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Identification of Multiple Clones of Extended-Spectrum Cephalosporin-Resistant *Streptococcus pneumoniae* Isolates in the United States

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We characterized 12 isolates of *Streptococcus pneumoniae* with various levels of susceptibility to penicillin and extended-spectrum cephalosporins by antimicrobial susceptibility patterns, serotypes, ribotypes, chromosomal DNA restriction patterns by pulsed-field gel electrophoresis, multilocus enzyme electrophoresis patterns, penicillin-binding protein (PBP) profiles, and DNA restriction endonuclease cleavage profiles of *pbp1a*, *pbp2x*, and *pbp2b*. Seven cefotaxime-resistant (MIC, ≥2 μg/ml) serotype 23F isolates were related on the basis of ribotyping, pulsed-field gel electrophoresis, and multilocus enzyme electrophoresis, but they had two slightly different PBP patterns: one unique to strains for which the MIC of penicillin is high (4.0 μg/ml) and one unique to strains for which the MIC of penicillin is low (0.12 to 1.0 μg/ml). The *pbp1a* and *pbp2x* fingerprints were identical for the seven isolates; however, the *pbp2b* fingerprints were different. An eighth serotype 23F isolate with high-level resistance to cephalosporins was not related to the other seven isolates by typing data but was a variant of the widespread, multiresistant serotype 23F Spanish clone. The PBP profiles and fingerprints of *pbp1a*, *pbp2x*, and *pbp2b* were identical to those of the Spanish clone isolate. An additional serotype 6B isolate with high-level resistance to cephalosporins had unique typing profiles and was unrelated to the serotype 23F cephalosporin-resistant isolates but was related on the basis of genetic typing methods to a second serotype 6B isolate that was cephalosporin susceptible. The serotype 6B isolates had different PBP profiles and fingerprints for *pbp1a*, but the fingerprints for *pbp2x* and *pbp2b* were the same.

Penicillin-resistant strains of *Streptococcus pneumoniae*, first isolated in the late 1960s in Australia, are now prevalent throughout the world and are often associated with resistance to other antimicrobial agents (1, 25). In the United States, previous surveys revealed only sporadic occurrences of penicillin-resistant pneumococcal isolates (prevalence, ≤5% [23, 46]); however, more recent surveys show much higher rates of infection caused by resistant pneumococci (14, 16, 47). Although the optimal therapy for infections caused by penicillin-intermediate or -resistant strains of pneumococci has not been established, several reports indicate that cefotaxime or ceftriaxone is the drug of choice for initial empiric therapy for meningitis and sepsis caused by this pathogen (7, 14, 31).

Most pneumococcal isolates that are resistant to penicillin also have increased resistance to extended-spectrum cephalosporins, including cefotaxime and ceftriaxone. The MICs of penicillin are generally equal to or two to four times higher than the MICs of the cephalosporins. Recently, we (45) and others (3, 14, 22) reported on infections caused by pneumococcal isolates that have not responded to cephalosporin therapy and for which the MICs of cefotaxime and ceftriaxone are 2 to 32 μg/ml. These MICs are in excess of the MICs of penicillin for these isolates, which is the opposite of most previously characterized strains. Additional isolates that demonstrate this reversed ratio of MICs of penicillin and MICs of extended-spectrum cephalosporins have continued to appear in several Southeastern states (4). Most of these isolates have high-level resistance to penicillin, cefotaxime, and ceftriaxone, are serotype 23F, and appear to be related by ribotype (4, 45).

This study examined cephalosporin-resistant pneumococcal isolates by rRNA gene restriction pattern analysis (ribotyping), multilocus enzyme electrophoresis (MLEE), and chromosomal DNA restriction analysis by pulsed-field gel electrophoresis (PFGE) to determine the degree of overall genetic variation among strains, examined the penicillin-binding proteins (PBPs) of the isolates by comparing the electrophoretic mobilities of the PBPs on sodium dodecyl sulfate (SDS)-polyacrylamide gels (PBP profiles), and compared the DNA fingerprints of *pbp1a*, *pbp2x*, and *pbp2b* to determine the relatedness of the *pbp* genes. The results of this investigation provide evidence that resistance to extended-spectrum cephalosporins has emerged on several occasions in different clonal populations of pneumococci in the United States.

MATERIALS AND METHODS

Bacterial strains and conditions of growth. The properties of the pneumococcal strains examined are listed in Table 1. Strains were kindly provided by Robert J. Leggiadro (LeBonheur Children's Medical Center, University of Tennessee, Memphis), Paul H. Edelstein (University of Pennsylvania Medical Center, Philadelphia), and Sharon Busch (Smith-Kline Beecham Clinical Laboratories, West Norriton, Pa.). The organisms were identified by susceptibility to ethylhydrocuprein and bile solubility (13); they were serotyped on the basis of capsular swelling (Quellung reaction) with type-specific antisera (Childhood and Vaccine Preventable Diseases Branch, Centers for Disease Control and Prevention). Unless otherwise stipulated, pneumococci were grown in C + y medium, a casein-based chemically defined medium supplemented with 0.2% yeast extract (26), at 35 to 37°C in an atmosphere containing 5.0% CO_2.

Susceptibility testing. The antimicrobial susceptibility profiles of the isolates were determined by broth microdilution with cation-adjusted Mueller-Hinton broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.) supplemented with 3 to 5% lysed horse blood as described in the National Committee for Clinical Laboratory Standards publication M7-A3 (40). *S. pneumoniae* ATCC 49619 was used for quality control.

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TABLE 1. Properties of cephalosporin-resistant clinical isolates of *S. pneumoniae*

Strain	MIC (μg/ml) of:			Phenotype for[a]:				Place of isolation	Year of isolation	Source[b]
	Penicillin	Cefotaxime	Ceftriaxone	Chloramphenicol	Tetracycline	Trimethoprim-sulfamethoxazole	Erythromycin			
CS107	0.5	4.0	2.0	S	S	R	S	Georgia	1991	CSF
CS110	1.0	4.0	4.0	S	S	R	S	Tennessee	1991	CSF
CS117	0.5	4.0	2.0	S	S	R	S	Tennessee	1991	BLD
CS111	0.25	32.0	16.0	S	S	R	R	Tennessee	1991	BLD
CS109	4.0	8.0	4.0	S	S	R	S	Tennessee	1991	CSF
CS119	4.0	4.0	4.0	S	S	R	S	Georgia	1992	BLD
CS122	4.0	8.0	4.0	S	S	R	S	South Carolina	1992	SPU
CS108	0.25	0.25	0.25	S	S	R	S	Ohio	1991	SPU
CS105	2.0	1.0	1.0	R	R	R	S	Spain	1987	NK[c]
CS126	2.0	8.0	4.0	R	R	R	R	Pennsylvania	1992	NK
CS125	4.0	4.0	2.0	S	S	R	R	Pennsylvania	1992	SPU
CS112	1.0	2.0	1.0	S	S	R	R	New Jersey	1991	SPU
CS103[d]	0.015	≤0.06	≤0.06	S	S	S	S	New York	1985	NK
R6[d]	0.015	≤0.06	≤0.06	S	S	S	S	New York	NK	NK

[a] R, resistant; S, susceptible.
[b] CSF, cerebral spinal fluid; BLD, blood; SPU, sputum.
[c] NK, not known.
[d] Susceptible control.

Ribotyping. Genomic DNA was prepared in situ in agarose blocks as previously described (35). Patterns of DNA encoding rRNA were obtained by restricting DNA samples with *Hin*dIII (Life Technologies, Gaithersburg, Md.), electrophoresing the fragments on 0.8% agarose gels, and transferring the fragments onto nylon membranes (MagnaGraph; Micron Separations, Inc., Westboro, Mass.), and this was followed by hybridization with end-labeled 16+23S rRNA from *Escherichia coli* (Sigma Chemical Co., St. Louis, Mo.). The ribotyping was repeated with some isolates, and the membrane-bound DNA fragments were hybridized with a digoxigenin-labeled probe that was detected by an enzyme-catalyzed color reaction (2). Isolates showing two or more band differences were given different types (designated by Roman numerals); those with a single band difference (either in size or number of bands) were considered subtypes.

MLEE. Horizontal starch gel electrophoresis and enzyme assays were performed as described previously (35). Electrophoretic variants of each enzyme assayed were considered alleles of that enzyme and assigned different numbers. Each unique combination of alleles in the strains was designated an electrophoretic type. Genetic relationships among electrophoretic types were determined by the average linkage method of clustering from a matrix of pairwise coefficients of weighted distance (43) by using an SAS program described by Jacobs (21). Strains were delineated at a genetic distance of 0.1 and assigned number and letter designations reflecting their relative relatedness.

PFGE. Genomic DNA was prepared in situ in agarose blocks (30), and the equilibrated DNA was digested with *Sma*I (Life Technologies). The fragments were resolved by PFGE in 1% agarose (SeaKem GTG agarose; FMC Bioproducts, Rockland, Maine) in 0.5× Tris-borate-EDTA buffer for 20 h at 14°C and 6 V/cm with a CHEF-DRII device (Bio-Rad Laboratories, Richmond, Calif.). The parameter was an initial pulse time of 1 s increased to 20 s. Gels were stained with ethidium bromide, destained in distilled water, and photographed with UV illumination. Isolates with identical restriction profiles were assigned the same type. Isolates that differed by one to four bands were assigned a subtype. Isolates with more than four band differences were interpreted as indicative of strain differences.

Purification of chromosomal DNA and fingerprinting of genes encoding PBP2b, PBP2x, and PBP1a. Pneumococcal chromosomal DNA was purified on cesium chloride-ethidium bromide gradients essentially as described previously (11). However, following the extraction of the ethidium bromide with cesium chloride-saturated 1-butanol, the DNA solution was diluted with 3 volumes of heat-sterilized ultrapure water and precipitated with 2 volumes of ethanol at 4°C for 15 min. The DNA was dried under a vacuum and resuspended in sterile water at a concentration of approximately 200 μg/ml. Segments of the genes encoding PBP2b, PBP2x, and PBP1a were amplified from chromosomal DNA by PCR (11).

Amplification of the gene encoding PBP2b with the previously described oligonucleotide primers Pn2bup and Pn2bdown (11) yielded a 1.5-kb product that included the region encoding the transpeptidase domain of the enzyme (6). The *pbp2x* product (28), obtained with the previously described oligonucleotide primers Pn2xup and Pn2xdown (37), was a 2.0-kb fragment. The genes encoding PBP2b and PBP2x were each amplified in a 100-μl reaction mixture containing ~1 μg of chromosomal DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.5 μM oligonucleotide primers, 200 μM deoxynucleoside triphosphates, and 2.5 U of native *Taq* DNA polymerase (Boehringer Mannheim Corporation, Indianapolis, Ind.). PCR cycling conditions were denaturation at 94°C for 7 min; 35 cycles of 94°C for 2 min, 50°C for 1 min, and 72°C for 2 min; and a final extension period of 72°C for 7 min.

The gene encoding PBP1a (32) was amplified as a 2.24-kb product with the oligonucleotides Pn1afor (dATAAGGGACAAAAATCAATGCCAC; nucleotides 819 to 842) and Pn1arev (dATGTTATGGTTGTGCTGGTTGAGG; nucleotides 3108 to 3085). The composition of the 100-μl reaction mixture was the same as that used for the amplification of the genes encoding PBP2b and PBP2x, except that the $MgCl_2$ was increased to 2.0 mM. PCR cycling conditions for *pbp1a* were denaturation at 94°C for 7 min; 35 cycles of 94°C for 2 min, 54°C for 1 min, and 72°C for 3 min; and a final extension period of 72°C for 8 min. Amplified fragments were purified with Wizard PCR Preps DNA purification minicolumns (Promega Corp., Madison, Wis.) according to the manufacturer's instructions. PCR products were eluted from the minicolumns in 50 μl of heat-sterilized water.

Gene fingerprinting was performed as previously described (6). Briefly, approximately 20 ng of purified PCR product was digested for at least 2 h at 37°C with 20 U of a restriction enzyme (*Sty*I for the *pbp2b* gene fragment and *Hin*fI for the *pbp2x* and *pbp1a* gene fragments) in a mixture (final volume, 10 μl) containing 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, and either 100 or 50 mM NaCl, respectively (Gibco-BRL; Grand Island, N.Y.). Five microliters of an end-labeling mixture was added. For *Sty*I-digested DNA, this was 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 6 mM dithiothreitol, 0.1 mM dATP, 0.1 mM dTTP, 0.1 mM dGTP, and 0.6 μCi of [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol) and 0.1 U of Klenow fragment per μl. For *Hin*fI-digested DNA, the radiolabeled nucleotide was [α-^{32}P]dTTP, so the unlabeled dTTP was replaced with 0.1 mM dCTP in this reaction mixture. After 10 min at room temperature, 4 μl of a 6× gel-loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 40% sucrose in sterile water was added (42). The size standard consisted of pBR322 DNA that was digested with *Hpa*II in a buffer containing 20 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$ and that was end labeled with [α-^{32}P]dCTP as described above (6).

Labeled restriction fragments were fractionated by applying 6 μl of each sample to a nondenaturing 5 to 10% gradient polyacrylamide gel containing a 19:1 ratio of acrylamide to *N,N′*-methylenebisacrylamide. The 10% acrylamide solution contained 75% glycerol. To ensure uniform well formation, a 5% polyacrylamide stacking gel containing an increased amount of the catalyst was used. Samples were electrophoresed at 4°C and 100 V in a Protean II slab gel chamber (Bio-Rad Laboratories) until the bromophenol blue marker dye migrated to within 3.0 cm of the bottom of the gel (requiring approximately 21 h). Following electrophoresis, the gels were not fixed but instead were directly transferred to Whatman no. 1 filter paper, dried under a vacuum, and autoradiographed with Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

Labeling, separation, and detection of PBPs. PBPs were labeled as previously described (35), except that the whole cells were suspended in 14 μl of 50 mM phosphate buffer (pH 7) and 6 μl of 0.2% Triton X-100 in the same buffer. The cells were radiolabeled with 15 to 20 μg of ^{3}H-benzylpenicillin (specific activity, 29.1 Ci/mM; Merck Sharp & Dohme Research Laboratories, Rahway, N.J.) per ml, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (35).

TABLE 2. Typing characteristics of cephalosporin-resistant clinical isolates of *S. pneumoniae*

Strain	Serotype	Ribotype	Type and subtype by[a]:		PBP pattern	Fingerprint profile for:		
			MLEE	PFGE		*pbp1a*	*pbp2x*	*pbp2b*
CS107	23F	I	1.d	A.5	1	1	1	1
CS110	23F	I	1.a	A.2	1	1	1	2
CS117	23F	I	1.a	A.4	1	1	1	2
CS111	23F	I	1.a	A.1	1	1	1	2
CS109	23F	I	1.b	A	2	1	1	3
CS119	23F	I	1.c	A	2	1	1	4
CS122	23F	I	1.c	A.3	2	1	1	4
CS108	23F	I	1.a	A.6	3	2	2	5
CS105	23F	II	3.a	B.1	4	3	3	6
CS126	23F	II	3.b	B.2	4	3	3	6
CS112	6B	III	2.a	C.1	5	4	4	3
CS125	6B	III	2.b	C.2	6	5	4	3
CS103[b]	23F	IV	1.e	D	7	4	5	2
R6[b]	NT	V	4	E	7	4	5	2

[a] See Materials and Methods for explanation of types and subtypes.
[b] Susceptible control.

RESULTS

Phenotypic characterization studies. The properties of the organisms used in this study are presented in Table 1. They demonstrate three β-lactam phenotypes: penicillin- and cephalosporin-susceptible strains, penicillin-resistant strains for which the MICs of cefotaxime and ceftriaxone are equal to or 1 to 2 dilutions lower than their MIC of penicillin, and cephalosporin-resistant strains (MIC of cefotaxime and ceftriaxone, ≥2.0 μg/ml) for which the MIC of cefotaxime is equal to or 1 or more dilutions higher than the MIC of penicillin. The cephalosporin-resistant phenotype contains strains for which the MIC of penicillin is both intermediate (0.1 to 1.0 μg/ml) and high level (≥2 μg/ml). Six of the cephalosporin-resistant isolates were also resistant to trimethoprim-sulfamethoxazole. Three of these strains were isolated in Tennessee in 1991, and the other three were from nearby states. Isolate CS126 was resistant to penicillin, cefotaxime, ceftriaxone, chloramphenicol, tetracycline, erythromycin, and trimethoprim-sulfamethoxazole and resembled the Spanish serotype 23F multiresistant clone, except that the latter clone is more susceptible to cefotaxime and ceftriaxone. Isolates CS125, CS112, and CS111 were resistant to trimethoprim-sulfamethoxazole and erythromycin.

Serotyping, ribotyping, MLEE, and DNA macrorestriction analysis. Eight of the nine cephalosporin-resistant isolates were serotype 23F, and of these, seven isolates (CS107, CS110, CS117, CS111, CS109, CS119, and CS122) were indistinguishable by ribotyping with *Hin*dIII (Table 2) (Fig. 1). These seven isolates also had similar macrorestriction DNA patterns upon examination by PFGE with *Sma*I (Fig. 2) and differed by only 1 of 20 enzymes when assayed by MLEE (Fig. 3). Isolate CS108, which was also serotype 23F, was penicillin intermediate and susceptible to the cephalosporins. It had ribotype and MLEE profiles (Fig. 1 and 3) similar to those of the cephalosporin-resistant isolates, but there were at least three band differences in the PFGE patterns (Fig. 2). The eighth cephalosporin-resistant serotype 23F isolate (CS126) had the same ribotype as that of and PFGE and MLEE profiles similar to those of the multiresistant serotype 23F Spanish isolate (CS105). The typing profiles of these two isolates were very different from those of the other serotype 23F isolates, which were designated the Tennessee clone. The cephalosporin-resistant serotype 6B isolate (CS125) had a ribotype identical to that of and MLEE and PFGE profiles similar to those of a serotype 6B isolate (CS112) with lower MICs of penicillin and cephalosporins. The penicillin-susceptible serotype 23F control strain, CS103, had ribotype and PFGE profiles different from those of the other serotype 23F isolates but surprisingly had the same MLEE allele profile as that of isolate CS107. Strain R6 was clearly distinguishable from the other study isolates.

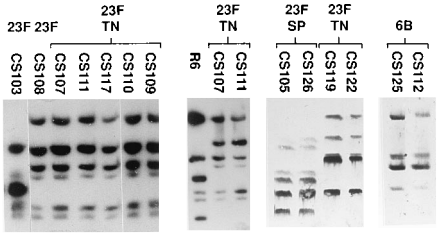


FIG. 1. Ribotyping patterns of pneumococcal isolates. Autoradiographs of electroblots of *Hin*dIII-cleaved *S. pneumoniae* DNA fragments hybridized with ^{32}P-labeled *E. coli* 16+23S rRNA (CS103 through CS109) and electroblots of *Hin*dIII-cleaved DNA fragments hybridized with a digoxigenin-labeled probe (R6 through CS112). TN, Tennessee; SP, Spain.

PBP profiles and *pbp* fingerprints. The PBP profiles (Fig. 4) and *pbp* fingerprints (Fig. 5) of the nine cephalosporin-resistant isolates were different from those of penicillin- and cephalosporin-susceptible pneumococci, with the exception of the PBP2b profiles and the *pbp2b* fingerprints of isolates CS110, CS117, and CS111. The Tennessee cephalosporin-resistant serotype 23F isolates had two slightly different PBP patterns: one unique to strains (CS107, CS110, CS117, and CS111) with MICs of penicillin that are intermediate (0.12 to 1.0 μg/ml) and one unique to strains (CS109, CS119, and CS122) with MICs of penicillin that are high level (4.0 μg/ml). The *pbp1a* and *pbp2x* fingerprints appeared identical for all seven of these isolates, while the *pbp2b* fingerprints varied. Isolates CS119 and CS122 had identical *pbp2b* fingerprints, isolates CS110, CS117, and CS111 had *pbp2b* fingerprints indistinguishable from that of isolate R6, isolate CS107 had a unique *pbp2b* fingerprint, and isolate CS109 had the same *pbp2b* fingerprint as those of serotype 6B isolates CS112 and CS125. The cephalosporin-susceptible, penicillin-intermediate pneumococcal isolate (CS108) that had ribotype and MLEE profiles similar to

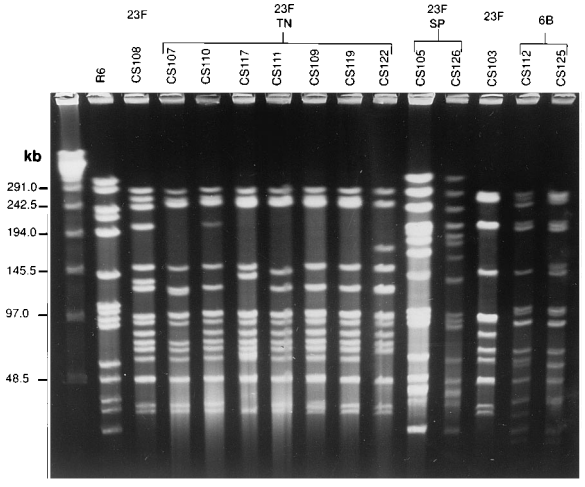


FIG. 2. PFGE separation of *Sma*I-generated restriction fragments of the chromosome of the pneumococcal isolates. Molecular size markers (lambda DNA ladder) were run in the first lane. TN, Tennessee; SP, Spain.

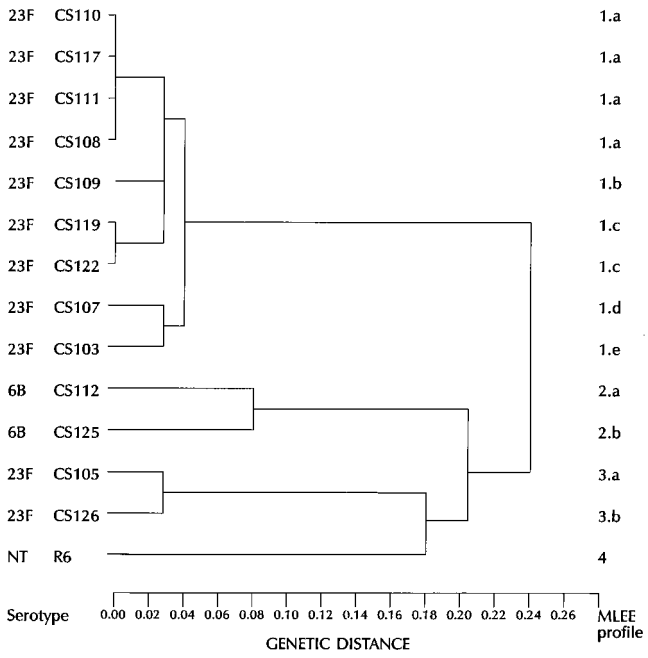


FIG. 3. Dendrogram depicting genetic distances and overall relatedness of pneumococcal isolates on the basis of MLEE.

those of the cephalosporin-resistant Tennessee isolates had a unique PBP profile and three unique *pbp* fingerprints. The cephalosporin-resistant serotype 23F isolate (CS126) had a PBP pattern identical to that of the multiresistant Spanish isolate (CS105). Both of these isolates appeared to have identical *pbp1a*, *pbp2x*, and *pbp2b* fingerprints that were very different from the other gene fingerprints. The two serotype 6B isolates (CS125 and CS112) had slightly different PBP patterns. The fingerprints were the same for *pbp2x* and *pbp2b* but were different for *pbp1a*. The *pbp1a* fingerprint for strain CS112 was indistinguishable from the *pbp1a* fingerprint of the penicillin-susceptible isolates CS103 and R6.

DISCUSSION

High-level resistance of *S. pneumoniae* to extended-spectrum cephalosporins has occurred concurrently in several pneumococcal clones in the United States, three of which (two serotype 23F and one 6B) were examined in this study. In each case, isolates with high-level resistance to cephalosporins were related by serotype, ribotype, MLEE profile, and DNA restriction pattern to isolates for which the MICs of cefotaxime and ceftriaxone are lower. This suggests that resistance to cephalosporins has emerged within preexisting clones de novo, and additional resistant clones can be expected to appear throughout the United States.

Resistance of pneumococci to penicillin occurs when alterations in the organism's PBPs reduce their affinity for β-lactam agents (17). The genes encoding the PBPs are mosaics in which sequences of the native (susceptible) *pbp* genes have been replaced with blocks of highly altered DNA from other pneumococci or streptococcal species (9, 29, 33). The mosaic genes are the end result of one or several recombination events that follow the uptake of DNA by transformation (8–10, 29). Since pneumococci are naturally transformable, transformation provides an efficient mechanism for distributing the mosaic resistance genes among genetically distinct populations of pneumococci. Indeed, epidemiologic evidence suggests that pneumococcal resistance to β-lactams is spread both by dissemination of resistant organisms (clonal spread) and by dissemination of *pbp* mosaic genes (horizontal spread) (9, 19).

Molecular typing studies of penicillin-resistant pneumococci from several countries have demonstrated that, in general, the majority of strains circulating within a geographic area are derivatives of a relatively small number of clonal lineages (39), even though the MICs of β-lactam drugs for these isolates cover a range. The difference in MICs is likely due to gene transfer of resistance determinants and point mutations causing additional changes in the genes. Selection for the resultant resistant strains, presumably under response to different antimicrobial and other selective pressures, results in a population of pneumococcal isolates with varied resistance to β-lactam antibiotics. Thus, pneumococcal resistance is a combination of the spread of resistant clones and the spread of resistance genes within those clonal lineages.

Several molecular typing techniques—MLEE, ribotyping, and macrorestriction pattern analysis of chromosomal DNA by PFGE—were used to determine the genetic relatedness of the isolates in this study. Three distinct lineages of cephalosporin-resistant isolates emerged. The first group, from Tennessee and surrounding areas in the Southeast, was highly related by serotyping, MLEE, ribotyping, and PFGE. These isolates also had ribotype and MLEE profiles similar to those of a cephalosporin-susceptible, penicillin-intermediate serotype 23F isolate, although the latter organism demonstrated unique *pbp* fingerprints. The Tennessee serotype 23F clone was quite distinct from a highly cephalosporin-resistant serotype 23F multiresistant isolate from Pennsylvania that was highly related by all three typing methods to the widespread multiresistant serotype 23F Spanish clone. The Spanish clone and its derivatives have been isolated from many areas in the United States (35, 37), the United Kingdom (6), South Africa (24, 35), and Mexico (24). The third lineage was represented by a highly cephalosporin- and penicillin-resistant serotype 6B isolate that was related by the three typing methods to a more susceptible serotype 6B isolate. Thus, for each highly cephalosporin-resis-

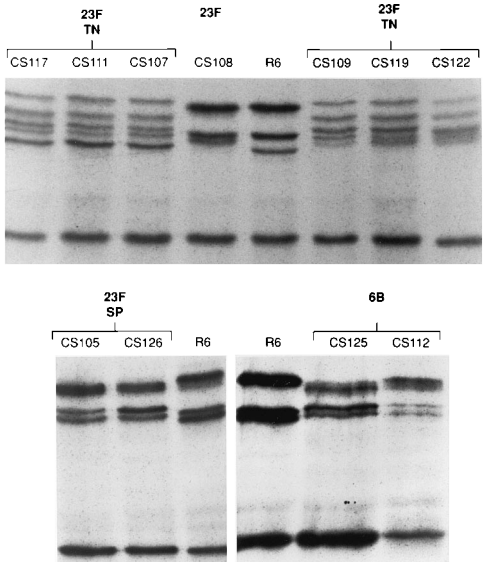
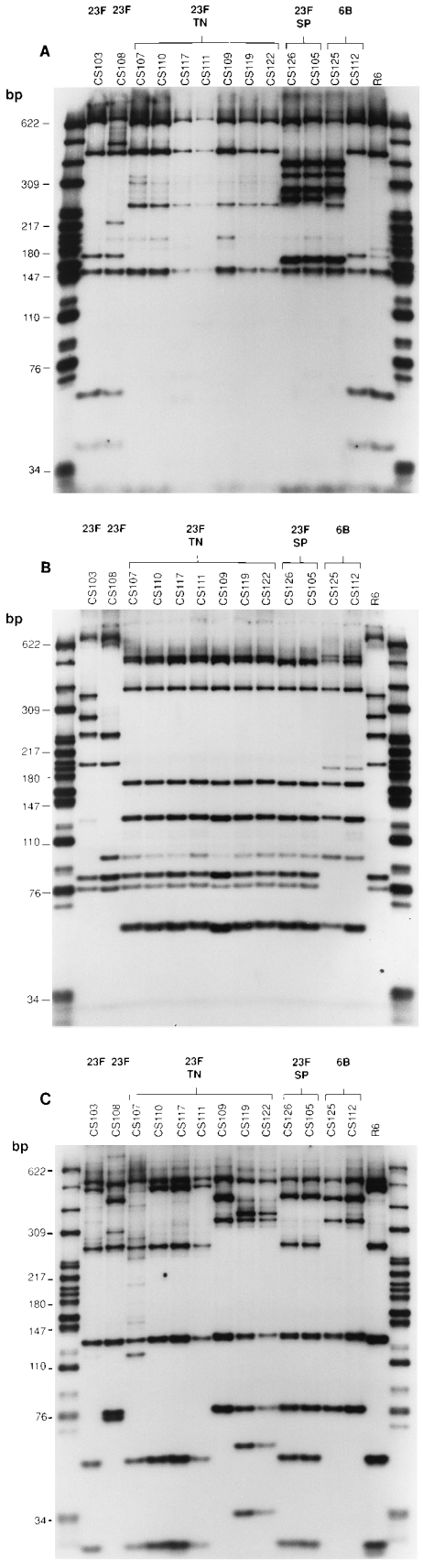


FIG. 4. Fluorographs of electrophoretic profiles of polyacrylamide gels (3.6% stacking gels and 7.5% separating gels) of PBPs of penicillin- and cephalosporin-resistant isolates of *S. pneumoniae*. TN, Tennessee; SP, Spain.



tant lineage, we have been able to identify a related cephalosporin-susceptible counterpart.

In this study, we used DNA fingerprinting (restriction digestion analysis of PCR products obtained after amplification of distinct regions of the *pbp* genes) to provide a more discriminating method for differentiating the *pbp* genes of β-lactam-resistant pneumococci than SDS-PAGE and fluorography (6, 8, 17). Previous studies using DNA fingerprinting and sequencing of *pbp* genes indicated the presence of extensive heterogeneity in natural populations of penicillin-resistant pneumococci, suggesting that resistant strains have arisen independently many times (9, 19). At least three of the five high-molecular-weight PBPs are consecutively altered, with increasing levels of resistance to penicillin (9). An altered *pbp2x* appears to be a prerequisite for higher resistance levels involving the acquisition of modified, low-affinity variants of *pbp2b* and *pbp1a* in clinical isolates (10, 12, 27, 28, 38). High-level resistance to penicillin can be achieved by successive alterations of the genes encoding PBPs 2x, 2b, and 1a (11). Penicillin-resistant isolates also show increased resistance to the cephalosporins (1, 25). However, for high-level resistance to extended-spectrum cephalosporins, only altered *pbp2x* and *pbp1a* are required (5, 38). PBP2b is not a target for this class of β-lactam antibiotics (20). Since both *pbp1a* and *pbp2x* are closely linked on the chromosome, they can be transferred simultaneously to recipient strains of pneumococci during transformation events (15, 38). Consequently, the levels of resistance to penicillin do not necessarily correlate with the levels of resistance to the cephalosporins.

The *pbp1a* and *pbp2x* fingerprints were the same for all of the cephalosporin-resistant Tennessee isolates, but their *pbp2b* fingerprints were different, as would be expected from their different MICs of penicillin. PBP2b is clearly visible in the radiolabeled penicillin-assayed PBP profiles (Fig. 4) of isolates CS110, CS117, and CS111, indicating a higher affinity for penicillin than that found for PBP2b in isolates CS109, CS119, and CS122 (36). Subsequent sequencing, however, of *pbp2b* of strain CS111 showed that it (and presumably CS110 and CS117) possessed a mosaic gene structure, although this was not detected by fingerprinting, as the distribution of *Sty*I sites was not altered from that in *pbp2b* from strain R6 (5). It is interesting that the fingerprint of *pbp2b* of the highly penicillin-resistant isolate CS109 is identical to the fingerprint of *pbp2b* of isolates CS125 and CS112 of the 6B clone while the *pbp2b* fingerprints of isolates CS119 and CS122 are identical but different from other *pbp2b* fingerprints. *pbp* fingerprinting and PBP profiles clearly showed that the penicillin-intermediate, cephalosporin-susceptible isolate (CS108) had quite different *pbp* genes, although overall genomic typing methods showed that this isolate appeared to be related to the highly cephalosporin-resistant Tennessee isolates. Although genetic diversity among isolates in a given serotype has been demonstrated in several studies (44, 48), the degree of diversity within serotypes remains unclear. While isolate CS108 is not a variant of the Tennessee clone, as it differs in three *pbp* fingerprints, the overall genetic similarity suggests that the horizontal transfer of *pbp* genes may have contributed to the various levels of β-lactam resistance.

FIG. 5. (A) *Hin*fI fingerprints of *pbp1a*. The ^{32}P-labeled *Hin*fI fragments from *pbp1a* of the pneumococcal isolates were fractionated on a 5 to 10% polyacrylamide gradient gel and detected by autoradiography. (B) *Hin*fI fingerprints of *pbp2x*. (C) *Sty*I fingerprints of *pbp2b*. The molecular size markers (pBR322 digested with *Hpa*II) are in the first and last lanes (all panels). TN, Tennessee; SP, Spain.

None of the three genes of the two related multiresistant Spanish isolates (CS126 and CS105) could be distinguished by fingerprinting. The four- to eightfold difference in the level of cephalosporin resistance for CS126 and CS105 would appear to be due to alterations or point mutations in both *pbp1a* and *pbp2x* of CS126 that do not affect the *Hin*fI restriction sites; sequence information and genetic analysis of these genes are necessary to determine the genetic differences responsible for the increase in the level of cephalosporin resistance.

In the serotype 6B isolates (CS125 and CS112) in which the fingerprints of *pbp2x* and *pbp2b* were the same, the two- to fourfold difference in resistance to both penicillin and the cephalosporins is possibly due to an altered mosaic *pbp1a*, as was demonstrated by the unique gene fingerprint and lower molecular weight of PBP1a in isolate CS125. Although the *pbp1a* fingerprint of isolate CS112 was indistinguishable from the *pbp1a* fingerprints of penicillin- and cephalosporin-susceptible R6 and CS103 isolates, the sequencing of *pbp1a* would be necessary before we could say that the genes are the same. Also, differences in *pbp2x* and *pbp2b* not discerned by gene fingerprinting could account for the differences in susceptibility to penicillin and the cephalosporins.

In each of these three clones, high-level resistance to the extended-spectrum cephalosporins was associated with uniquely altered *pbp1a* and *pbp2x* that resulted in proteins that expressed low affinity for the cephalosporins (36). In all three clones, the *pbp* genes were similar enough so that restriction endonuclease fingerprinting was unable to distinguish differences in the *pbp* genes that could account for the differences in resistance levels to both penicillin and the cephalosporins; direct sequencing of the *pbp* genes will be necessary to find these differences. However, the high degree of relatedness suggests that these isolates originated from a common ancestor, most likely by nucleotide substitutions. Point mutations in *pbp2x* and *pbp2b* that are responsible for the decreased affinity of the PBP2x and PBP2b proteins to β-lactam antibiotics have been described for laboratory mutants of pneumococci (18, 27), and in a clinical isolate, a similar substitution in *pbp2x* that was found in the laboratory mutants selected for increased resistance to cefotaxime was shown to result in the decreased affinity of PBP2x for extended-spectrum cephalosporins (5).

In summary, these studies demonstrate that pneumococci with low-level resistance to cephalosporins were closely related to isolates with high-level resistance to cephalosporins. These data, in conjunction with recent DNA sequence data (5), provide strong evidence for the development in pneumococci of resistance to β-lactams by horizontal transfer of mosaic *pbp* genes into susceptible isolates and by secondary modifications involving nucleotide substitutions. The concurrent occurrence of high-level resistance to extended-spectrum cephalosporins in several pneumococcal clones in the United States suggests that cephalosporin-resistant pneumococci are likely to be isolated with increasing frequency because of the strong selective pressure resulting from the high utilization of β-lactams for the treatment of pneumococcal infections in the United States (12, 34, 41).

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