Penicillin-resistant *Streptococcus pneumoniae* isolates were first detected in the 1960s and have been isolated from patients in many parts of the world, including South Africa, Australia, the United States, and Europe. There is evidence that penicillin resistance in pneumococci is mainly due to alterations in the structural genes encoding the penicillin-binding proteins (PBPs), thus resulting in PBPs with decreased affinity for penicillin (4, 10). Nucleotide sequence analysis of the DNA regions encoding the penicillin-binding domains of PBP 2B and PBP 2X from resistant isolates strongly suggests that these alterations of PBPs might be due to the acquisition of exogenous PBP-encoding DNA through transformation and recombination (5, 14). These events might have occurred independently on more than one occasion in various parts of the world. Resistant isolates may then be transported to geographically distant areas, where they may spread rapidly, as described in Iceland (23).

In France, the first penicillin-resistant isolate was found in 1978, and resistant pneumococci were rare until 1987 (7). The proportion of isolates resistant to penicillin increased yearly thereafter to 6.6% in 1989 and 25% in 1993 (8). A nationwide multicenter study of children with acute otitis media (1) revealed that 37.5% of pneumococci isolated from the nasopharyngeal flora of children were resistant (1). The geographical distribution of resistant isolates was uneven, with a predominance in urban areas, where the proportion was as high as 50% in some cities. The majority of resistant isolates (65%) belonged to the 23F serogroup, suggesting a common clonal origin (1). Here, we report an analysis of clonality involving the study of a panel of clinical penicillin-resistant and -susceptible isolates of *S. pneumoniae* obtained from children (age, <6 years) in various parts of France. The penicillin-resistant 23F isolates appear to be closely related and clearly different from susceptible 23F isolates. Thus, the emergence of resistant 23F isolates might have been due to rapid clonal spreading of bacteria in the commensal nasopharyngeal flora of children.

**MATERIALS AND METHODS**

**Bacterial strains and culture media.** We used clinical isolates of *S. pneumoniae*, obtained between November 1992 and March 1993 from nasopharyngeal samples from children (age, <6 years) from throughout France with acute otitis media. They included 24 isolates of serotype 23F susceptible to penicillin G for which MICs ranged from ≤0.0075 to 0.03 mg/liter and 24 isolates of serotype 23F resistant to penicillin G (MIC, 2 mg/liter), with most isolates being multidrug resistant (resistant to erythromycin, tetracycline, chloramphenicol, and co-trimoxazole). Twenty-three penicillin-susceptible isolates (MIC, ≤0.0075 to 0.03 mg/liter) belonging to various serotypes were used as controls. The isolates (listed in the legends to Fig. 1 and 4) were selected according to the MICs for the isolates and their serotypes and geographic distributions from among a collection of 632 isolates obtained during a national survey of 1,200 children with acute otitis media (1). We also used penicillin-susceptible *S. pneumoniae* ATCC 33400 (serotype 1; MIC, ≤0.0075 mg/liter; isolated in the United States in 1981), penicillin-resistant *S. pneumoniae* 456 (MIC, 1 to 2 mg/liter; isolated in Spain in 1984) (9), penicillin-susceptible unencapsulated strain R6 (MIC, 0.008 mg/liter; isolated in the United States in 1930), and a penicillin-resistant 23F strain, strain BM4200 (MIC, 0.5 mg/liter; isolated in France in 1978) (2). Pneumococci were grown on Columbia agar plates containing 5% blood or in brain heart infusion medium (Diagnostic Pasteur, Marne-la-Coquette, France). Serotyping was carried out by the quellung reaction with specific antisera provided by the Statens Serum Institute (Copenhagen, Denmark). The MIC of penicillin G was determined on 5% sheep blood Mueller-Hinton agar (Diagnostik Pasteur).

**PFGE.** Pulsed-field gel electrophoresis (PFGE) was performed as described previously (15). DNA was digested overnight with 20 U of Smal or Apal (New England Biolabs Inc., Beverly, Mass.), and fragments were separated on a 1% agarose gel by PFGE (CHEF mapper DRII; Bio-Rad Laboratories, Richmond, Calif.) for 24 h at 14°C at 7 V/cm, with pulse times of 10 to 30 s and an angle of 120°. Gels were visualized with ethidium bromide under UV light.

**Computer-assisted analysis of the DNA fingerprints.** Coefficients of similarity (CS) (CS is the number of shared bands × 2 × 100/total number of bands in the two samples) or the Dice index were determined for each isolate by using the computer program Bio-Profil (Vilber Lourmat, Marne-la-Vallée, France), and a dendrogram was calculated by the unweighted pair-group method. Strains with a CS value of more than 80% were considered to belong to the same lineage.

**DNA polymorphism of the PBP genes.** The genes encoding PBP 1A (pbp1A), PBP 2X (pbp2X), and PBP 2B (pbp2B) of *S. pneumoniae* were amplified from chromosomal DNA by PCR with previously described oligonucleotide primers (3, 6, 19). These primers amplified DNA fragments of 2,409 bp (pbp1A), 2,056 bp (pbp2X), and 1,505 bp (pbp2B). Amplification was performed with a Perkin-Elmer (Norwalk, Conn.) 9600 DNA thermal cycler with an initial denaturation
Genetic polymorphism of \textit{S. pneumoniae} 23F isolates by PFGE. The clonality of \textit{S. pneumoniae} 23F, the serotype which is most often associated with penicillin resistance in France, was studied by PFGE. Forty-eight 23F isolates from nasopharyngeal samples from children from various regions of France with acute otitis media were analyzed. We compared the DNA polymorphism of 24 penicillin-susceptible 23F isolates with that of 24 penicillin-resistant 23F isolates (MIC, 2 mg/liter). Chromosomal DNA was digested with \textit{SmaI} or \textit{ApaI}, generating about 14 or 15 fragments in each case, as illustrated for \textit{SmaI} in Fig. 1. The results obtained with \textit{ApaI} were consistent with those obtained with \textit{SmaI} (data not shown). The restriction profiles of resistant 23F isolates were all similar and were different from those of susceptible 23F isolates (Fig. 1A and B), which were more heterogeneous. A dendrogram was constructed by computer analysis of the DNA fingerprints (Fig. 2). The isolates segregated into two clusters corresponding to the susceptible and the resistant 23F isolates. By using a cutoff value of 80% similarity, at least 14 different lineages were found among the susceptible 23F isolates, suggesting clonal diversity in this serotype. In contrast, only one lineage was identified for the group of resistant 23F isolates. In this lineage, there were three clusters of identical isolates (four, four, and five isolates, respectively), which were isolated from various and in some cases distant parts of the country (Fig. 2). The penicillin-resistant serotype 23F strain 456, isolated in Spain in 1984, was related to the lineage of resistant isolates, (CS, 70%) and to one penicillin-susceptible isolate (isolate 1752). In addition, the first penicillin-resistant strain, strain BM4200 (MIC, 0.5 mg/liter), isolated in France in 1978, had a restriction pattern different from that of the multidrug-resistant isolates (data not shown). The genetic polymorphism of 14 penicillin-susceptible isolates of 14 different serotypes (including the 23F serotype) was analyzed (Fig. 1C). Each strain produced a different banding pattern, demonstrating the high discriminatory power of the PFGE analysis. These results suggest that penicillin-resistant 23F isolates from all over the country have a common clonal origin, whereas the penicillin-susceptible 23F isolates do not.

Genetic polymorphisms of \textit{php1A}, \textit{php2B}, and \textit{php2X} genes in \textit{S. pneumoniae} 23F. We then studied the genetic polymorphisms of the genes encoding the PBPs implicated in the penicillin resistance of \textit{S. pneumoniae}. Intragenic fragments of the \textit{php1A} (2,409 bp), \textit{php2X} (2,056 bp), and \textit{php2B} (1,505 bp) genes from the 48 23F isolates were amplified by PCR and digested with \textit{AluI} or \textit{HinII}; the results for representative isolates are illustrated in Fig. 3. The \textit{AluI} and \textit{HinII} restriction patterns of the three amplified \textit{php} fragments were similar for the 24 French penicillin-resistant isolates and Spanish strain 456, but differed from those of the penicillin-susceptible isolates. Most of the patterns obtained for these penicillin-susceptible isolates were also identical; \textit{php2X}, however, gave at
least three different profiles. Among the 24 susceptible 23F isolates, there was one exception (isolate 1835; MIC, 0.0075 mg/liter), for which pbp1AAluI and HinI restriction profiles were identical to those for the resistant isolates (data not shown). Twenty-three penicillin-susceptible non-23F isolates belonging to 13 different serotypes were also analyzed, as illustrated in Fig. 4 for representative isolates. The pbp1A and pbp2B restriction patterns were similar to those for susceptible 23F isolates, whereas at least three different restriction patterns were found for pbp2X (Fig. 4). Thus, the genes encoding PBP 1A, PBP 2B, and PBP 2X in penicillin-resistant isolates (MIC, 2 mg/liter) are closely related or identical and are distantly related to those found in penicillin-susceptible isolates.

Penicillin affinities of PBPs in S. pneumoniae 23F. We tested the penicillin affinity of S. pneumoniae PBPs for the 48 23F isolates. Bacterial proteins were labeled with [3H]penicillin and were separated by SDS-PAGE. The results for representative isolates are illustrated in Fig. 5. As expected, the penicillin
affinities of PBP 1A, PBP 2B, and PBP 2X were reduced for the 24 resistant isolates and for Spanish strain 456. All resistant isolates displayed similar PBP patterns (Fig. 5).

**DISCUSSION**

We have shown in the present work that penicillin-resistant isolates of *S. pneumoniae* 23F are genetically closely related, suggesting a common clonal origin. The PFGE restriction patterns of some of the resistant 23F isolates from diverse regions of France were identical (Fig. 1), indicating that clones have spread throughout the country in the commensal nasopharyngeal flora of children. In contrast, penicillin-susceptible 23F isolates were genetically diverse: at least 14 distinct lineages were distinguished by PFGE (Fig. 1). None of these lineages gave restriction patterns resembling those of the penicillin-resistant 23F isolates or those of the penicillin-susceptible isolates of unrelated serotypes (Fig. 1). Resistant 23F isolates are therefore not closely related to susceptible 23F or non-23F isolates. Our results suggest that the emergence of penicillin-resistant 23F isolates in France is due to the rapid spread of a recently introduced 23F clone. Reichmann et al. (20), using multilocus enzyme electrophoresis, discriminated six lineages among resistant 23F isolates displaying intermediate or high-level penicillin resistance. One lineage corresponds to the multiply resistant Spanish 23F clone and to highly resistant isolates from France, Germany, and Hungary (20). This also agrees with several reports indicating that resistant 23F clones can spread to distant areas (18, 19). A resistant 23F clone has been shown to have spread from Spain to the United States (Ohio) (19), where it was rapidly disseminated (18). Similarly, a genetic relationship has been observed between resistant 23F clones from Spain and South Africa (12, 21). The dissemination of resistant clones of *S. pneumoniae* has also been described for the penicillin-resistant clones of the 6B serotype, which has been propagated from Spain to Iceland, where it is now prevalent (23). This clone is also related to resistant 6B clones isolated in Alaska and Texas (24). Interestingly, the PFGE restriction patterns of our resistant 23F isolates resemble that of an epidemic 23F strain isolated in Barcelona, Spain, in 1984 (9), suggesting that these clones might have a common origin.

The results obtained by PFGE were confirmed by studying the restriction patterns of three PBP genes (*pbp1A, pbp2B, and pbp2X*). These restriction patterns were identical in all resistant 23F clones, including the Spanish strain 456, confirming the close relatedness of these isolates. Multiple transformation events producing resistant clones are expected to generate genetic heterogeneity of the PBP genes (5, 9, 10, 16), but there was no evidence for this in our study. The susceptible 23F and non-23F isolates also displayed relatively similar PBP gene restriction patterns, consistent with previous reports (5, 11, 16, 25). However, a low level of genetic polymorphism was observed for *pbp2X* in these susceptible isolates. Such genetic polymorphism has been described for highly susceptible strains belonging to serotypes 6 and 19 (22). In addition, we found a highly susceptible 23F isolate exhibiting a *pbp1A* restriction pattern identical to that found in resistant 23F isolates, suggesting horizontal gene transfer in this strain without detectable alteration of the MIC of penicillin. The PBPs of resistant 23F isolates, including the Spanish strain 456, displayed low affinities for penicillin. The migration profiles of PBPs from resistant 23F isolates were similar, further eliminating the possibility that these resistant isolates had been generated independently of each other by multiple genetic events. In conclusion, our results demonstrate that the emergence of penicillin-
resistant 23F isolates accounts for the majority of penicillin-resistant S. pneumoniae in France and is the result of the clonal expansion of isolates rapidly spreading in the nasopharyngeal flora of children.

ACKNOWLEDGMENTS

We thank Patrice Courvalin for the gift of strain BM4200 and Edouard Bingen for helpful discussion and for the gift of strain R6 and the Spanish epidemic strain.

This work was supported by INSERM and the University René Descartes Paris V.

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