ($R6_T$) obtained with *pbp2x* after PCR-derived mutagenesis are indicated below; only nucleotides and amino acid residues that differ from those of R6 are shown. $R6_{T2x\text{-}M3}$, R6 transformants obtained with *S. oralis* M3 *pbp2x* with 0.13 μg of cefotaxime per ml; $R6_{T2x\text{-}R1}$, R6 transformant obtained with R6 *pbp2x* with 0.13 μg of cefotaxime per ml (class R1); $R6_{T2x\text{-}R2}$, R6 transformants obtained after multiple rounds of selection and transformation with PCR-amplified R6 *pbp2x* with 0.2 μg of cefotaxime per ml (class R2).

suggests that more than one mutation is required for the elevated level of resistance. Another four rounds of selection with cefotaxime concentrations greater than 0.3 μg/ml gave only negative results. (iii) The homologous *pbp2x* gene of a susceptible strain, *S. oralis* M3, was amplified by PCR and was used for the selection of resistant transformants in *S. pneumoniae* R6. The *S. oralis pbp2x* gene is approximately 20% divergent in nucleotide sequence from that of the penicillin-susceptible *S. pneumoniae* R6 strain, resulting in more than 10% altered amino acids, but it is highly related (less than 3% divergence) to a main class of mosaic *pbp2x* genes of penicillin-resistant and high-level cefotaxime-resistant clinical isolates of *S. pneumoniae* (26). The number of transformants obtained after three successive rounds of PCR amplification, transformation, and selection was similar to that obtained with the *S. pneumoniae* R6 *pbp2x* gene, and selection with greater than 0.15 μg of cefotaxime per ml gave only negative results.

In all three approaches—that is, one round of PCR amplification of the R6 *pbp2x* gene and then transformation and selection (R1), several rounds of the same selection procedure (R2), and selection with the *S. oralis* M3 *pbp2x* gene—the same *pbp2x* codon 550 was affected in the resistant transformants analyzed (Fig. 1). The mutation selected in the R1 transformant resulted in a change from a Thr to an Ala at codon 550 (Thr550→ Ala) (ACG to GCG). The mutation obtained after multiple rounds of selection at the highest possible concentrations was Thr550→ Gly (ACG to GGG); i.e., two changes in the same codon had been selected consecutively. With the strain M3 *pbp2x* gene, the mutation obtained was identical to the one obtained with the strain R6 gene: ACG to GCG, i.e., Thr550→ Ala. Surprisingly, after the three rounds of selection, only a small part of the M3 *pbp2x* gene remained in the transformant (between codons 541 and 550), as judged from the nucleotide alterations surrounding the mutation, and all of the remaining sequence was that of the recipient R6 strain (Fig. 1). In other words, three successive transformations sufficed to reduce the potential mosaic structure to the immediate environment of the important point mutation selected for. Since mosaic PBP genes appear to be fairly stable within a resistant pneumococcal clone (10, 20, 22), this result suggests that transformation events are not frequent outside of the laboratory or that it is important to maintain the mosaic makeup of the gene, which results in a corresponding mosaic protein for functional reasons.

The MIC of cefotaxime increased from 0.02 μg/ml for the susceptible R6 strain first to 0.16 μg/ml with the mutation Thr550→ Ala and then to 0.3 μg/ml with the mutation Ala550 → Gly. PBP 2x with a single mutation at the C-terminal end of the penicillin-binding domain between residues 596 and 601 that were isolated at primary mutations in cefotaxime-resistant laboratory mutants conferred cefotaxime MICs of only 0.06 to 0.08 μg/ml, and the Thr550→ Ala mutation occurred at later selection steps only (17; unpublished data). The Thr550 in the *S. pneumoniae* PBP 2x corresponds to the Thr301 in the DD-peptidase of *Streptomyces* sp. strain R61, in which analysis of refined crystal structures revealed an important role of the OH side chain of Thr301 in cefotaxime binding (14). Our results confirm the importance of this residue for cefotaxime resistance. Both mutations had almost no effect on ampicillin or piperacillin susceptibility, except that the Thr550→Ala mutation resulted in an increased susceptibility to oxacillin, an effect not seen with the glycine residue at the same position (Table 1). In fact, all cefotaxime-resistant laboratory mutants containing the Thr550→Ala substitution that were described recently (17) had lost cross-resistance to oxacillin (unpublished data), and the same mutation in a high-level cefotaxime-resistant clinical isolate resulted in the loss of resistance to benzylpenicillin (3). It is hoped that the successful crystallization of a PBP 2x derivative will provide the basis for understanding the effects of the mutations on differential β-lactam binding on the structural level (2).

Several attempts by PCR to select for further *pbp2x* mutations conferring even higher resistance levels failed. It is interesting that the mosaic *pbp2x* genes of all penicillin-resistant *S.*

TABLE 1. MICs for R6 derivatives with altered PBP 2b or PBP 2x

| Strain | MIC (μg/ml)[a] | | |
|---|---|---|---|
| | Piperacillin | Oxacillin | Cefotaxime |
| R6 | 0.04 | 0.09 | 0.025 |
| $R6_{T2x\text{-}R1}$ | 0.03–0.04 | <0.02 | 0.16 |
| $R6_{T2x\text{-}R2}$ | 0.03–0.04 | 0.09 | 0.3 |
| $R6_{T2b\text{-}P506}$ | 0.04–0.05 | ND | ND |
| $R6_{T2b\text{-}R1}$ | 0.08 | 0.09 | 0.025 |
| $R6_{T2b\text{-}23F}$ | 0.08 | ND | ND |
| $R6_{T2x\text{-}R2/2b\text{-}R1}$ | 0.08 | ND | 0.3 |

[a] MICs were determined by agar dilution with narrow concentrations of the respective antibiotic. Transformants of *S. pneumoniae* R6 ($R6_T$) contained the PBP 2x mutation Thr550→Ala ($R6_{T2x\text{-}R1}$) or Thr550→Gly ($R6_{T2x\text{-}R2}$), the PBP 2b mutation Gly617→Ala ($R6_{T2b\text{-}P506}$) or Thr446→Ala ($R6_{T2b\text{-}R1}$), or the mosaic class B PBP 2b of the penicillin-resistant type 23F clinical isolate 2349 ($R6_{T2b\text{-}23F}$) or the respective mutation in both, PBP 2x and PBP 2b ($R6_{T2x\text{-}R2/2b\text{-}R1}$); ND, not determined.

*pneumoniae* clones analyzed in our laboratory (more than 20, including the multiresistant clone 23F) also conferred resistance to cefotaxime at a concentration of approximately 0.16 to 0.2 μg/ml but did not contain the Thr550→Ala mutation (unpublished data). On the other hand, the *pbp2x* genes of two high-level cephalosporin-resistant *S. pneumoniae* solates (3) or viridans group streptococci (unpublished data) did not contain the Gly550 mutation, but nevertheless, they conferred resistance to cefotaxime at a concentration of up to 0.5 μg/ml. This demonstrates that another mutation, the combination of several mutations, or the mosaic makeup of the protein must be responsible for the cefotaxime resistance that was selected outside of the laboratory. One of the high-level cephalosporin-resistant *S. pneumoniae* isolates contained the Ala550 mutation (3), strongly suggesting that in this particular case either one of the last two possibilities must be true.

**Selection of PCR-derived mutated PBP 2b.** So far, selection of a low-affinity PBP 2b in a susceptible wild-type background has not been reported. However, a correlation between the MICs of a variety of β-lactams and the concentration required to half-saturate PBP 2b indicated that this PBP is the main physiologically important target (32). In agreement with this, the gene for PBP 2b was the first PBP gene (although not the primary gene) affected during selection for the piperacillin-resistant laboratory mutants (12). We therefore tried to transform the *pbp2b* allele of the piperacillin-resistant mutant P506 (Gly617→Ala) directly into the R6 strain using piperacillin for selection. Resistant transformants were indeed obtained, although the increase in resistance was only marginal (Table 1). Since this experiment nevertheless demonstrated that a low-affinity PBP 2b can be selected in the R6 strain, the PCR mutagenesis approach was also applied to *pbp2b* in order to identify mutations that confer more significant levels of resistance.

At piperacillin concentrations of 0.04 μg/ml, the MIC for the R6 strain, the number of transformants obtained was approximately 200 when PCR-amplified *pbp2b* of the R6 strain was used as donor DNA (which is roughly fourfold more than that when competent cells and no DNA were used). When the *pbp2b* gene of such a resistant transformant was subjected to PCR amplification and tested again, transformants were readily selectable at a frequency comparable to the one obtained with a mutated *pbp2x* gene and cefotaxime ($>10^4$), demonstrating that an altered *pbp2b* gene is responsible for the increase in the level of resistance. The same high number of transformants was obtained when the *pbp2b* gene of a clinical isolate, *S. pneumoniae* 2349, a representative of the multiresistant Spanish clone 23F, was used as donor DNA. The *pbp2b* gene of this clone is a mosaic gene of class B (5), and the experiment shows that it also contains an alteration selectable with piperacillin.

The PCR-derived mutation in the *pbp2b* gene of the transformant resulted in a Thr446→Ala change (ACC to GCC) directly adjacent to the Ser443SerAsn-triad, the SXN box that is conserved in all PBPs (7). This mutation is identical to that found in the *pbp2b* genes of all clinical isolates investigated so far, including that of the 23F clone, but is absent from penicillin-susceptible *S. pneumoniae* and *Streptococcus mitis* isolates, strongly suggesting that this mutation is a prerequisite for the development of resistance in clinical isolates (4, 28).

The Thr446→Ala mutation conferred a twofold increase in piperacillin resistance when it was transformed into the R6 strain (MIC, 0.08 μg/ml), which is identical to the MIC obtained with *pbp2b* of the highly penicillin-resistant strain *S. pneumoniae* 2349. Distinct mutations were seen in piperacillin-resistant laboratory mutants (12), and the *pbp2b* allele of the mutant P506 (Gly617→Ala) conferred resistance of only 0.04 to 0.05 μg/ml (Table 1). The MICs of oxacillin and cefotaxime remained unchanged in all cases. For a double mutant, PBP 2b-Ala446/PBP 2x-Gly550, the MICs of piperacillin and cefotaxime were not higher compared with those for the respective single mutants, documenting the specificity of each of the mutations (Table 1).

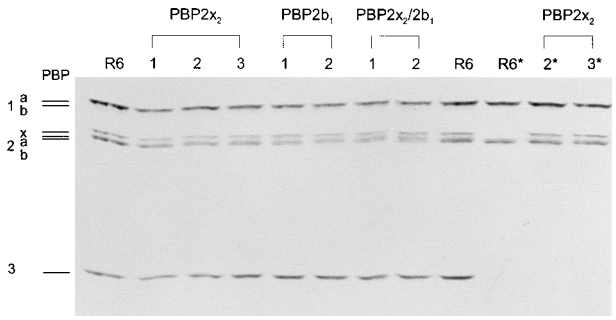


FIG. 2. PBP profiles of *S. pneumoniae* R6 transformants containing various PBP 2x and PBP 2b mutations. Cell lysates of the parental strain R6 and the transformants were incubated with [$^3$H]propionylampicillin and PBPs were detected after SDS-PAGE and fluorography. *, cell lysates were preincubated with 0.05 μg of cefotaxime per ml prior to radioactive labeling. Lanes 1, 2, and 3, independently isolated transformants obtained with different PCR-mutagenized PBP genes. PBP mutations are as follows: PBP $2x_2$, Thr550→Gly; PBP $2b_1$, Thr446→Ala; PBP $2x_2/2b_1$, double mutant. The positions of the PBPs are indicated on the left.

**Affinity changes in mutated PBP 2x and PBP 2b.** The PBP profiles of the transformants obtained with mutated *pbp2x* and *pbp2b* genes were visualized in cell lysates by using standard concentrations of [$^3$H]propionylampicillin (Fig. 2). The different transformants tested all looked identical: an affinity change in PBP 2x or PBP 2b was only barely detectable. Since the radioactive β-lactam is a penicillin derivative, i.e., does not belong to the class of selective compounds related to cefotaxime, and since its concentration is not exactly known because of the chemical synthesis involved, we tried to detect affinity changes (i) in PBP 2x after preincubation with nonradioactive cefotaxime and (ii) in PBP 2b using lower dilutions of the radioactive antibiotic. As can be seen in Fig. 2, the PBP 2x of the susceptible R6 strain can no longer be labeled after preincubation with cefotaxime, whereas the two mutant PBP 2x's were perfectly visible; i.e., the mutation conferred a reduced affinity for cefotaxime. In contrast, with a low concentration of [$^3$H]propionylampicillin, the altered PBP 2b clearly had a decreased affinity for this class of antibiotics and could not be labeled, whereas the mutated PBP 2x as well as the R6 PBP 2x were perfectly visible under these conditions (Fig. 3). Under these conditions, PBPs 1a and 1b in all strains are also not fully saturated; they therefore appear to be much less labeled compared with that in the fluorogram shown in Fig. 2.

**Effect of PBP mutations on growth and lysis.** The growth of none of the transformants was significantly affected in liquid medium; the generation time was between 36 and 39 min in all cases, including the R6 strain, as determined in three different experiments (Fig. 4). In addition, the lytic response of the various mutants was tested for the following reason. Cefotaxime has been shown to induce a tolerant response only but no lysis, and this was related to the fact that it does not interact with PBP 2b (13). The mutations in PBP 2b that confer low affinity, i.e., that prevent interaction with a β-lactam, should mimic the nonlytic response to any β-lactam, but mutations in PBP 2x should not. Induction of lysis with piperacillin was tested in R6 derivatives containing (i) the PCR-derived PBP 2b

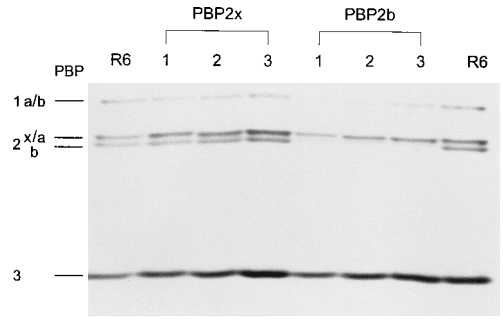


FIG. 3. Effects of PBP 2x and PBP 2b mutations on piperacillin affinity. PBPs in cell lysates were labeled with [$^3$H]propionylampicillin. Only 1/30 of the standard concentration was used in order to detect potential affinity changes (approximately $3 \times 10^4$ cpm). Results for strain R6 (susceptible laboratory strain *S. pneumoniae* R6), R6 transformants, (lane 1, $R6_{T2x\text{-}M3}$; lane 2, $R6_{T2x\text{-}R1}$ [Thr550→Ala]; lane 3, $R6_{T2x\text{-}R2}$ [Thr550→Gly]), and *pbp2b* (lane 1, $R6_{T2b\text{-}R1}$ [Thr446→Ala]; 2, $R6_{T2b\text{-}P506}$ [Gly617→Ala]; lane 3, $R6_{T2b\text{-}23F}$) are shown.

mutation, (ii) the *pbp2b* gene of the clinical isolate, and (iii) the PBP 2x Thr550→Gly mutation (Fig. 4). All cultures were treated with piperacillin at the same cell density. The parental R6 strain as well as the PBP 2x mutant had already lysed at the lowest piperacillin concentration used (0.02 μg/ml, corresponding to half the MICs for the strains), and at concentrations of 0.04 μg/ml and higher, rapid lysis occurred at similar rates in both strains (Fig. 4a and c). In contrast, the $R6_{T2b\text{-}R1}$ transformant (PBP 2b-Ala446) was less affected at corresponding concentrations (MIC, 0.08 μg/ml). This effect was even more pronounced in the R6 transformant containing the *pbp2b* gene of the clinical isolate (MIC, 0.08 μg/ml) in which lysis was strikingly reduced and in which the onset of lysis also appeared to be much delayed (Fig. 4d).

**Concluding remarks.** The mutations identified in PBP 2x and PBP 2b in the present study demonstrate the importance of the respective amino acid positions for interaction with β-lactams, Thr550 in PBP 2x and Thr446 in PBP 2b, both of which have previously been identified as important for the development of resistance in laboratory mutants and/or clinical isolates (17, 28). One interesting aspect concerning the biological effects of these mutations is the fact that they result only in

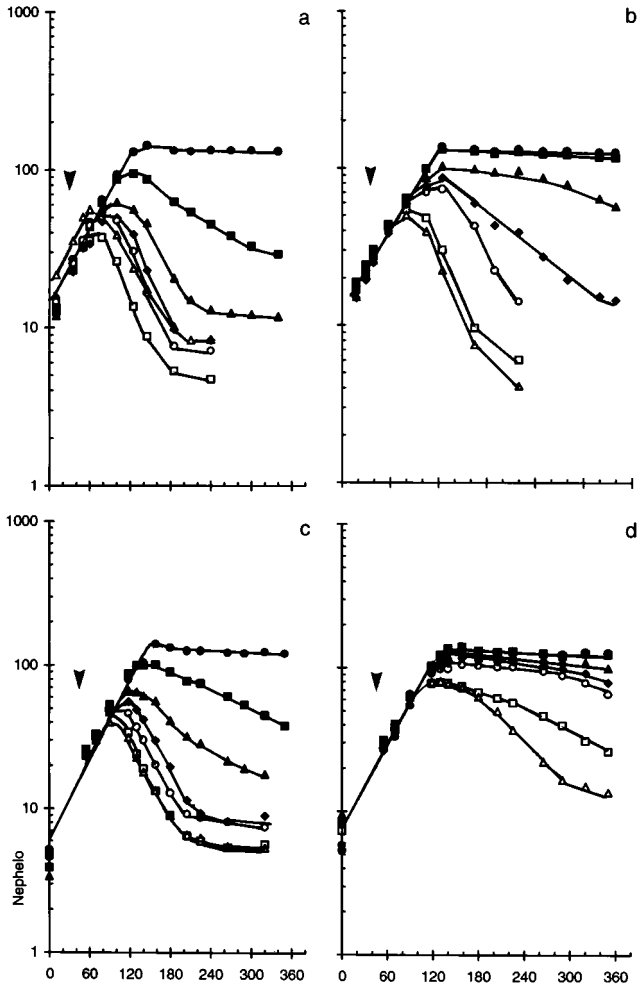


FIG. 4. Piperacillin-induced lysis in strain R6 derivatives containing low-affinity PBP 2x or PBP 2b. Overnight cultures grown in C medium were diluted 1:30 in prewarmed medium. Growth was monitored by nephelometry (Nephelo). At a nephelometry value of 25, the culture was divided into several aliquots and piperacillin was added to give final concentrations of 0 μg/ml (●), 0.02 μg/ml (■), 0.05 μg/ml (▲), 0.08 μg/ml (◆), 0.11 μg/ml (○), 0.5 μg/ml (□), and 1 μg/ml (△). (a) *S. pneumoniae* R6; (b) R6 with the PBP 2b mutation Ala446; (c) R6 with the PBP 2x mutation Gly550; (d) R6 transformant obtained with *pbp2b* of the highly penicillin-resistant 23F clinical isolate *S. pneumoniae* 2349. Arrowheads indicate addition of antibiotic.

a decrease in affinity to the selective antibiotic, i.e., cefotaxime and piperacillin, respectively, and, correspondingly, an increase in the level of resistance to the selective β-lactam. In both PBPs, the mutation is located directly adjacent to one of the three major homology boxes known in all penicillin-interacting enzymes, PBPs and β-lactamases, that form the active cavity (7): the Lys547ThrGly box in the case of PBP 2x and the Ser443SerAsn box in the case of PBP 2b. Thus, different classes of β-lactam antibiotics select for changes in different PBPs, and while cefotaxime has been known to be highly selective for PBP 2x mutations (17), the results presented here demonstrate for the first time that piperacillin selects for PBP 2b alterations; i.e., either one of the two PBPs represents a primary target for a distinct class of β-lactam antibiotics.

The levels of resistance achieved with one single point mutation in a PBP appear far from striking, especially in the case of PBP 2b, in which the Thr446 mutation conferred only a twofold increase in piperacillin resistance. However, one should remember that the high MICs documented for clinical isolates are the result of multiple alterations in at least four PBP genes. The second phenotype associated with the low-affinity PBP 2b, i.e., the reduced lytic response, may well be of much higher clinical significance, especially since it can be selected directly in a susceptible strain. In agreement with this, it has been noted before that in clinical isolates, penicillin resistance and defective lysis upon penicillin treatment are frequently associated (19). Most importantly, the cyclic antibiotic exposure that is generally used in the clinical setting has recently been shown to select primarily for lysis-defective mutants rather than for penicillin-resistant ones (21). Lysis-defective *S. pneumoniae* strains were isolated from several patients, and it has been suggested that this property adversely affects the course of meningitis, documenting the clinical relevance of this phenotype (31). It would be interesting to see whether lysis deficiency in these strains is indeed related to a low-affinity PBP 2b. Another aspect of a reduced lytic response upon penicillin treatment is related to the fact that penicillin resistance in pneumococci is an acquired property that involves gene transfer, and a lysis-defective strain may well have a better chance of taking up and incorporating DNA fragments encoding resistance determinants.

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