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Five Independent Combinations of Mutations Can Result in Low-Affinity Penicillin-Binding Protein 2x of *Streptococcus pneumoniae*

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Penicillin-binding protein 2x (PBP 2x) of *Streptococcus pneumoniae* is one of the high-molecular-weight PBPs involved in the development of intrinsic β-lactam resistance. Point mutations in the PBP 2x genes (*pbpX*) have now been characterized in five independent spontaneous laboratory mutants in order to identify protein regions which are important for interaction with β-lactam antibiotics. All mutant genes contained two to four mutations resulting in amino acid substitutions within the penicillin-binding domain of PBP 2x, and none of the mutants carried an identical set of mutations. For one particular mutant, C606, carrying four mutations in *pbpX*, the mutations at positions 601 and 597 conferred first- and second-level resistance when introduced into the susceptible parent strain *S. pneumoniae* R6. However, the other two mutations, at amino acid positions 289 and 422, which were originally selected at the fifth and sixth isolation steps, did not contribute at all to resistance in similar experiments. This suggests that they are phenotypically expressed only in combination with mutations in other genes. Three PBP 2x regions were mutated in from two to all four mutants carrying a low-affinity PBP 2x. However, in a fifth mutant containing a PBP 2x with apparent zero affinity for β-lactams, the three mutations in *pbpX* mapped at entirely different positions. This demonstrates that different mutational pathways exist for remodeling this PBP during resistance development.

Intrinsic penicillin resistance in pneumococci and various other pathogenic bacteria is mediated by the production of low-penicillin-affinity variants of essential high-molecular-weight penicillin-binding proteins (PBPs), the target proteins of β-lactam antibiotics (11, 25, 30, 34). These PBPs are believed to be essential enzymes functioning during late stages of murein biosynthesis. They interact with β-lactams by enzymatically forming a covalent penicilloyl (cephalosporoyl) complex which is enzymatically inactive. β-Lactams are recognized by PBPs because of their structural similarity to substrate molecules (i.e., muropeptides), and inhibitors as well as substrates interact with the same active-site serine (for a review, see reference 8). Therefore, mutations responsible for low affinity must be carefully positioned within the protein in order to still allow for its actual in vivo function.

We have recently isolated a series of spontaneous, independent mutants with stepwise increasing resistance to cefotaxime (17). All higher-level-resistance mutants contained low-affinity variants of PBP 2x and, in some cases, of PBP 2a as well. Reduction in the penicillin binding of PBP 2x at different selection steps was detected when mutants from different mutant families were compared, indicating that the development of resistance does not follow a strictly predetermined pathway.

The gene coding for the 750-amino-acid PBP 2x (*pbpX*) has been sequenced, and the mutations in one of the mutants (C506) have been mapped (18). We have now determined the sequence of mutations that occurred with increasing cefotaxime resistance in one mutant family and analyzed their contribution to resistance development. Furthermore, mutations in the *pbpX* genes of another four independent mutants, obtained after five or six selection steps, were characterized.

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MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The penicillin-susceptible laboratory strain *Streptococcus pneumoniae* R6 is an unencapsulated derivative of the Rockefeller University strain R36A (1). The cefotaxime-resistant mutants, all derived from the R6 strain, are independent, spontaneous mutants, as described previously (17). The mutant family C006 is composed of six mutants with increasing levels of resistance, from C106 (first-step isolate) to C606 (sixth-step isolate). Mutants C501, C503, C604, and C505 were obtained after five or six selection steps (indicated by the first digit) in mutant families 1, 3, 4, and 5, respectively (indicated by the last digit).

The phages Phagescript SK (Stratagene, San Diego, Calif.) and M13mp8/9 (22) were propagated in *Escherichia coli* JM103 (21), and derivatives of the plasmids pJDC9 (4) and pR28 (20) were propagated in *E. coli* DH5. Transformation of *S. pneumoniae* (32) and of *E. coli* (3) followed published procedures.

Isolation of DNA. Details of the isolation of chromosomal DNA and the preparation of phage and plasmid DNA have been described recently (2, 18).

Isolation and sequencing of *pbpX* genes. The 1.5- to 2-kb fraction of an *Eco*RI-*Pst*I digest of chromosomal C606 DNA was subcloned into the *Eco*RI-*Pst*I vector fragment of pR28. The recombinant plasmid pCG16 containing a 1.8-kb *Eco*RI-*Pst*I *pbpX* fragment was identified by restriction analysis. After subcloning into the vector Phagescript derived from M13 phages, the fragment was sequenced by using a set of unidirectional deletion derivatives obtained by the method of Guo et al. (9). The 3′ region of the *pbpX* gene was amplified by the polymerase chain reaction (PCR) technique with the primers 624 (pGCTTTTT..ATTGC; nucleotides 4 to 23) and 623 (pATTCCC...TGAC; nucleotides 786 to 767) and cloned into the vector pJDC9. Sequencing was carried out with M13 universal primers (28).

*Eco*RI-*Pst*I *pbpX* fragments of mutant family C006 were cloned by using the same strategy, and clones were identified

by colony hybridization (27). The mutations were identified after subcloning into M13mp8/mp9 and the sequencing of the following DNA fragments known to carry mutations in C606: (i) 200-bp *Hin*dII, (ii) 810-bp *Hin*dII-*Pst*I, and (iii) 460-bp *Eco*RV-*Pst*I (18).

pbpX genes of the independent mutants C501, C503, C604, and C505 were amplified by PCR (26), using the primers Pn2xUP (pC-478–GTGGG...ATGG-503) and Pn2xDOWN with an introduced *Eco*RI site (pG-2503–GCG...ATTA-2533) (19). Thirty cycles of 1 min of denaturation at 95°C, 2 min of annealing at 52°C, and 6 min of extension at 72°C were used. The amplified DNA bands were isolated from agarose gels by the method of Vogelstein and Gillespie (33). The ends were filled with Klenow polymerase, kinased, and restricted with *Eco*RI. The fragments were cloned into M13mp8/mp9 phages. At least two independent isolates were sequenced in order to resolve possible errors introduced by gene amplification by using a set of primers along the *pbpX* sequence.

Colony hybridization. Colony hybridization was performed essentially as described by Sambrook et al. (27). Colonies carrying the desired recombinant plasmids were detected with a nonradioactive labeling and detection kit (Boehringer, Mannheim, Germany), using a digoxygenin-labeled 460-bp *Eco*RV-*Pst*I DNA fragment of the R6 strain.

Detection of PBPs by Western blotting (immunoblotting) and immunostaining. Cell lysates of *S. pneumoniae* mutants, prepared as described previously (17), were incubated with 100 μg of benzylpenicillin per ml for 30 min at 37°C. Prolonged incubation times had no influence on the detectability of PBPs. Conditions of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein transfer onto nitrocellulose membrane, and immunostaining with rabbit anti-benzylpenicilloic acid antiserum have been described previously (10).

RESULTS

Mutations in PBP 2x genes of mutant family C006. The mutant family C006, as described recently, consists of five spontaneous mutants, derived from the sensitive laboratory strain R6 (17), with successively increasing resistance to cefotaxime; a sixth-step mutant, C606, has since been isolated. C stands for cefotaxime, the first digit indicates the selection step, and the last digit indicates the mutant family. A distinct affinity change in PBP 2x was apparent in the second-step mutant, C206; a drastic decrease in the affinity of PBP 2a was noted at the fourth selection step in mutant C406 (17). Since the mutants had been isolated as spontaneous mutants rather than after mutagenesis, one point mutation per selection step, with some of the mutations mapping in the PBP 2x gene, is to be expected. The positions of mutations in the C606 *pbpX* were determined first. The presence of these mutations in mutants C106 to C506 was then determined by sequencing the respective gene fragments in order to see at which selection step the mutations occurred (Tables 1 and 2). The C606 PBP 2x gene contained four mutations, three of which were found in C506 (18). Thus, the mutation at amino acid position 422 was introduced during the sixth step. The other mutations appeared at steps one and two, mapping curiously closely together at amino acid positions 597 and 601; the third mutation, Met-289 to Thr, was introduced at step five.

Contribution of the C606 *pbpX* mutations to resistance development. PBP 2x genes of C106, C206, C506, and C606 carrying one up to four mutations were used in transformation experiments, with *S. pneumoniae* R6 used as the acceptor strain. As shown in Table 1, transformants obtained with *pbpX* containing the first one or two mutations of C106 or C206 had MIC values identical to those of the donor strains, confirming that these two mutations are responsible for first- and second-level cefotaxime resistance. PBPs 2x of the transformants were of lower affinity, similar to the PBPs 2x of the donor strains (not shown).

TABLE 1. Amino acid substitutions in PBP 2x of mutant family C006

Mutant	Amino acid substitution at following position				MIC (μg/ml)	
	289	422	597	601	Mutant	Transformant[a]
R6	M	G	G	G	0.02	
C106	M	G	G	V	0.16	0.16
C206	M	G	D	V	0.32	0.32
C306	M	G	D	V	0.64	
C406	M	G	D	V	0.64	
C506	T	G	D	V	1.28	0.3
C606	T	D	D	V	1.28	0.3

[a] MIC for R6 transformant obtained with the *pbpX* of the respective mutant.

However, when *pbpX* genes of C506 or C606 with three or four mutations were used in corresponding transformation experiments, no transformants with MIC values higher than that of C206 could be obtained. Furthermore, with donor DNA containing only the third (Thr-289) or only the third and fourth (Met-422) mutations, resistant transformants could not be selected at all. These results imply that the complete transfer of resistance level can be achieved as long as one (and only one) gene is responsible for a given MIC, as is the case with the PBP 2x mutations in C106 and C206.

However, the third and fourth mutations apparently contribute to resistance only when introduced into a background of already low-level resistance. Experiments using C306 or C406 as the acceptor strain were unsuccessful; with both mutants, no competent cells could be isolated and the transformation frequency was below detectability. Whether this phenotype correlates with the low-affinity PBP 2a already present in C306 or whether other genes are involved is not known.

Mutations in PBP 2x genes of independent cefotaxime-resistant mutants. In addition to mutant C606, another four independent mutants isolated after five or six selection steps were available (17). The mutants C501, C503, and C604 carried a low-affinity PBP 2x similar to that of C506. However, when labeled with penicillin at concentrations as high

TABLE 2. Mutations in *pbpX* of independent mutants

Mutant	Nucleotide changes[a]
C501	A-1900–CG to GCG,TT-2051–G to TGG
C503	C-1528–GT to TGT, A-1900–CG to GCG, GG-2042–T to GAT
C604	A-1900–CG to GCG, TC-2039–A to TTA, GG-2054–A to GAA
C606	AT-1118–G to ACG, GG-1517–T to GAT, GG-2042–T to GAT, GG-2054–A to GTA
C505	C-1459–TT to TTT, C-1624–AG to AAG, AC-1829–C to AGC

[a] Numbering refers to the *pbpX* sequence with the A-253–GT start codon, as published in reference 17.

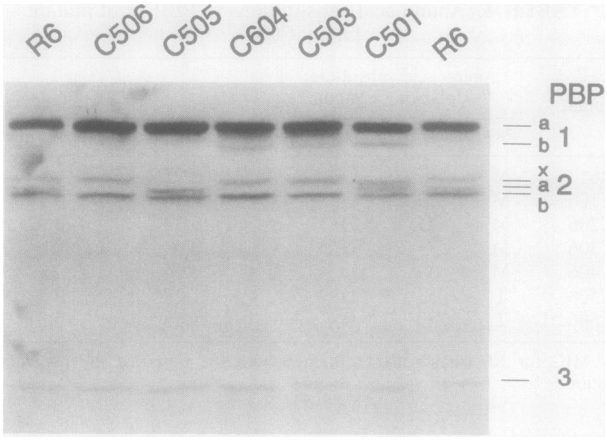

FIG. 1. PBPs in independent cefotaxime-resistant mutants after labeling with high concentrations of penicillin. Cell lysates of the parent strain R6 and the five mutant derivatives were labeled with 100 μg of benzylpenicillin per ml. After SDS-PAGE and blotting onto nitrocellulose, PBPs were revealed by using anti-benzylpenicilloyl antibodies and immunostaining. PBPs are indicated on the right.

as 100 μg/ml, PBP 2x could readily be detected (Fig. 1); the same result was obtained with C606 (not shown). In contrast, PBP 2x of mutant C505 could not be detected even under these conditions, as was the case when radioactive [^{3}H]propionylampicillin was used for labeling (17), indicating that this protein may be modified differently. It can also be seen that three of the mutants contained an apparent zero-affinity PBP 2a. In some experiments, a band with the mobility of PBP 1b appeared on the Western blot, as shown in Fig. 1. Since PBP 1b is not well stained with anti-benzylpenicilloyl antibodies (10), we believe that it represents a degradation product of PBP 1a known to have the same electrophoretic mobility (unpublished data) but not related to cefotaxime resistance.

Mutations in PBP 2x of the cefotaxime-resistant mutants were determined after the cloning of PCR-amplified *pbpX* gene fragments into M13. All mutations found were nonsynonymous changes (Table 2 and Fig. 2). None of the mutants contained an identical set of mutations, but the same GG-2042–T to GAT mutation (equals Gly-597 to Asp) occurred in C503 and C606, and three mutants showed an alteration of A-1900–CG to GCG (Thr-550 to Ala). In three protein regions, Gly-422 to Arg-426, Thr-550, and Ser-596 to Gly-601, the PBP 2x mutations of at least two and up to four mutants were located. On the other hand, mutations of PBP 2x from mutant C505 (with the zero-affinity PBP 2x) contained three mutations at positions totally different from those of the other four mutants (Fig. 2), confirming the phenotypic distinctions, as shown in Fig. 1, on the genetic level.

DISCUSSION

Analysis of PBP 2x genes from independent laboratory mutants revealed that an affinity change in PBP 2x can be achieved by different sets of mutations, although the selection mode was identical for all five mutants. The main difference was between the positions of mutations in the low-affinity PBPs 2x of the mutants C501, C503, C606, and C604 and those in the zero-affinity PBP 2x of C505. The mutations in the C505 PBP 2x indicate a different three-dimensional remodeling than exists in the other mutant PBP 2x (zero versus low affinity) and suggest that different steps of penicillin interaction are affected in the two mutant classes. The overall mechanism of the interaction of penicillin with PBPs follows a three-step model (8): $E + I \overset{K}{\rightleftarrows} EI \overset{k_2}{\rightarrow} EI^* \overset{k_3}{\rightarrow} E + P^*$, with E being the enzyme (PBP), I being the inhibitor (β-lactam), and P* being the inactive product. Resistance to a β-lactam can be induced by mutations that affect the formation of the reversible complex (recognition of the antibiotic, K), the formation of the covalent complex (catalytic step, k_2), or the turnover (k_3). The answer as to which reaction step is altered in which mutant type has to await detailed kinetic studies, the prerequisite being the isolation of PBP 2x in sufficient amounts.

Each of the four mutants with a low-affinity PBP 2x contained two to four mutations in the PBP 2x penicillin-binding domain. All but one of the mutations fall into three regions within the C-terminal half of the penicillin-binding domain.

All four mutant proteins contained one or two amino acid substitutions between residues 596 to 601 at the extreme C-terminal end of the penicillin-binding domain. The two mutations Gly-597 to Val and Gly-601 to Asp in the C606 PBP 2x, which were introduced during the first and second selection step, respectively, could be demonstrated to be solely responsible for low-level cefotaxime resistance. The same exchange of Gly-597 to Val was found in PBP 2x from one penicillin-resistant clinical isolate, and PBP 2x from another four resistant isolates contained amino acid substitutions in the same region (19). As shown earlier (18), low-affinity variants of related PBPs from other bacterial species carry alterations at homologous sites; examples are *E. coli* PBP 3 in some classes of cephalexin-resistant laboratory mutants (12, 13) and *Neisseria gonorrhoeae* PBP 2 (29) and *S. pneumoniae* PBP 2b (7) from penicillin-resistant clinical strains. This region may be important for interaction with β-lactams in general rather than being specific for cefotaxime. The mutations at position 601 predict an altered secondary structure, whereas the other mutations changed the hydrophobicity (not shown). Since it is still unclear where in relation to the active center these residues are positioned three-dimensionally, their impact on the overall arrangement of the active center is not clear.

The same Thr-to-Ala exchange directly adjacent to the KSG triad was found in three of the mutant proteins. Curiously, the reverse substitution, Ala-235 to Thr, at the homologous site of TEM β-lactamase resulted in an enzyme with an extended substrate profile that could hydrolyze cefotaxime, an antibiotic which is not a substrate for the original protein. Likewise, substitutions of Gly-236 to Ser or Glu-237 to Lys resulted in increased hydrolysis rates for third-generation cephalosporins and hence to increased resistance against these antibiotics in strains carrying the "new" β-lactamase (for a review, see reference 5). One might therefore speculate that the substitution of Thr-550 to Ala in PBP 2x is directly related to the selection for cefotaxime resistance. Interestingly, mutant C501, which has the Thr-to-Ala mutation, is rather specifically resistant to cefotaxime, whereas mutant C506, which lacks this mutation (but which has the two C-terminal substitutions), is resistant to other cephalosporins and penicillins as well (17). The K(H)T(S)G triad is part of the active center in β-lactamases (6, 14, 23, 24) and in the low-molecular-weight PBP of *Streptomyces* strain R61 (16), whose three-dimensional structures are known. Since this box is conserved in all high-molecular-weight PBPs (15), it is generally assumed

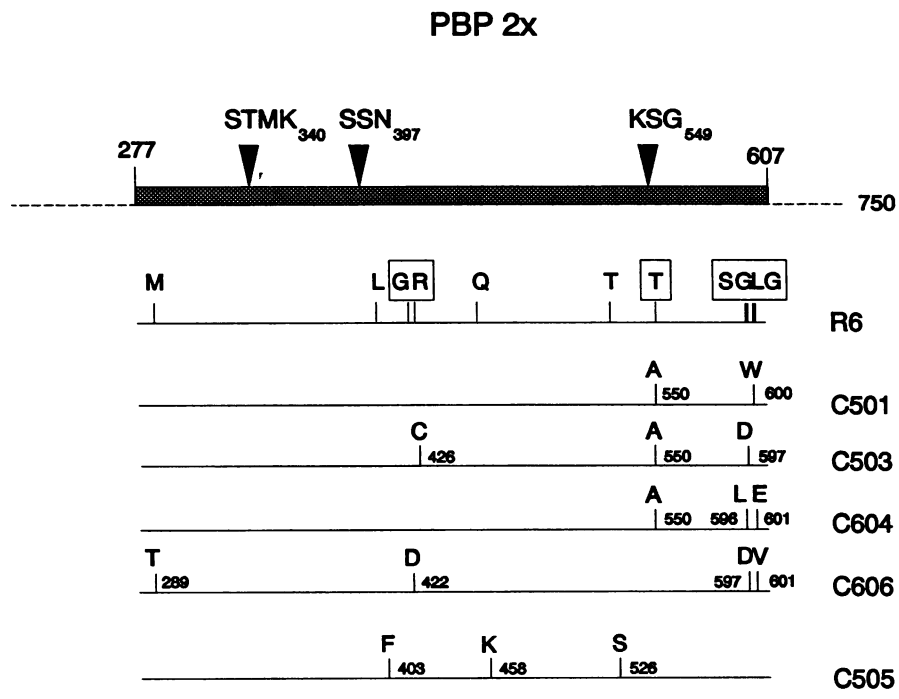

FIG. 2. Amino acid substitutions in the PBP 2x of five cefotaxime-resistant mutants. The penicillin-binding domain of PBP 2x (amino acids 277 to 607), with the three homology boxes (STMK [with the active-site serine], SSN, and KSG), is indicated above. The amino acid substitutions in the five mutants are shown. In the R6 sequence, positions at which two or more mutants are altered are boxed.

that here, too, these amino acids are located in a similar spatial relation to the active-site serine and contribute to the β-lactam-binding site. The importance of the three amino acid residues has been documented for *E. coli* PBP 3, in which an amino acid substitution within the KTG site at Thr-495 inactivates the enzyme without impairing its ability to bind penicillin (31). Mutations in the direct vicinity of the active center are especially critical if the PBP is not to lose its essential enzymatic function. This may be the reason why mutations in this region were found only at position 550, and in all three cases, the original Thr was replaced by the slightly more hydrophobic Ala.

Strains producing a PBP with reduced penicillin-binding capacity are resistant only up to the antibiotic concentration that inactivates another essential PBP. For example, *E. coli* strains with a mutated PBP 3 are substantially resistant to cephalexin, since PBP 3 is the primary target for cephalexin over a wide concentration range. In the *S. pneumoniae* mutant family C006, PBP 2x was mutated primarily, but PBP 2a had already become a low-affinity variant at the third selection step, and the third mutation in PBP 2x was introduced at the fifth selection step. This indicates that PBP 2a is the second target for cefotaxime. Therefore, it is not surprising that transformation of the C606 *pbpX* gene, which carries four mutations, into the susceptible R6 strain with the wild-type PBP 2a yielded transformants that were only as resistant as the second-step mutant C206. Likewise, C505, with the apparent zero-affinity PBP 2x (but with no severe defects in any other PBP), was not more resistant than the mutants with PBPs 2x of reduced affinity.

However, although the third PBP 2x mutation in the C006 family (Thr-289 in C506) resulted in a twofold increase in cefotaxime resistance (17), no resistant transformant of the R6 strain could be obtained at all when the donor DNA carried this mutation only. This suggests that its phenotype is expressed only in the context of the other mutations and that mutations are introduced in a certain succession during the remodeling of a PBP.

Some of the mutations may be merely compensating for better enzymatic function without decreasing the penicillin affinity to a great extent. For instance, the mutation in C606 PBP 2x Met-422 has only a little effect on affinity, and the increase in MIC between C506 and C606 is marginal. Similar observations have been described for *E. coli* PBP 3 (12). The impact of the different PBP 2x mutations on conformation and enzymatic function can be solved only after successful crystallization of the enzyme and X-ray analysis. Up to now, no satisfactory crystals have been obtained from high-molecular-weight PBPs. Cloning and expression of the *pbpX* gene is currently under investigation.

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