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In Vitro Selection of One-Step Mutants of *Streptococcus pneumoniae* Resistant to Different Oral β-Lactam Antibiotics Is Associated with Alterations of PBP2x

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Many oral penicillins and cephalosporins are used to treat clinical infections caused by *Streptococcus pneumoniae*. Therefore, using different β-lactams as selectors, we estimated the frequencies of one-step mutations leading to resistance. Resistant mutants were obtained from penicillin-susceptible, intermediately resistant, and penicillin resistant strains. For cefixime, cefuroxime, cefpodoxime, cefotaxime, and ceftriaxone, the frequencies of mutation ranged from 10^{-6} to 10^{-8} when resistant mutants were selected at 2- to 8-fold the MIC, and the MICs increased 2- to 16-fold. For ampicillin, ampicillin-sulbactam, amoxicillin, amoxicillin-clavulanic acid, cefaclor, and loracarbef, the frequencies of mutation were about 10^{-7} to 10^{-8}, and the MICs increased twofold at most. One to three resistance profiles of the resulting mutants were selected for each of the selecting antibiotics. Among those, some showed resistance to the cephalosporins associated with a 2- to 32-fold increase in susceptibility to the penicillins. Competition experiments showed a decreased affinity of PBP2x for cefpodoxime in all mutants. In some mutants that were more susceptible to amoxicillin, a decreased affinity of PBP2x for cefpodoxime was associated with an increased affinity for amoxicillin and a particular substitution of alanine for threonine at position 550 just after the KSG triad. From these results we infer (i) that among the β-lactams tested the penicillins, cefaclor, and loracarbef selected one-step resistant mutants less frequently and that they achieved a lower level of resistance, and (ii) that mutants with different profiles may have acquired different point mutations in PBP2x.

The most common mechanism of resistance to β-lactam antibiotics in pneumococci is linked to a decrease in the affinities of the penicillin-binding proteins (PBPs) for these antibiotics (5, 7, 11). It results from the recombination of the native PBP genes with PBP gene fragments from other streptococcal species, creating what are called mosaic PBPs (14). Cross-resistance between penicillins and cephalosporins is generally found in these strains, although at different levels (7, 15). Since oral penicillins and cephalosporins have been widely used to treat clinical infections caused by *Streptococcus pneumoniae*, one could expect that the extensive use of these compounds would select for either spontaneously resistant mutants from susceptible strains or more resistant mutants from already resistant strains. Thus, point mutations could occur in vivo in a gene coding either for a susceptible PBP or for a mosaic protein. The detection of such a mechanism in the absence of previous evidence of selection among the general population of resistant clinical isolates would be very difficult by using only standard susceptibility tests, and therefore, the rate of this type of event may be underestimated. Using susceptible, intermediately resistant, and resistant strains of pneumococci, we approached this problem by using different oral β-lactam antibiotics as selectors; we estimated the frequencies of mutation leading to decreased susceptibility to these compounds and determined if cross-resistance with other β-lactam antibiotics occurred.

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MATERIALS AND METHODS

Bacterial strains and selection of one-step resistant mutants. *S. pneumoniae* R6 is a susceptible derivative of the unencapsulated Rockefeller University strain R36A; *S. pneumoniae* 415 is a susceptible clinical isolate (MIC of penicillin, 0.015 μg/ml); *S. pneumoniae* 4387LL and *S. pneumoniae* 4411HL are clinical isolates which express intermediate resistance to penicillin G (0.25 μg/ml) and resistance to penicillin G (2 μg/ml), respectively. All clinical isolates were isolated at Hospital Saint Joseph, Paris, France.

The *S. pneumoniae* isolates were grown at 37°C on either Muller-Hinton agar containing 5% horse blood, or Todd Hewitt broth (Difco) with the addition of 5% yeast extracts. One-step resistant mutants were selected as follows. A total of 30 ml of an exponential culture at an optical density at 650 nm of 0.500 (approximately 10^8 to 10^9 CFU/ml) was harvested by centrifugation (5,000 × *g* at 4°C for 15 min). The cell pellet was resuspended in 1 ml of distilled ice-cold water, and the bacterial count from 200 μl ($\geq 10^9$ CFU/ml) was determined by serial dilutions on agar plates. Simultaneously, 200 μl was plated onto Mueller-Hinton agar containing 5% horse blood with concentrations equivalent to two-, four-, and eightfold the MIC of the β-lactam used as the selector. Incubation was for 3 to 7 days at 30°C. The mutation frequency was defined by the ratio of the number of colonies growing on the plates containing antibiotics compared with that obtained on the plate without antibiotic. The results are the means of at least two experiments. Generally, 10 to 20 colonies selected on the different concentrations of each antibiotic were picked to verify whether the selected resistant population was homogeneous with respect to the MIC of the selecting agent.

Susceptibility tests and antibiotics. MICs were determined on Muller-Hinton agar containing 5% horse blood with a Steers replicator device and an inoculum of 10^4 to 10^5 CFU per spot. MICs were read after 18 h at 37°C (3). The lowest antibiotic concentration which prevented visible growth was defined as the MIC. Antimicrobial compounds were kindly provided as follows: penicillin G, Specia, Paris France; amoxicillin and clavulanic acid, Beecham Sévigné, Paris, France; oxacillin and ampicillin, Bristol, Paris, France; sulbactam, Pfizer, Paris, France; loracarbef and cefaclor Lilly France s.a., Paris, France; cefuroxime, Glaxo, Paris, France; cefixime, Rhône-Poulenc, Paris, France; ceftriaxone, Roche Produits, s.a., Neuilly-sur-Seine, France; and cefotaxime and cefpodoxime, Roussel, Paris, France. Selections of mutants and determination of the MICs of amoxicillin-clavulanate and ampicillin-sulbactam were carried out in the presence of a fixed concentration of each inhibitor (2 μg/ml).

Analysis of PBPs and competition assays. In brief, analysis of PBPs was performed as described previously (16). The strains were grown to an optical density at 650 nm of 0.30 (~10^8 CFU/ml) in Todd-Hewitt broth containing 5% yeast extract. One-milliliter samples of cells were centrifuged (5,000 × *g* at 4°C

TABLE 1. Frequency of selection of resistant mutants from the susceptible strain *S. pneumoniae* R6, the intermediately resistant strain *S. pneumoniae* 4387LL, and the resistant strain *S. pneumoniae* 4411HL by different antibiotics using different multiples of the MICs of the selector

Selector[a]	R6S			4387LL			4411HL		
	Selection[b]	Range of frequency of selection	Range of fold[c] increased MIC	Selection	Range of frequency of selection	Range of fold increased MIC	Selection	Range of frequency of selection	Range of fold increased MIC
Cefuroxime	2–4	10^{-6}–10^{-7}	4	2	10^{-7}	4	2	10^{-7}	2–4
	8	10^{-8}	8–16	4	10^{-8}	8	4	10^{-8}	4–8
Ceftriaxone	2–4	10^{-6}–10^{-7}	2–8	2	10^{-7}	2	2	10^{-7}	2
	8	10^{-8}	8–16	4	10^{-8}	4	4	10^{-8}	4
Cefotaxime	2–4	10^{-6}–10^{-7}	2–8	2	10^{-7}	2	2	10^{-7}	2
	8	10^{-8}	8–16	4	10^{-8}	4	4	10^{-8}	4–8
Cefpodoxime	2–4	10^{-6}–10^{-7}	2–16	2	10^{-7}	2	2	10^{-7}	2
	8	10^{-8}	8–16	4	10^{-8}	4	4	10^{-8}	4–8
Cefixime	2–4	10^{-6}–10^{-7}	2–16	2	10^{-7}	2	2	10^{-7}	2–4
	8	10^{-8}	8–16	4	10^{-8}	4	4	10^{-8}	4–8
Loracarbef	2	10^{-7}–10^{-8}	2–4	2	10^{-8}	2	2	10^{-7}–10^{-8}	2–4
Cefaclor	2	10^{-7}–10^{-8}	2	2	10^{-8}	2	2	10^{-8}	2
Amoxicillin	2	10^{-8}	2	2	10^{-8}	2	2	10^{-8}	2
Amox+Clav	2	10^{-8}	2	2	10^{-8}	2	2	10^{-8}	2
Ampicillin	2	10^{-8}	2	2	10^{-8}	2	2	10^{-8}	2
Amp+Sul	2	10^{-8}	2	2	10^{-8}	2	2	10^{-8}	2

[a] The MICs for the wild-type strains R6, 4387LL, and 4411HL are presented in Fig. 2; the MICs of the penicillins were identical in the absence or presence of an inhibitor of β-lactamase. Amox + clav, amoxicillin-clavulanate; Amp + Sul, ampicillin-sulbactam.
[b] Multiple of the MIC of the selector at which the mutants were selected.
[c] Ranges of multiples of the MIC of the selector for the mutants.

for 15 min) and were resuspended in 20 μl of 50 mM sodium phosphate buffer (pH 7.0). Ten microliters containing different concentrations of antibiotics were added, and incubation was carried out for 10 min at 37°C. Ten microliters of [^{3}H]benzylpenicillin (25 Ci/mmol; final concentration, 5 or 10 μg/ml) was added, and the incubation was continued for 10 min at 37°C. Finally, 200 μg of nonlabelled penicillin G in 5 μl was added along with 10 μl of 50 mM sodium phosphate buffer (pH 7) containing 0.5% of Triton X-100. Complete lysis occurred after 5 min following incubation at 37°C.

Polyacrylamide gel electrophoresis (16) was performed with slab gels containing 0.1% sodium dodecyl sulfate, and an acrylamide/bisacrylamide ratio of 30:1.1. The final concentrations of polyacrylamide were 5 and 7.5% in the stacking and the separating gels, respectively. The numbers and intensities of the bands on the fluorograms were estimated by densitometry to determine 50% saturation of PBPs.

PCR experiments and DNA sequencing. A 683-bp *pbp2x* fragment from *S. pneumoniae* (10) was amplified by PCR. DNA was prepared from 20 ml of an exponential-phase culture as described previously (10). DNA was subjected to PCR by using the oligonucleotide primers 5′-CGCGGATCCTATGGAACTCA TGTATA-3′ and 5′-GCGAATTCTTAGTCTCCTAAAGT-3′, the sequences of which correspond to positions 1571 to 1586 and are complementary to positions 2240 to 2253, respectively. Reactions were performed in a DNA thermal cycler (PHC-2 techne, Paris, France): 40 cycles of 1 min of denaturation at 92°C, 1 min of annealing at 52°C, and 1 min of extension at 72°C. Amplified *pbp2x* PCR fragments isolated from agarose gels were purified by using the Gene Clean II kit (Bio 101 Inc., Vista, Calif.) and were digested with *Eco*RI and *Bam*HI (Boehringer Mannheim), whose restriction sites are present at only one copy each in the oligonucleotide primers (underlined sequence). They were cloned into the corresponding sites of M13mp18 and M13mp19 phages after overnight ligation at 16°C (12). The recombinant phages were transformed into *Escherichia coli* JM101 and were sequenced by using the T7 sequencing kit (Pharmacia, Paris, France).

RESULTS

Selection of mutants and MICs. The selection of one-step resistant mutants derived from the penicillin-susceptible strains *S. pneumoniae* R6 and 415, the intermediate penicillin-resistant strain *S. pneumoniae* 4387LL, and the penicillin-resistant strain *S. pneumoniae* 4411HL was performed with increasing concentrations of each antibiotic chosen as the selectors (Table 1). Mutants could be selected with concentrations only equal to twofold the MICs of the penicillins, cefaclor, and loracarbef for the wild-type strain and two- to eightfold the MICs of the other cephalosporins. Similar results were obtained for strains 415 and R6. Therefore, only the results obtained for strain R6 are presented.

For the different oral penicillins used, amoxicillin, amoxicillin-clavulanate, ampicillin, and ampicillin-sulbactam, the frequencies of selection of one-step resistant mutants derived from the different wild-type strains were similar (about 10^{-8}) (Table 1). For the oral cephalosporins, cefaclor, and loracarbef, mutants were selected at frequencies in the range of those found with the penicillins. With the other oral cephalosporins, cefixime, cefuroxime, cefpodoxime, as well as the parenteral β-lactams ceftriaxone and cefotaxime, and according to the concentration of antibiotic used for selection, the frequencies of selection of resistant mutants ranged from 10^{-6} to 10^{-8} for those derived from strain R6 and from 10^{-7} to 10^{-8} for those derived from strains 4387LL and 4411HL. For example, for R6 with ceftriaxone, one population of mutants was selected (on plates containing ceftriaxone at concentrations of two to four times the MIC) at a frequency of 10^{-6} to 10^{-7} and another (on plates containing ceftriaxone at eight times the MIC) at a frequency of 10^{-8}. Resistance to the selector was increased only two-fold for the penicillins, whereas for the cephalosporins, it increased in a range of 2- to 16-fold according to the concentration of antibiotic used for selection (Table 1). Among the cephalosporins, cefaclor and loracarbef showed the lowest increase in MICs.

Cross-resistance to the different β-lactam antibiotics was examined for many mutants selected with the different compounds (Fig. 1). Among those selected either on cefpodoxime or on cefixime, cefuroxime, ceftriaxone, and cefotaxime, two or three types of resistance profiles were found. The most representative types of resistance profiles found after selection with cefpodoxime are shown for strains R6 (Fig. 1A), 4387LL (Fig. 1B), and 4411HL (Fig. 1C). In general, the levels of resistance of these mutants to penicillins, loracarbef, and cefaclor were lower than those to the other cephalosporins. A very unexpected type of mutant was selected from the three parental strains. The MICs of the cephalosporins for the mutant in-

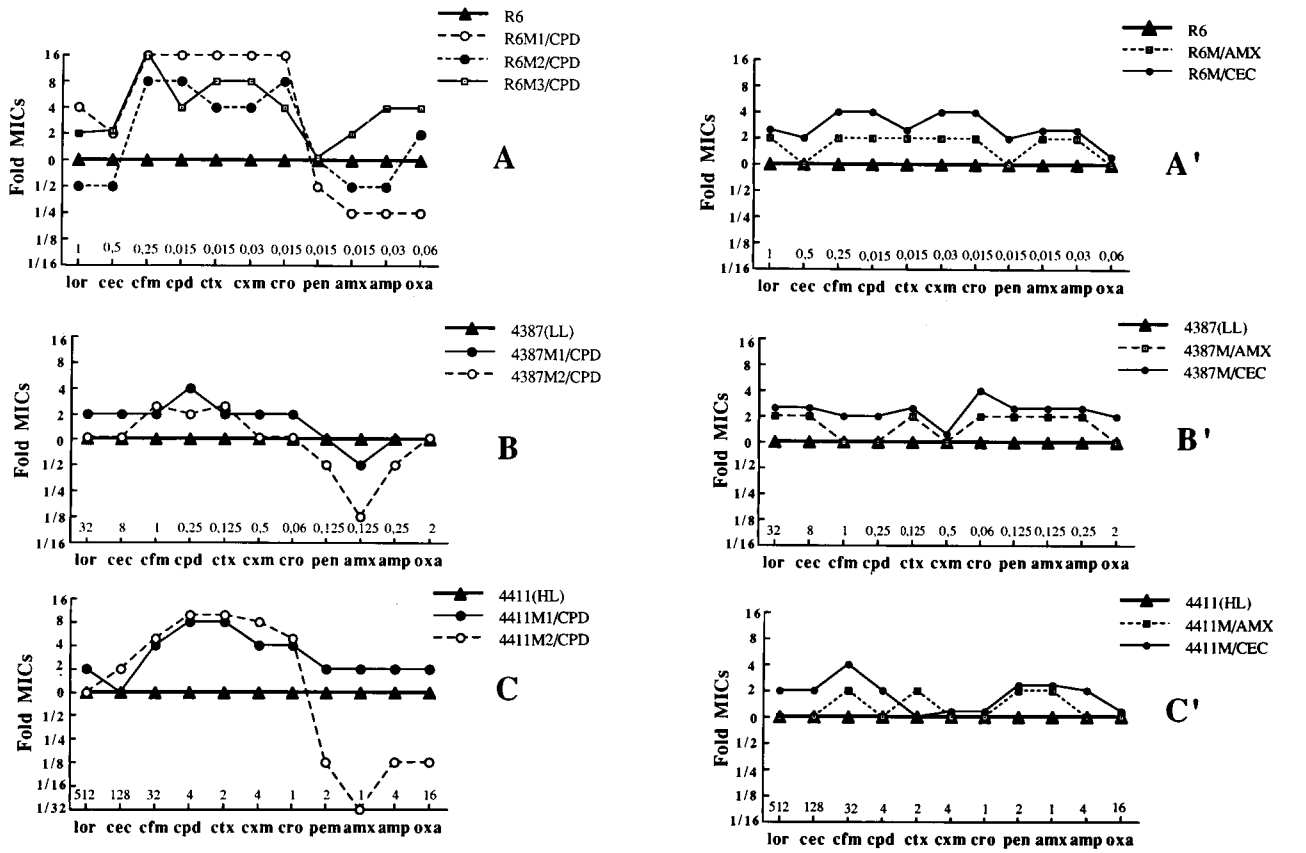

FIG. 1. Susceptibility profiles of different one-step mutants (M) derived from susceptible *S. pneumoniae* R6, intermediately resistant *S. pneumoniae* 4387LL, and resistant *S. pneumoniae* 4411HL. Selection was done either on cefpodoxime (M/CPD; A, B, and C), cefaclor (M/CEC; A′, B′, and C′), or amoxicillin (M/AMX; A′, B′, C′). ▲, wild-type strain, the MIC for which is considered as zerofold increased for all antibiotic tested. MICs (in micrograms per milliliter) for the wild-type strain are given above the antibiotic tested. lor, loracarbef; cec, cefaclor; cfm, cefixime; cpd, cefpodoxime; ctx, cefotaxime; cxm, cefuroxime; cro, ceftriaxone; pen, penicillin G; amx, amoxicillin; amp, ampicillin; oxa, oxacillin.

creased, but the MICs of the penicillins decreased. This was particularly striking for the mutants selected from the high-level penicillin-resistant strain 4411HL (Fig. 1C).

One type of profile was found for the mutants selected on amoxicillin, amoxicillin-clavulanate, ampicillin, and ampicillin-sulbactam, and another, similar type was obtained for the mutants selected on cefaclor and loracarbef. The representative profiles corresponding to the mutants selected with either amoxicillin or cefaclor for strains R6, 4387LL, and 4411HL are presented in Fig. 1A′, B′, and C′, respectively. Very similar profiles were observed, regardless of the parental strain. Increases in the MICs of the cephalosporins and the penicillins were no more than two- to fourfold. According to the profiles found for these mutants, it is likely that these mutants differ from those selected with the other cephalosporins.

Analysis of PBPs. To better understand the mechanism of resistance of the different types of mutants selected after one-step mutations, the affinities of the PBPs were studied by competition assays for cefpodoxime and amoxicillin. These assays were performed with the mutants whose susceptibility profiles are presented in Fig. 1A, B, and C selected with cefpodoxime and derived from R6, 4387LL, and 4411HL, respectively. An example of a competition assay with PBPs of R6 and 4411HL and their cefpodoxime-resistant derivatives R6M1 and 4411M2 is presented in Fig. 2. For R6M1 and 4411M2, for which the MICs of cefpodoxime increased 16- and 8-fold, respectively

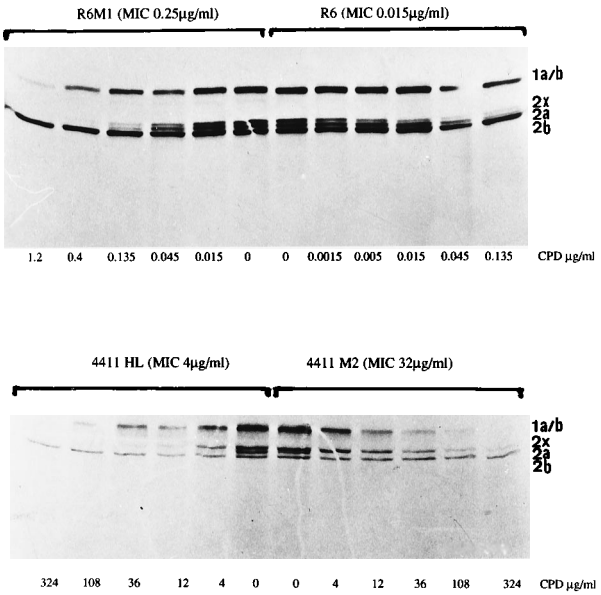

FIG. 2. Saturation by cefpodoxime (CPD) of PBPs of *S. pneumoniae* R6 (MIC, 0.015 µg/ml) and 4411HL (MIC, 4 µg/ml) and their respective one-step cefpodoxime-resistant mutants R6M1 (MIC, 0.25 µg/ml) and 4411M2 (MIC, 32 µg/ml).

TABLE 2. Saturation (50%) of PBP2x's from different spontaneous resistant mutants of *S. pneumoniae* selected on cefpodoxime

Strain	Cefpodoxime		Amoxicillin	
	MIC (μg/ml)	50% saturation of PBP2x (μg/ml)	MIC (μg/ml)	50% saturation of PBP2x (μg/ml)
R6 (WT)[a]	0.015	0.01	0.015	0.015
R6M3	0.06	0.03	0.03	0.015
R6M2[2]	0.125	0.045	0.007	0.005
R6M1[2]	0.25	0.09	0.003	0.006
4387LL (WT)	0.25	0.15	0.25	0.35
4387M2[b]	0.5	0.75	0.06	0.25
4387M1[b]	1	1.5	0.125	0.35
4411HL (WT)	4	1.3	1	1
4411M2[b]	32	12	0.03	0.04
4411M1	32	12	2	2

[a] WT, wild-type strain.

[b] Mutants resistant to cefpodoxime for which the MIC of amoxicillin is decreased.

(Table 2), the only change that was found was a decreased affinity of PBP2x.

The levels of saturation (50%) of the PBP2x's of the different strains are presented in Table 2. In comparison with the wild-type strains, the different mutants showed a decreased affinity of PBP2x only for cefpodoxime, which correlated well with the increased MICs of this compound for the mutants. Interestingly, for some of these cefpodoxime-resistant mutants (R6M1, R6M2, 4387M1, 4387M2, and 4411M2), the MICs of amoxicillin were decreased (Table 1). Among these, the PBP2x of the cefpodoxime-resistant mutant 4411M2, for which the MIC of amoxicillin decreased the most significantly, had a decreased affinity for cefpodoxime and the affinity of its PBP2x for amoxicillin increased significantly. For the other mutants cited above, the decreased MICs of amoxicillin were less important and no significantly decreased affinity of PBP2x for amoxicillin could be observed (Fig. 1; Table 2).

For the mutants selected on amoxicillin and whose susceptibility profiles are shown in Fig. 1, the modestly increased MICs of the penicillins as well as those of the cephalosporins were associated with only an apparent slight decrease in the affinity of their PBP2x's for cefpodoxime and amoxicillin (twofold at most; data not shown).

Point mutation present in PBP2x's of the cephalosporin-resistant, amoxicillin-hypersusceptible mutants. A PCR fragment of a region of *pbp2x* previously known to code for amino acid substitutions and associated with resistance in in vitro-selected, multiple-step, cefotaxime-resistant mutants from strain R6 (9) was amplified from different strains: the susceptible strain R6, the resistant strain 4411HL, and their respective one-step mutants R6M1 and 4411M2, which are more resistant to cefpodoxime and more susceptible to amoxicillin. Sequencing of these PCR products showed that only one, but an identical, mutation was present in the two mutants (Fig. 3): alanine for threonine at position 550 immediately following the KSG triad. It should be noted that the partial amino acid sequences of the PBP2x's of 4411HL and its derivative 4411M2, which are presented in Fig. 3, resemble that of the mosaic PBP2x previously described for resistant strains isolated in other parts of Europe (13).

In contrast, for strain R6M3, which expresses resistance to cefpodoxime but which is not more susceptible to amoxicillin, the sequence of the PCR product showed that glutamic acid substituted for glutamine at position 552 was located three amino acids after the KSG triad (Fig. 3).

DISCUSSION

One-step mutants derived from penicillin-susceptible, intermediately resistant, and penicillin-resistant *S. pneumoniae* strains were selected on different penicillins or cephalosporins, many of which are used orally.

The purpose was to define the risk of selection of such mutants, considering that previous studies did not screen such an extended panel of antibiotics, did not determine the frequencies of selection, and did not examine cross-resistance. In previous studies, generally only the mutants obtained after multiple rounds of selection were examined (2, 4, 8). Our purpose was also to determine if such mutants could be selected from intermediately resistant and resistant strains in which the natural resistance is known to be due to the presence of mosaic PBPs (13, 14).

One-step mutants obtained from the penicillin-susceptible strains, as well as those obtained from the intermediately resistant and penicillin-resistant *S. pneumoniae* strains, could be separated according to their resistance profiles. Those selected with the penicillins (amoxicillin, amoxicillin-clavulanate, ampicillin, and ampicillin-sulbactam) were very similar. They showed relatively low frequencies of selection and a cross-resistance to penicillin and cephalosporins, with a modest increase in MICs (fourfold or less). The frequencies of selection of and the increases in the MICs for these mutants were in the range of those selected with loracarbef and cefaclor. In contrast, mutants selected with the extended-spectrum cephalosporins (cefpodoxime, cefuroxime, cefixime, cefotaxime, and ceftriaxone) showed higher frequencies of selection and different profiles of resistance, and the increases in the MICs of the cephalosporins were larger for these mutants. For these mutants only small increases in the MICs of the penicillins, cefaclor, and loracarbef were found, and some of them were even more susceptible to penicillins.

Analysis of PBPs showed that one-step resistant mutants selected with the cephalosporins were associated with a change in affinity of only one PBP, i.e., PBP2x, as described previously for other multiple-step resistant mutants selected with cefotaxime (9, 10). As shown for some of our mutants, it is likely that the different profiles are linked to different amino acid

R6	530 -	HSTGKPTVTV	PGQNVALKSG	TAQIADEKNG	GYLVGLTDYI	FSAVSMSPAE - 579
R6M3				**E**		
R6M1				**A**		
4411HL		Y II	V		S N	V T N
4411M2		Y II	V	**A**	S N	V T N

FIG. 3. Partial predicted amino acid sequence of PBP2x of strains R6, R6M1, R6M3, 4411HL, and 4411M2. Alanine (GCG) was substituted for threonine (ACG) at position 550 in R6M1 and 4411M2, and glutamic acid (GAG) was substituted for glutamine (CAG) at position 552 in R6M3 (shown in boldface type). Only amino acids different from those in the amino acid sequence of the susceptible strain R6 are shown. Numbering corresponds to that of the predicted PBP2x amino acid sequence of Laible et al. (10).

substitutions in PBP2x. In fact, the same substitutions could be responsible for the similar resistance profiles observed for the susceptible, the intermediate resistant, as well as the resistant strains. The substitution of alanine for threonine at position 550 just after the conserved KSG triad (6) could modify the active site of the PBP2x present in the susceptible R6 strain as well as that of the mosaic PBP2x present in the resistant strains, resulting in an increased resistance to cephalosporins and an increased susceptibility to penicillins. This was associated with a decreased affinity of PBP2x for cefpodoxime and, at least in the mosaic PBP2x, with a noticeable increased affinity for amoxicillin. Since this substitution, although described previously, was associated with additional mutations in the PBP2x's of multiple-step resistant derivatives of R6, its involvement in the particular susceptibility profile toward penicillins was not emphasized (9). The presence of an alanine at the same position on a mosaic PBP2x from a resistant clinical isolate was recently considered to be responsible for a similar profile, i.e., resistance to cefotaxime and increased susceptibility to penicillin (1).

Finally, many of the mutants that we studied were selected with concentrations of only four times the MIC or less for the parental strains. Thus, one could hypothesize that the higher the concentration of antibiotic at the site of infection, the lower will be the chance to select for such spontaneous mutants from either susceptible, intermediately resistant, or resistant strains.

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