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Genetic Analysis of Clinical Isolates of *Streptococcus pneumoniae* with High-Level Resistance to Expanded-Spectrum Cephalosporins

TRACEY J. COFFEY,[1] MAGGIE DANIELS,[1] LINDA K. McDOUGAL,[2] CHRISTOPHER G. DOWSON,[1] FRED C. TENOVER,[2] AND BRIAN G. SPRATT[1]*

Molecular Microbiology Group, School of Biological Sciences, University of Sussex, Brighton, United Kingdom,[1] and Nosocomial Pathogens Laboratory Branch, Hospital Infections Program, Centers for Disease Control and Prevention, Atlanta, Georgia[2]

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Streptococcus pneumoniae **CS109 and CS111 were isolated in the United States in 1991 and have high levels of resistance to expanded-spectrum cephalosporins (MICs of 8 and 32 μg of cefotaxime per ml, respectively). CS109, but not CS111, also showed high-level resistance to penicillin. As both strains expressed the serotype 23F capsule, were very closely related in overall genotype, and possessed identical or closely related mosaic *pbp1a*, *pbp2x*, and *pbp2b* genes, it is likely that they have arisen from a recent common ancestor. High-level resistance to expanded-spectrum cephalosporins was entirely due to alterations of penicillin-binding proteins (PBPs) 1a and 2x, since a mixture of the cloned *pbp1a* and *pbp2x* genes from the resistant strains could transform the susceptible strain R6 to the full level of cephalosporin resistance of the clinical isolates. Both PBP1a and PBP2x of these strains were more resistant to inhibition by cephalosporins than those of typical highly penicillin-resistant isolates. The *pbp1a* genes of CS109 and CS111 were identical in sequence, and the fourfold difference in their levels of resistance to cephalosporins was due to a Thr-550→Ala substitution at the residue following the conserved Lys-Ser-Gly motif of PBP2x. This substitution was also the major cause of the 16-fold-lower resistance of CS111 to penicillin. The *pbp2x* gene of CS111, in an appropriate genetic background, could provide resistance to 16 μg of cefotaxime per ml but only to 0.12 μg of benzylpenicillin per ml. Removal of the codon 550 mutation resulted in a *pbp2x* gene that provided resistance to 4 μg of cefotaxime per ml and 4 μg of benzylpenicillin per ml. The Thr-550→Ala substitution in CS111 therefore appears to provide increased resistance to expanded-spectrum cephalosporins but a loss of resistance to penicillin.**

Penicillin-resistant and multiply antibiotic-resistant pneumococci have been encountered with increasing frequency during the last decade (1, 3, 17, 18, 22, 24). Resistance to penicillin is due to the development of altered forms of some of the high-M_r penicillin-binding proteins (PBPs) that have decreased affinity for the antibiotic (8, 13, 34, 37). Penicillin-resistant isolates show increased resistance to other β-lactam antibiotics (1, 17). For example, the MICs of the expanded-spectrum cephalosporins cefotaxime and ceftriaxone for penicillin-resistant pneumococci are usually about equal to or slightly less than the MIC of benzylpenicillin. Pneumococci with high-level resistance to expanded-spectrum cephalosporins (MICs of ceftriaxone and cefotaxime as high as 16 and 32 μg/ml, respectively) have recently been identified in the United States (4, 11, 26, 32). The emergence and likely increase in the incidence of high-level cephalosporin resistance is of considerable concern, as it further limits the available options for the therapy of serious pneumococcal infections.

The altered PBP genes of penicillin-resistant pneumococci are very different in sequence from those of penicillin-susceptible isolates and are believed to have arisen by interspecies recombinational events mediated by genetic transformation (7–9, 20, 23). Altered (mosaic) *pbp1a*, *pbp2x*, and *pbp2b* genes are invariably found in pneumococci that are resistant to ≥0.1 μg of benzylpenicillin per ml (8). Genetic analysis using the cloned *pbp1a*, *pbp2x*, and *pbp2b* genes has shown that high-level resistance to penicillin (MICs > 8 μg/ml) can result from alterations of only these three PBPs (2, 8).

We have shown previously that intermediate-level resistance to expanded-spectrum cephalosporins in a serogroup 6 isolate of *Streptococcus pneumoniae* from Spain (MIC, 4 μg of cefotaxime per ml) was entirely due to the expression of altered forms of PBP1a and PBP2x that had decreased affinity for these antibiotics (28). In this report we analyze two clinical isolates that have very high resistance to expanded-spectrum cephalosporins and show that pneumococci for which the MIC of cefotaxime is at least 32 μg/ml can result from alterations of only PBP1a and PBP2x. We also identify the key role of a substitution at the residue following the conserved Lys-Ser-Gly motif (15) of PBP2x in the different levels of resistance of these strains to both cephalosporins and penicillins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* was routinely grown at 37°C in an atmosphere of 95% air–5% CO_2 on brain heart infusion agar (Difco, Detroit, Mich.) with 4,000 U of catalase/ml or on the same agar plus 5% defibrinated sheep blood (BHI/B). The properties of the pneumococcal strains are shown in Table 1. The antimicrobial susceptibility profiles of the clinical isolates were determined by broth microdilution using cation-adjusted Mueller-Hinton broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.) supplemented with 3 to 5% lysed horse blood as described by the National Committee for Clinical Laboratory Standards (30).

Transformation. The nonencapsulated, penicillin-susceptible *S. pneumoniae* R6 was used as the recipient for genetic transformation and was made competent in c+y medium as described previously (28). The role of the altered *pbp* genes in cephalosporin resistance was analyzed initially by the two-step procedure used previously (28), in which strain R6 is transformed to intermediate-level cephalosporin resistance with the cloned *pbp2x* gene and the resulting transformant is transformed to a higher level of resistance with the cloned *pbp1a* gene. In subsequent experiments a one-step procedure was used: 0.25-μg amounts of the cloned *pbp1a* and *pbp2x* genes (replicative-form DNA of M13 recombinants

* Corresponding author. Molecular Microbiology Group, School of Biological Sciences, University of Sussex, Brighton BN1 9QG, United Kingdom. Phone: (1273) 678309. Fax: (1273) 678433. Electronic mail address: b.g.spratt@sussex.ac.uk.

TABLE 1. Properties of pneumococcal strains[a]

Strain	Serotype	MIC (μg/ml) of:			Susceptibility to:			Yr of isolation	Origin
		PenG	Cefotaxime	Ceftriaxone	Chlor	Tet	Ery		
R6	NT	0.016	0.016	0.016	S	S	S	1930s	United States
CS109	23F	4	8	4	S	S	S	1991	United States
CS111	23F	0.25	32	16	S	S	(R)	1991	United States
Hun159	19A	16	2	2	R	R	R	1993	Hungary
SP264	23F	1	0.5	0.5	R	R	S	1989	Spain
SP971	6B	2	0.5	0.5	R	R	S	1984	Spain

[a] NT, nonencapsulated strain; S, susceptible; R, resistant; (R), low-level resistant; PenG, penicillin G; Chlor, chloramphenicol; Tet, tetracycline; Ery, erythromycin.

containing the *pbp* genes) were mixed and added to 400 μl of competent cells of strain R6. The bacteria were incubated at 30°C for 30 min and, after 2 h at 37°C to allow expression of antibiotic resistance, they were plated on BHI/B containing twofold-increasing concentrations of ceftriaxone or cefotaxime. The MICs of β-lactam antibiotics of the resulting transformants were determined as described previously (28).

Gene fingerprinting. The *pbp1a*, *pbp2x*, and *pbp2b* genes were amplified from chromosomal DNA by PCR using primers and conditions described previously (5). The amplified fragments were purified from agarose gels with GeneClean (Bio 101, Inc., La Jolla, Calif.), digested with *Hin*fI and *Mse*I plus *Dde*I (for the *pbp1a* and *pbp2x* genes) or *Hin*fI and *Sty*I (for the *pbp2b* gene), end labelled with α-^{32}P-deoxynucleoside triphosphate, fractionated on a 6% nondenaturing polyacrylamide gel, and autoradiographed, as described previously (5).

DNA sequencing. The *pbp1a*, *pbp2x*, and *pbp2b* genes were amplified from chromosomal (or plasmid) DNA by PCR with one 5′-biotinylated and one nonbiotinylated primer, as described previously (16). The biotinylated and nonbiotinylated DNA strands were separated with avidin-coated paramagnetic beads (Dynal UK, Ltd., Wirral, United Kingdom) and were sequenced with a series of oligonucleotides that prime at intervals on each DNA strand (16).

Site-directed mutagenesis. The *pbp2x* gene of CS109 was amplified by PCR (5) using the proofreading UlTma DNA polymerase (Perkin-Elmer, Branchburg, N.J.) to minimize the introduction of errors. The 2-kb PCR product was transferred into pALTER-1 and Ser-701 was converted to Gly-701 by using the oligonucleotide MUT-1 (5′-dTTTTCCAGCCATACATGTC-3′) and the Altered Sites II in vitro mutagenesis system (Promega, Madison, Wis.) according to the manufacturer's protocol. The desired mutants were identified by sequencing. The entire *pbp2x* gene of one of the mutants was sequenced to verify that no additional mutations had occurred and was recloned in M13mp18.

REP-PCR and pulsed-field gel electrophoresis. PCR using repetitive element primers (REP-PCR) was performed as described by Versalovic et al. (36). Genomic DNA for pulsed-field gel electrophoresis was prepared in situ in agarose blocks (21), and the equilibrated DNA was digested with *Sma*I. The fragments were resolved by pulsed-field electrophoresis in 1% agarose (SeaKem GTG agarose; FMC Bioproducts, Rockland, Maine) in 0.5× Tris-borate-EDTA buffer for 20 h at 14°C at 6 V/cm in a CHEF-DR II system (Bio-Rad Laboratories, Hercules, Calif.). The parameters were an initial pulse time of 1 s increased to 20 s.

Multilocus enzyme electrophoresis and ribotyping. Preparation of cytoplasmic extracts of *S. pneumoniae*, horizontal starch gel electrophoresis, and enzyme staining was performed as described previously (25). For ribotyping, genomic DNA was digested with *Hin*dIII and, after electrophoresis on 0.8% agarose gels, the fragments were transferred to nylon membranes (MagnaGraph; Micro Separations Inc., Westboro, Maine) and hybridized with end-labelled 16S–23S rRNA from *Escherichia coli* (Sigma Chemical Co., St. Louis, Mo.).

Nucleotide sequence accession numbers. The sequences have been submitted to the EMBL/GenBank data library under the accession numbers Z49094 to Z49097.

RESULTS

Characteristics of cephalosporin-resistant isolates. The two isolates analyzed in this study (CS109 and CS111) were serotype 23F pneumococci recovered from patients with invasive disease in Tennessee in 1991. The isolates were very closely related, as the patterns of DNA fragments obtained by pulsed-field gel electrophoresis of *Sma*I-digested chromosomal DNA were almost identical (Fig. 1). CS109 and CS111 were indistinguishable by REP-PCR and by ribotyping with *Hin*dIII, and when analyzed by multilocus enzyme electrophoresis they differed at only one of the 20 enzymes that were assayed (α-naphthyl propionate esterase).

Although CS109 and CS111 strains were serotype 23F, they did not appear to be closely related to the widespread Spanish serotype 23F multiresistant clone (27), as the REP-PCR profiles (data not shown) and the patterns of *Sma*I fragments were very different (Fig. 1). They also differed from the Spanish 23F clone at 7 of 20 enzyme-encoding loci and in their antibiotic resistance profiles (Table 1). Isolate CS109 showed high-level resistance to penicillin, cefotaxime, and ceftriaxone, whereas isolate CS111 showed only intermediate-level resistance to penicillin, but very high resistance to cefotaxime and ceftriaxone. Both isolates were susceptible to chloramphenicol and tetracycline; CS111 demonstrated low-level resistance to erythromycin.

Mosaic *pbp1a*, *pbp2x*, and *pbp2b* genes in strains CS109 and CS111. The *pbp1a*, *pbp2x*, and *pbp2b* genes of CS109 and CS111 were amplified by PCR, and gene fingerprints were obtained with frequently cutting restriction enzymes (Fig. 2). With the exception of the *pbp2b* gene of strain CS111, which was indistinguishable from that of strain R6, the fingerprints of all three genes were different from those of penicillin-susceptible pneumococci, suggesting that the *pbp1a* and *pbp2x* genes (and the *pbp2b* gene of strain CS109) of the resistant isolates possessed the mosaic structure invariably found in pneumo-

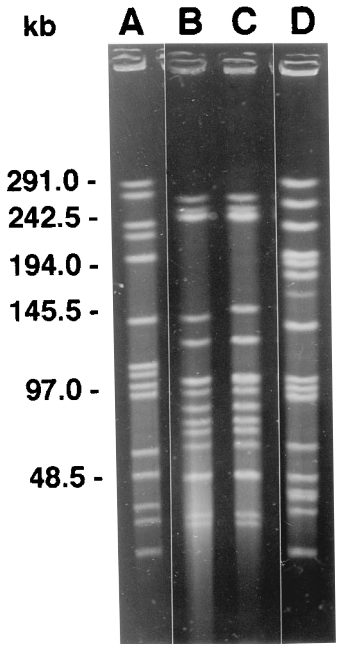

FIG. 1. Pulsed-field gel electrophoresis separation of *Sma*I restriction fragments of *S. pneumoniae* strains. Lane A, strain R6; lane B, CS111; lane C, CS109; lane D, Spanish 23F clone. The sizes of molecular markers (phage λ DNA ladder) are indicated on the left.

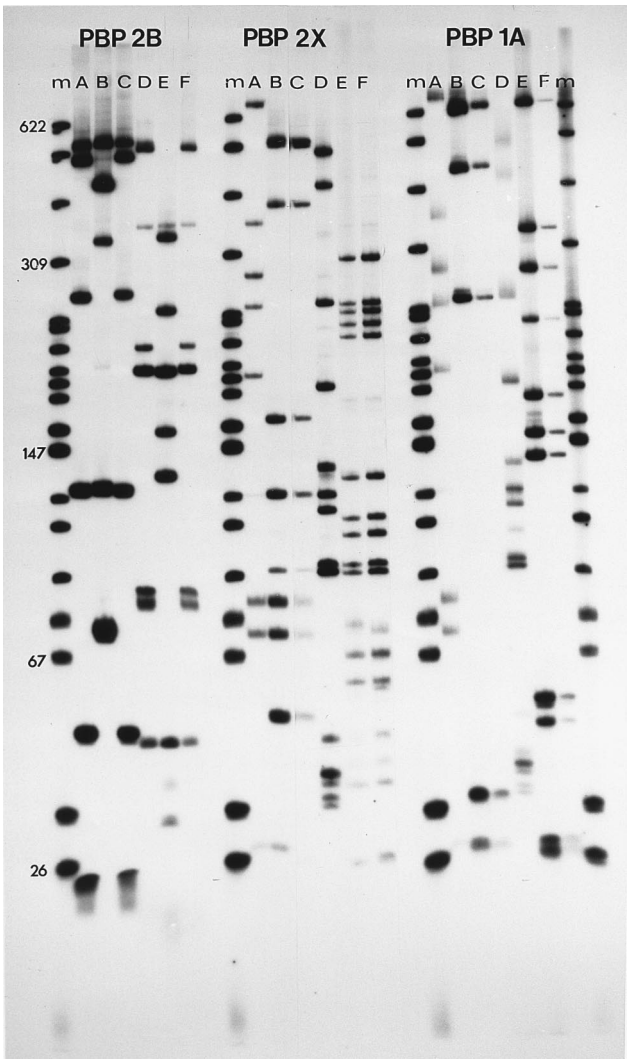

FIG. 2. Fingerprints of the *pbp* genes of cephalosporin-resistant pneumococci. The *pbp2b* gene was digested with *Sty*I (lanes A to C) or *Hin*fI (lanes D to F). The *pbp2x* and *pbp1a* genes were digested with *Hin*fI (lanes A to C) or *Dde*I plus *Mse*I (lanes D to F). Lanes A and D, strain R6; lanes B and E, CS109; lanes C and F, CS111. Lane m, molecular size markers (pBR322 digested with *Hpa*II). The sizes of the visible pBR322 *Hpa*II fragments are 622, 527, 404, 309, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, and 26 bp. Slight differences in the *Dde*I-*Mse*I fragments from the *pbp2x* genes of CS109 and CS111 within the 50- to 80-bp region (lanes E and F) were probably gel artifacts, as no differences were observed in several other experiments.

cocci with intermediate- or high-level resistance to penicillin. Sequencing of the *pbp2b* gene of strain CS111 showed that it also possessed mosaic structure, although this was not detected by fingerprinting, as the distribution of *Sty*I or *Hin*fI sites was not altered in relation to that in the *pbp2b* gene from strain R6. The *pbp1a* and *pbp2x* genes of strains CS109 and CS111 could not be distinguished by fingerprinting, but their *pbp2b* genes were clearly distinct (Fig. 2). The *pbp1a* and *pbp2x* gene fingerprints of strains CS109 and CS111 (and the *pbp2b* gene of CS109) were different from any in our database of fingerprints of *pbp* genes from β-lactam-resistant pneumococci isolated worldwide.

Role of altered *pbp* genes in the resistance of strains CS109 and CS111 to expanded-spectrum cephalosporins. The *pbp1a* and *pbp2x* genes of strains CS109 and CS111 were amplified by PCR and were cloned into M13mp18. Replicative-form DNA of the resulting phage was used to transform *S. pneumoniae* R6 to increased levels of resistance to cefotaxime. The *pbp2x* gene from either strain was able to transform strain R6 to an intermediate level of cefotaxime resistance (Table 2). Strain R6 could be transformed to a higher level of resistance by using a mixture of equal amounts of the cloned *pbp1a* and *pbp2x* genes. The MICs of ceftriaxone and cefotaxime for the resulting transformants ($R6^{CS109/2x/1a}$ and $R6^{CS111/2x/1a}$) were the same as those for the resistant clinical isolates from which the *pbp* genes were cloned (Table 2 [the genetic nomenclature for transformants of strain R6 is described in footnote *a*]). High-level cephalosporin resistance in CS109 and CS111 was therefore entirely due to alterations of PBP2x and PBP1a.

TABLE 2. Transformants constructed to analyze the role of PBPs in cephalosporin resistance

Strain or transformant[a]	MIC (μg/ml) of:		
	Cefotaxime	Ceftriaxone	PenG[b]
R6	0.016	0.016	0.016
$R6^{CS109/2x}$	0.5	0.25	0.032
$R6^{CS111/2x}$	0.5	0.25	0.032
$R6^{CS109/2x/1a}$	8	4	0.064
$R6^{CS111/2x/1a}$	32	16	0.064
$R6^{CS109/2x/CS111/1a}$	8	4	0.064
$R6^{CS111/2x/CS109/1a}$	32	16	0.064
$R6^{CS111/2x/SP264/1a}$	2	1	0.064
$R6^{CS111/2x/SP971/1a}$	2	1	0.064
$R6^{SP264/2x/CS111/1a}$	1	0.5	0.064
$R6^{SP971/2x/CS111/1a}$	1	1	0.064
$R6^{ancestral/2x/CS111/1a}$	8	4	0.064
$R6^{double/2x/CS109/1a}$	32	16	0.064

[a] $R6^{CS109/2x/1a}$ corresponds to a transformant of R6 containing the *pbp2x* and *pbp1a* genes of strain CS111. Similarly, $R6^{CS111/2x/CS109/1a}$ contains the *pbp2x* gene from CS111 and the *pbp1a* gene from CS109. The ancestral *pbp2x* gene lacking the codon 550 and 701 mutations (ancestral) and the *pbp2x* gene with both mutations (double) are described in the text.

[b] PenG, penicillin G.

Strain CS111 was fourfold more resistant to cefotaxime and ceftriaxone than strain CS109. A mixture of equal amounts of the cloned *pbp1a* gene of strain CS111 and the cloned *pbp2x* gene of strain CS109 was used to transform the susceptible strain R6 to increased resistance to cefotaxime. The resulting transformants ($R6^{CS109/2x/CS111/1a}$) showed the lower level of cephalosporin resistance characteristic of strain CS109 (Table 2). Conversely, transformants of strain R6 obtained with equal quantities of the cloned *pbp1a* gene of strain CS109 and the cloned *pbp2x* gene of strain CS111 had the higher level of cephalosporin resistance characteristic of strain CS111 (Table 2). The higher degree of resistance to cefotaxime and ceftriaxone of strain CS111 compared with CS109 was therefore due to a difference in their *pbp2x* genes rather than their *pbp1a* genes.

We next investigated whether the exceptionally high level of cephalosporin resistance of strain CS111 was due solely to the production of an altered PBP2x that had extremely low affinity for cephalosporins or whether it also required an altered PBP1a with unusually low affinity for cephalosporins. The cloned *pbp1a* genes of two typical high-level penicillin-resistant pneumococci (strains SP971 and SP264, members of the successful Spanish serotype 6B [29, 31] and 23F [27] multiresistant clones, respectively), which have substantial levels of cross-resistance to expanded-spectrum cephalosporins (Table 1), were therefore used, together with an equal amount of the cloned *pbp2x* gene from strain CS111, to transform strain R6 to

increased resistance to cefotaxime. The maximum level of resistance of the resulting transformants ($R6^{CS111/2x/SP264/1a}$ and $R6^{CS111/2x/SP971/1a}$) was 2 μg of cefotaxime per ml (Table 2). Similarly, transformation of strain R6 with a mixture of the *pbp1a* gene from strain CS111 and the *pbp2x* genes from the highly penicillin-resistant pneumococci produced transformants ($R6^{SP264/2x/CS111/1a}$ and $R6^{SP971/2x/CS111/1a}$) that had a maximum MIC of 1 μg of cefotaxime per ml (Table 2). Our results suggest that both PBP2x and PBP1a of strain CS111 have unusually low affinity for expanded-spectrum cephalosporins compared with those of typical highly penicillin-resistant pneumococci.

Nucleotide sequences of the *pbp1a*, *pbp2x*, and *pbp2b* genes of strains CS109 and CS111. The *pbp1a* genes of strains CS109 and CS111 were identical in sequence (data not shown). The sequence differed from that of the susceptible strain R6 at 11.2% of nucleotide sites, resulting in 56 differences in the amino acid sequence of PBP1a. There were only 16 nucleotide differences within the first 938 bp (1.7% divergence) and one difference within the last 237 bp (0.4% divergence), but the region between codons 313 and 641 differed at 226 sites (22.9% divergence).

The *pbp2x* genes of strains CS109 and CS111 were almost identical (differing only at two nucleotide sites), but they differed from the *pbp2x* gene of strain R6 at 14.4% of nucleotide sites (Fig. 3). The front part of the gene (codons 84 to 249 [the first 83 codons were not sequenced]) differed from that of strain R6 at 3.6% of sites, whereas the remainder of the gene, including the whole region that encodes the transpeptidase domain, differed at 18.6% of sites. The two differences between the *pbp2x* genes of strains CS109 and CS111 were both nonsynonymous substitutions, and the PBP2x sequences of the isolates therefore differed at two amino acid residues (Fig. 4). Residue 550, immediately following the conserved Lys-Ser-Gly sequence motif (15), was Ala in PBP2x of strain CS111, whereas this residue in strain CS109 was the same as that (Thr) in the penicillin-susceptible strain R6. Conversely, residue 701 in strain CS111 was the same as that (Gly) in strain R6, whereas there was a Ser at this position in PBP2x from strain CS109.

The *pbp2b* gene of CS111 was almost identical to those of penicillin-susceptible isolates except for the presence of a 20.7%-diverged region from codon 618 to the end of the gene. The *pbp2b* gene from CS109 contained the identical downstream diverged region but also differed from susceptible strains by 12.1% between codons 332 and 615 (Fig. 5).

Role of alterations of *pbp2x* in resistance to cephalosporins. As the mosaic *pbp2x* genes of strains CS109 and CS111 were identical in sequence (except at two positions) but differed extensively from the *pbp2x* genes of penicillin-susceptible pneumococci, it is almost certain that they are derived from the same ancestral mosaic PBP gene. The most parsimonious explanation is that selection for increased resistance to β-lactam antibiotics resulted in two different nonsynonymous mutations in the ancestral mosaic gene, leading to the Gly-701→Ser alteration in strain CS109 and the Thr-550→Ala alteration in strain CS111. The presumed ancestral mosaic gene was therefore constructed by converting the Ser-701 codon of the *pbp2x* gene of CS109 back to a Gly codon (Fig. 4). Equal amounts of the cloned *pbp1a* gene of strain CS111 and the modified *pbp2x* gene were used to transform strain R6 to cefotaxime resistance. The MICs of cefotaxime and ceftriaxone for the resulting transformants ($R6^{ancestral/2x/CS111/1a}$) were 8 and 4 μg/ml, respectively (Table 2; Fig. 4). The ancestral *pbp2x* gene (when combined with the *pbp1a* gene of CS111) therefore provided high levels of cephalosporin resistance, which were equal to those of strain CS109 but fourfold less than those of strain CS111.

We next examined whether PBP2x containing both the Thr-550→Ala and the Gly-701→Ser substitutions would provide greater resistance to expanded-spectrum cephalosporins than that exhibited by strain CS111. The transformant $R6^{CS109/2x/1a}$ was transformed to a higher level of resistance to cefotaxime with the cloned *pbp2x* gene of strain CS111. Recombinational crossovers that occur upstream of codon 550 and downstream of codon 701 (regions I and III in Fig. 4) result in transformants that express PBP2x with Ala-550 and Gly-701 (as in strain CS111). Alternatively, a crossover upstream of codon 550 combined with one between codons 550 and 701 (regions I and II in Fig. 4) results in transformants that express PBP2x containing both Ala-550 and Ser-701 (Fig. 4). Partial sequencing of the *pbp2x* genes from six transformants obtained from agar plates containing 8 μg of cefotaxime per ml (a concentration that prevents the growth of the recipient strain, $R6^{CS109/2x/1a}$) showed that five had the desired double substitution (Ala-550 and Ser-701) whereas the other had Ala-550 combined with Gly-701. However, the MICs of cefotaxime and ceftriaxone (Table 2) for these two classes of transformants ($R6^{double/2x/CS109/1a}$) and $R6^{CS111/2x/CS109/1a}$) were identical (32 μg of cefotaxime and 16 μg of ceftriaxone per ml). The combination of the Thr-550→Ala and Gly-701→Ser alterations did not therefore provide increased resistance over that afforded by the PBP2x gene of CS111.

Genetic analysis of penicillin resistance of CS109 and CS111. The 16-fold-higher level of penicillin resistance of strain CS109 compared with CS111 could have been due to differences in their *pbp2x* and/or *pbp2b* genes. To analyze the contribution of the differences in *pbp2x*, $R6^{CS109/2x/1a}$, $R6^{CS111/2x/1a}$, $R6^{ancestor/2x/CS111/1a}$, and $R6^{double/2x/CS111/1a}$, which are all susceptible to penicillin (MICs, 0.064 μg/ml) as they have a normal high-affinity PBP2b, were transformed to an increased level of resistance to penicillin with the cloned *pbp2b* gene from a clinical isolate with very high resistance to penicillin (Hun159; MIC, 16 μg/ml; Table 1). As these transformants express identical low-affinity forms of PBP1a and -2b, any variation in their MICs of penicillin should be due to differences in their *pbp2x* genes. Transformants containing the *pbp2x* gene of CS111 ($R6^{CS111/2x/1a/Hun159/2b}$) had MICs of penicillin of 0.12 μg/ml, whereas those possessing the *pbp2x* gene of CS109 ($R6^{CS109/2x/1a/Hun159/2b}$) had MICs of 8 μg/ml (Table 3). Transformants containing the putative ancestral *pbp2x* gene were as resistant to penicillin as those containing the *pbp2x* gene of CS109 (Table 3). The introduction of the Thr-550→Ala substitution in PBP2x of CS111, which results in increased resistance to cephalosporins, appears to cause a loss of resistance to penicillin.

The contribution of the *pbp2b* gene to the difference in the penicillin resistance of strains CS109 and CS111 was examined by transforming $R6^{CS109/2x/1a}$ and $R6^{CS111/2x/1a}$ to increased penicillin resistance by using the cloned *pbp2b* genes from CS109 and CS111. As expected from the experiments described above, transformants of $R6^{CS111/2x/1a}$ obtained with either *pbp2b* gene had only low levels of resistance to penicillin (Table 3). The MIC of penicillin for transformants of $R6^{CS109/2x/1a}$ obtained with the *pbp2b* gene of CS111 was 1 μg/ml, whereas the MIC for transformants obtained with the *pbp2b* gene of CS109 was 4 μg/ml (Table 3). The *pbp2b* gene of CS109 can therefore provide higher levels of resistance to penicillin than that of CS111.

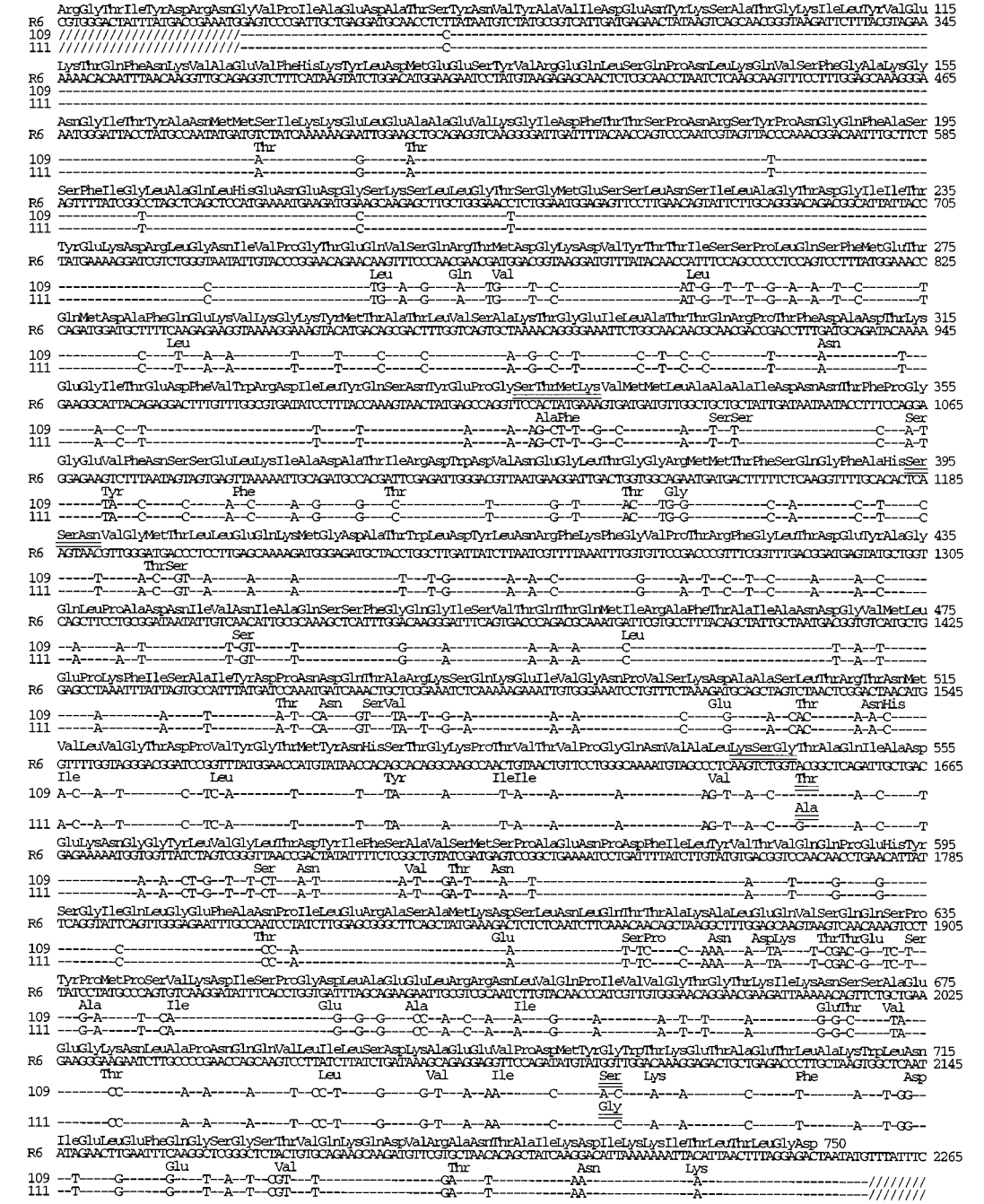

FIG. 3. Partial sequences of the *pbp2x* genes of cephalosporin-resistant pneumococci. The sequence of the *pbp2x* gene and the amino acid sequence of PBP2x are shown for strain R6. The nucleotide and amino acid sequences are numbered at the end of each line on the basis of data in reference 19. Positions at which *pbp2x* sequences of strains CS109 and CS111 differ from the R6 sequence are shown. Positions at which the PBP2x amino acid sequence of CS109 differs from that of R6 are also shown. The PBP2x amino acid sequence of strain CS111 is identical to that of CS109, except at residues 550 and 701 (doubly underlined). The active-site serine, Ser-X-Asn, and Lys-Ser-Gly conserved sequence motifs (15, 35) are also doubly underlined.

DISCUSSION

Genetic analysis established that the high level of resistance of strains CS109 and CS111 to cefotaxime and ceftriaxone was due to the production of altered low-affinity forms of only PBP1a and -2x, in contrast to intermediate- or high-level resistance to penicillin (2), which requires reductions in the affinities of PBP1a, -2x, and -2b. The *pbp2b* gene of *S. pneumoniae* has very low affinity for cephalosporins, and inactivation of this PBP appears not to be involved in the killing action of cefotaxime or ceftriaxone at physiologically relevant concentrations (14). Consequently, resistance to expanded-spectrum cephalosporins will not necessarily correlate with resistance to penicillins. This is clearly seen with the transformant $R6^{CS111/2x/1a}$, for which the MIC of cefotaxime was 32 μg/ml, as this transformant possesses low-affinity forms of PBP2x and PBP1a, but the MIC of benzylpenicillin was only 0.064 μg/ml,

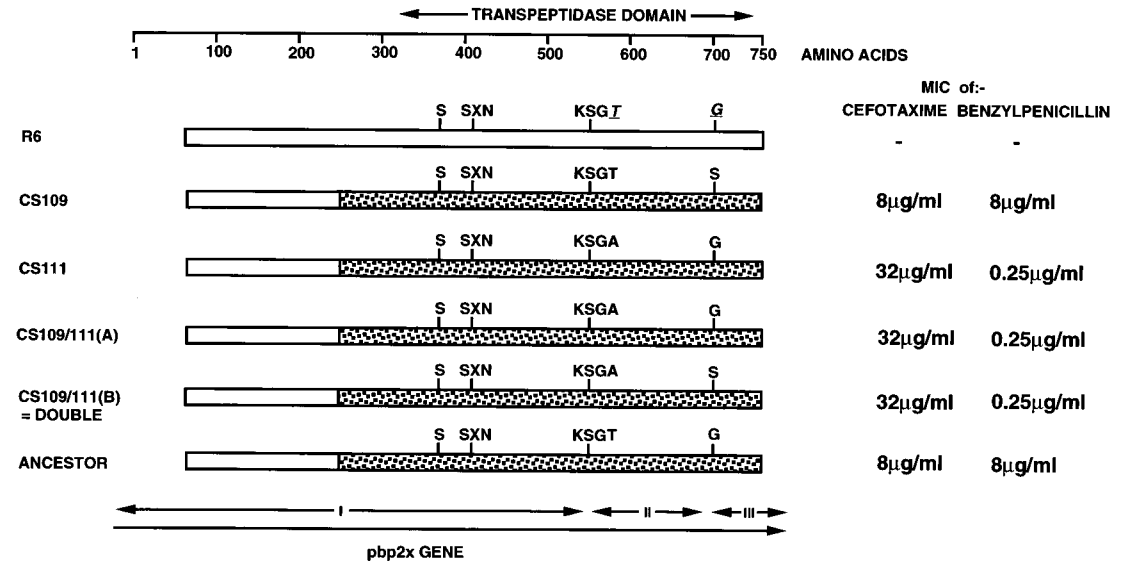

FIG. 4. Mosaic PBP2x of cephalosporin-resistant pneumococci and transformants. The positions of the active-site serine residue (S), the Ser-X-Asn motif (SXN), and the Lys-Ser-Gly motif (KSG) are indicated (15, 35). The residues that differ in PBP2x sequences of strains CS109 and CS111 are underlined and italicized in the diagram of PBP2x of strain R6. The MICs of cefotaxime and benzylpenicillin correspond to the highest levels of resistance that the illustrated PBP2x can provide when expressed together with PBP1a and/or PBP2b from highly penicillin- or cephalosporin-resistant pneumococci. The MICs of cefotaxime are those of transformants of strain R6 expressing PBP1a of strain CS111 (or CS109) and the illustrated PBP2x. The MICs of benzylpenicillin are those of transformants of R6 expressing PBP2b of strain Hun159, PBP1a of strain CS111 (or CS109), and the illustrated PBP2x. CS109/111(A) and CS109/111(B) are the two types of PBP2x resulting from recombinational crossovers between regions I and III or regions I and II, respectively, of the *pbp2x* gene (see the text). The sequences of PBP2x of CS109, CS111, CS109/111(A), and CS109/111(B) are identical except at residues 550 and/or 701, but, as indicated by the stippling, the sequences of their transpeptidase domains differ extensively from the sequence of PBP2x of strain R6.

as the transformant possesses a normal PBP2b (Table 2). Presumably, highly cephalosporin-resistant, but penicillin-susceptible, clinical isolates of this type could emerge in nature by the horizontal transfer of the *pbp2x* and *pbp1a* genes from isolates like CS111 into susceptible pneumococci. As demonstrated previously (28), horizontal transfer of cephalosporin resistance can be readily achieved in a single step by transformation using chromosomal DNA, presumably because the *pbp2x* and *pbp1a* genes are closely linked on the pneumococcal chromosome (12).

Susceptibility of pneumococci to β-lactam antibiotics is generally carried out with oxacillin discs. High-level resistance to oxacillin requires alterations of both PBP2x and PBP2b (10), and both CS109 and CS111 gave oxacillin diffusion zones of <20 mm and would be detected as resistant isolates. Highly cephalosporin-resistant, but penicillin-susceptible, pneumococci that possess a normal PBP2b (e.g., $R6^{CS111/2x/1a}$) have only low-level resistance to oxacillin but should still be recognized as β-lactam resistant with oxacillin discs.

Transformants of the antibiotic-susceptible strain R6, expressing the *pbp2x* gene of CS111 and the *pbp1a* gene of the multiresistant serotype 6B or 23F Spanish clones of *S. pneumoniae*, or the *pbp1a* gene of CS111 and the *pbp2x* gene of the

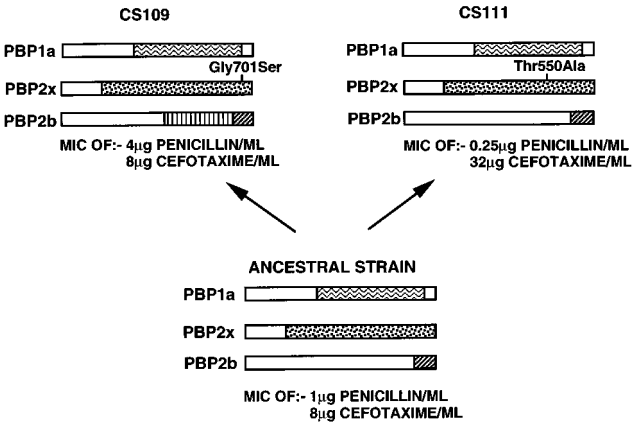

FIG. 5. Possible origins of strains CS109 and CS111. PBP1a, -2x, and -2b of CS109 and CS111 and the presumed common ancestor of these strains are shown (not to scale). The patterned portions represent parts of the PBPs that are encoded by regions that are highly diverged from the sequences of the corresponding genes from penicillin-susceptible pneumococci.

TABLE 3. Transformants constructed to analyze the role of PBPs in penicillin resistance

Transformant	MIC (μg/ml) of:		
	Cefotaxime	Ceftriaxone	PenG[a]
$R6^{CS109/2x/1a}$	8	4	0.064
$R6^{CS111/2x/1a}$	32	16	0.064
$R6^{ancestral/2x/CS111/1a}$	8	4	0.064
$R6^{double/2x/CS111/1a}$	32	16	0.064
$R6^{CS109/2x/1a/Hun159/2b}$	8	4	8
$R6^{CS111/2x/1a/Hun159/2b}$	32	16	0.12
$R6^{ancestral/2x/CS111/1a/Hun159/2b}$	8	4	8
$R6^{double/2x/CS111/1a/Hun159/2b}$	32	16	0.25
$R6^{CS109/2x/1a/2b}$	8	4	4
$R6^{CS111/2x/1a/2b}$	32	16	0.12
$R6^{CS109/2x/1a/CS111/2b}$	8	4	1
$R6^{CS111/2x/1a/CS109/2b}$	32	16	0.25
$R6^{ancestor/2x/CS111/1a/2b}$	8	4	1
$R6^{ancestor/2x/CS111/1a/CS109/2b}$	8	4	4
$R6^{double/2x/CS111/1a/CS109/2b}$	32	16	0.25
$R6^{double/2x/CS111/1a/CS111/2b}$	32	16	0.12

[a] PenG, penicillin G.

serotype 6B or 23F clones, did not show the high levels of cephalosporin resistance of CS111. The very high level of cephalosporin resistance in strain CS111 appears therefore to be due to the expression of altered forms of both PBP2x and PBP1a that have unusually low affinity for cefotaxime and ceftriaxone, compared with those of typical highly penicillin-resistant pneumococci. This implies that highly penicillin-resistant pneumococci, such as the successful multiresistant Spanish 6B (33) or 23F (27) clones, would require alterations of both PBP1a and PBP2x to achieve the levels of resistance to expanded-spectrum cephalosporins seen with strains CS109 and CS111. Variants of the Spanish 23F clone that have fourfold-increased resistance to ceftriaxone and cefotaxime have, however, recently been detected in the United States (26), and an analysis of one of these isolates will be presented elsewhere.

The fourfold-greater resistance of CS111 to cefotaxime and ceftriaxone, compared with CS109, was due to differences in *pbp2x*. There were two amino acid differences between PBP2x of CS109 and PBP2x of CS111, but the higher resistance of CS111 could be clearly ascribed to the Thr-550→Ala substitution. This substitution is of particular interest, as it occurs at the residue immediately following the conserved Lys-Ser-Gly motif (15). By analogy with the structures of serine β-lactamases and low-M_r PBPs, this motif is predicted to form a β-strand along one side of the β-lactam-binding pocket of high-M_r PBPs (15). Substitutions at this residue have not been found in the PBPs of previously examined β-lactam-resistant clinical isolates of *S. pneumoniae*, but the Thr-550→Ala substitution has been found in PBP2x of laboratory mutants selected for increased resistance to cefotaxime (19). Interestingly, the reverse substitution at the corresponding residue occurs in some expanded-spectrum serine β-lactamases (Ala-237→Thr) that have an increased ability to hydrolyze (and an increased affinity for) expanded-spectrum cephalosporins (6).

CS109 was 16-fold more resistant to penicillin than CS111. CS109 and CS111 possessed different *pbp2b* genes, but the resistance of CS111 to penicillin (MIC, 0.25 μg/ml) was not limited by the affinity of PBP2b, since for transformants expressing this PBP, together with low-affinity forms of PBP1a and -2x ($R6^{CS109/2x/1a/CS111/2b}$), the MIC of benzylpenicillin was 1 μg/ml. The penicillin resistance of CS111 was, however, limited by the affinity of PBP2x. The ancestral *pbp2x* gene, and the *pbp2x* gene of CS109, when combined with the *pbp1a* and *pbp2b* genes from highly penicillin-resistant pneumococci, resulted in transformants for which the MIC of benzylpenicillin was 8 μg/ml (Table 3). However, the *pbp2x* gene from CS111 could provide resistance to <0.25 μg/ml in the same genetic background. As the ancestral *pbp2x* gene differed from that of CS111 at only a single nucleotide site, the low level of penicillin resistance of CS111 must be a consequence of the Thr-550→Ala substitution in PBP2x.

The putative ancestral PBP2x, when expressed together with highly β-lactam-resistant forms of PBP1a and/or PBP2b, could result in transformants for which the MICs of cefotaxime and benzylpenicillin are 8 μg/ml each, whereas PBP2x from CS111 resulted in transformants for which the MIC of cefotaxime was 32 μg/ml but the MIC of benzylpenicillin was ≤0.25 μg/ml. The putative ancestral PBP2x appears therefore to have had very low affinity for both penicillin and expanded-spectrum cephalosporins. The Thr-550→Ala substitution in CS111 further reduces the affinity of PBP2x for expanded-spectrum cephalosporins but at the cost of a loss of its low affinity for penicillin.

Strains CS109 and CS111 appear to be very closely related and possess identical *pbp1a* genes and almost identical *pbp2x* genes. Their *pbp2b* genes are also identical, except that the gene from CS109 contains an additional region of a highly diverged sequence that has presumably been introduced by an interspecies recombinational event. It seems likely that CS109 and CS111 have arisen from a common ancestor that possessed identical mosaic *pbp1a*, *-2x*, and *-2b* genes (Fig. 5). The ancestor presumably possessed the *pbp2b* gene of CS111 and a *pbp2x* gene lacking the substitutions within codons 550 and 701. The MICs of benzylpenicillin and cefotaxime for transformants of this type ($R6^{ancestor/2x/CS111/1a/2b}$) were 1 and 8 μg/ml, respectively. In this scenario, CS111 would have arisen by the introduction of the Thr-550→Ala substitution in PBP2x, which provides increased resistance to expanded-spectrum cephalosporins but a loss of resistance to penicillin. Conversely, CS109 would have arisen by an interspecies recombinational event that introduced an additional diverged region into the *pbp2b* gene and by the Gly-701→Ser substitution in PBP2x (Fig. 5). The *pbp2b* gene of CS109 provides greater resistance to penicillin than that of CS111 (or the presumed ancestral strain), and the recombinational event in *pbp2b* was probably selected, as it further increases resistance to penicillin. The selection pressure for the Gly-701→Ser substitution in PBP2x of CS109 is unclear, as we were unable to detect any effect of this change on resistance to benzylpenicillin or cefotaxime (or ampicillin, amoxicillin, oxacillin, or ceftriaxone).

Finally, our results demonstrate that point mutations as well as recombinational events are important in the development of resistance to β-lactam antibiotics in pneumococci. Since all of the penicillin- or cephalosporin-resistant clinical isolates we have examined possess mosaic *pbp* genes, we believe that the initial steps in the emergence of resistance in pneumococci are predominantly due to interspecies recombinational events (8). However, as mosaic *pbp* genes that encode PBPs with decreased affinity for most (or all) β-lactam antibiotics have emerged and are now common in several countries, it is likely that further selection for resistance to penicillins or cephalosporins, or to newly introduced β-lactams, will occur by the introduction of point mutations in the preexisting pool of mosaic *pbp* genes to reduce further the affinity of these PBPs.

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