ANTIMICROBIAL RESISTANCE

Alterations to penicillin-binding proteins 1A, 2B and 2X amongst penicillin-resistant clinical isolates of *Streptococcus pneumoniae* serotype 23F from the nasopharyngeal flora of children

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Various amino acid substitutions were identified in the three major penicillin-binding proteins (PBP1A, PBP2B and PBP2X) of eight clinical isolates of *Streptococcus pneumoniae* serotype 23F collected from children. The particular changes related to the level of penicillin resistance. Alterations were detected in an isolate with a penicillin MIC as low as 0.06 mg/L. These results confirm that the level of penicillin resistance in pneumococci reflects with sequential alterations of PBPs in clinical isolates.

Introduction

The emergence and the rapid spread of penicillin-resistant *Streptococcus pneumoniae* presents a serious public health problem. The penicillin resistance is due to reduced affinity of one or more penicillin-binding proteins (PBPs). PBPs are the target enzymes of penicillin and catalyse the last steps of peptidoglycan biosynthesis. They are multifunctional enzymes containing a penicillin-binding domain homologous to that of β-lactamases, with three conserved motifs in a close spatial relationship. These sequences form the active centre and comprise the tetrad SXXX (which has the active site serine), the SXN box and the KTSIG triad [1]. PBPs form stable covalent complexes with β-lactam via the active serine of the SXXX box [2].

Amino acid sequence alterations cause conformational changes in PBPs and may alter the shape of the active site [3, 4]. In pneumococci, the high mol. wt PBPs 1A (79.7 kDa), 2B (82.3 kDa) and 2X (82.3 kDa) are those most involved in penicillin resistance [5, 6] and the three-dimensional structure of PBP2X has been determined recently, allowing a better understanding of its interactions with β-lactams [7].

Laboratory-selected mutants of pneumococci have only point mutations in their PBP genes, but resistance in clinical isolates is acquired via genetic transformation [8], yielding mosaic genes with regions diverging up to 25% from the homologous sequence of susceptible isolates, leading to substitution of c. 10% of the amino acids [1, 2, 9]. The sources of these DNA blocks are oral streptococci, including *S. oralis* and *S. mitis*, which harbour *php* genes homologous to those of *S. pneumoniae* [1, 9]. Most studies on the PBPs of wild-type clinical isolates have examined only one or two PBPs [4, 10–15]. The present work sequenced the three major *php* genes (*php1a*, *php2b* and *php2x*) in unrelated clinical pneumococci collected from the nasopharyngeal flora of young children in France, where the rate of penicillin-resistant pneumococci has increased considerably in recent years [16].

Materials and methods

Seven epidemiologically unrelated wild-type isolates of *S. pneumoniae* serotype 23F were collected between 1993 and 1998 from the nasopharyngeal flora of children aged <3 years in different parts of France. The first penicillin-resistant serotype 23F clinical strain (BM4200) to be isolated in France was also included; this organism dated from 1978 [17]. Minimal inhibitory concentrations (MICs) of penicillin G were determined on Mueller-Hinton Agar (bioMérieux, Marcy l’Etoile, France) containing lysed horse blood 5%. Incubation was at 37°C for 18 h in an atmosphere supplemented with CO2 5%. Serogroups were determined with specific antisera from the Statens Serum Institute (Copenhagen, Denmark). Pulsed-field gel electrophoresis of Smal-restricted chromosomal DNA was used to examine strain relatedness, as described previously for

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S. pneumoniae [18]. The penicillin-susceptible S. pneumoniae strain R6 was used as a control [19].

The nucleotide sequences of the penicillin-binding domains of the \( \text{pbp1a}, \text{pbp2b} \) and \( \text{pbp2x} \) genes were determined by PCR with the following primers:

1. \( \text{pbp1a}: \ 5'\text{GGTGCCCTTCCCTCAATCTCC3'} \) and \( 5'\text{TTTTCAGGCTTTGTAAACG3'} \);
2. \( \text{pbp2b}: \ 5'\text{GA TCTTCTAAATGATTTCAAGTGG3'} \) and \( 5'\text{GGAAT CCGGCCGACTCTC3'} \);
3. \( \text{pbp2x}: \ 5'\text{TCCAAAT CACAG ATTTCG3'} \) and \( 5'\text{TACAAATTTCCAGCCTGATG3'} \). PCR was performed in a Perkin-Elmer 9600 Thermocycler (Norwalk, CT, USA) with 40 cycles of 20 s at 94°C, 20 s at 55°C, 45 s at 72°C, and a final extension of 10 min at 72°C. The resulting amplicons were purified by filtration through an S-400 HR Microspin® purification column (Pharmacia Biotech, Uppsala, Sweden) and both strands were sequenced in an ABI-Prism 310 Sequencer (Perkin-Elmer, Foster City, Ca, USA), with a Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). Sequence alignments were performed with the multiple sequence alignment program Clustal W version 1.8 (Infobiogene). Sequences were compared with those of the susceptible reference strain R6.

Results and discussion

The study determined the sequences of the three major genes (\( \text{pbp1a}, \text{pbp2b} \) and \( \text{pbp2x} \)) involved in the penicillin resistance of \( S. \) pneumoniae in each of eight wild-type isolates of serotype 23F. All the isolates were collected from children. These included strain BM4200, which is the first example of a penicillin-resistant serotype 23F isolate from France. Although all were of serotype 23F, the eight isolates gave diverse PFGE profiles (Fig. 1) and varied in their level of penicillin resistance, with MICs from <0.0075 mg/L to 8 mg/L.

As previously reported for other strains [3, 12, 15], the nucleotide and peptide sequences of the genes and encoded PBPs were almost identical between the susceptible clinical strain 1261 (MIC <0.0075 mg/L) and R6 (~0.3% divergence) but – for the other clinical organisms – the proportion of peptide substitutions rose roughly in line with the level of penicillin resistance (Table 1). Similar substitution rates to those found here have been reported previously for penicillin-resistant strains, from serogroups 6 and 19 [4]. The divergence from the sequences for strain R6 was greatest in the highly resistant isolate 22861 (MIC 8 mg/L) in which all three PBPs were affected, with an overall substitution rate of 8% (Table 1). Nevertheless, a high number of substitutions was found in PBPs 2B and 2X of isolate 1513 (7.2% and 7.3%, respectively), which contrasts with the low MIC recorded (0.06 mg/L). Several of the sequence changes found in this latter isolate may be irrelevant to penicillin resistance.

The sequence of \( \text{PBP1A} \) was altered in an isolate with an MIC of 0.12 mg/L, a lower threshold value than that reported previously [15, 20]. Most of these alterations were adjacent to the motifs involved in the catalytic site, i.e., STMK, SRN, KTG, and included a T371A substitution inside the active site STMK motif (Fig. 2). This change is known to contribute to penicillin resistance [10, 15, 20]. The block NTGY (574–577) was found in the \( \text{PBP1A} \) sequence of all strains with penicillin MICs >0.5 mg/L, as described previously in strains from serogroups 6, 19 and 23 [15].

Most amino acid substitutions in \( \text{PBP2B} \) were located in the vicinity of the SVVK (192–195), and SSN (249–251) sites (Fig. 2). By analogy with the class \( \beta \)-lactamases of \( \text{Streptomyces albus} \), this region may form a pocket where the phenyl group of the 6’ acyl substituent of penicillin interacts with the PBP [3]. Two substitutions in \( \text{PBP2B} \) were associated with decreased affinity for penicillin: specifically all the isolates with MICs >0.06 mg/L had both substitutions T252A and

![Fig. 1. Pulsed-field electrophoresis of Smal-digested DNA of strains of \( S. \) pneumoniae belonging serogroup 23F. Lane 1, mol. wt marker (A); 2, strain R6; 3, strain 1261; 4, strain 1513; 5, strain 1465; 6, strain 1258; 7, strain 1053; 8, strain BM4200; 9, strain 1470; 10, strain 22861.](image-url)
Table 1. Nucleotide and peptide sequence substitutions in PBPs from eight clinical isolates of *S. pneumoniae* in relation to their levels of resistance to penicillin

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Origin</th>
<th>MIC (mg/L)</th>
<th>pbp1a length</th>
<th>pbp2b length</th>
<th>pbp2x length</th>
<th>Total length</th>
<th>GenBank accession nos</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6</td>
<td>France 1993</td>
<td>&lt;0.0075</td>
<td>11</td>
<td>4</td>
<td>7</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>1261</td>
<td>France 1993</td>
<td>&lt;0.0075</td>
<td>10</td>
<td>177</td>
<td>252</td>
<td>439</td>
<td>5</td>
</tr>
<tr>
<td>1513</td>
<td>France 1993</td>
<td>0.06</td>
<td>221</td>
<td>90</td>
<td>154</td>
<td>465</td>
<td>39</td>
</tr>
<tr>
<td>1465</td>
<td>France 1993</td>
<td>0.12</td>
<td>263</td>
<td>62</td>
<td>139</td>
<td>464</td>
<td>69</td>
</tr>
<tr>
<td>1258</td>
<td>France 1993</td>
<td>0.25</td>
<td>250</td>
<td>80</td>
<td>21</td>
<td>351</td>
<td>56</td>
</tr>
<tr>
<td>1053</td>
<td>France 1993</td>
<td>0.5</td>
<td>231</td>
<td>81</td>
<td>382</td>
<td>614</td>
<td>57</td>
</tr>
<tr>
<td>BM 4200</td>
<td>France 1978</td>
<td>0.5</td>
<td>230</td>
<td>155</td>
<td>294</td>
<td>679</td>
<td>48</td>
</tr>
<tr>
<td>1470</td>
<td>France 1993</td>
<td>2</td>
<td>203</td>
<td>10.6</td>
<td>13.1</td>
<td>11.6</td>
<td>48</td>
</tr>
</tbody>
</table>

E282G, as reported previously [4]. These changes are not found in susceptible *S. pneumoniae* strains nor in *S. mitis*, suggesting a role in the initial steps to penicillin resistance [4, 21]. Isolates 1258 and BM4200 had short mosaic sequences, AF5RPN or AF5VPN, adjacent to the SSRN motif of PBP2B, reflecting replacement of six contiguous residues (233–238). Therefore, these PBPs were products of class A *pbp2b* genes [11]. The other strains had multiple non-consecutive substitutions and presumably had class B *pbp2b* genes, as do resistant serogroup 23F strains from England and Spain [11].

A T338A mutation in the STMK motif of PBP2X was found in all the isolates with MICs >0.5 mg/L (Fig. 2). By means of site-directed mutagenesis, it has been demonstrated that this substitution plays an important role in determining the binding affinity between serine 337 and the β-lactam [13]. In strains 1513, 1465 and 1258, the substitution Q to E in position 552, next to the KSG conserved motif (547–549), is located in the β strand which spatially borders the active site S-337. This substitution too has been shown to play an additional role in determining the β-lactam-binding affinity [13]. The PBP2X sequences were almost identical in the most highly resistant isolates, 1470 (MIC 2 mg/L) and 22861 (MIC 8 mg/L), but additional unique mutations were also found in the latter isolate – these comprised M339F (within the STMK tetrad), A491G and Y595F.

Some isolates displayed particular patterns of PBP alterations. For example, in strain BM4200 (MIC 0.5 mg/L), the peptide sequences of the *pbp2b* and *pbp2x* products closely resembled those of isolate 1258, with an AF5VPN block for PBP2B, and of isolates 1470 and 22861 for PBP2X. Although not proven, this suggests that these strains have emerged via independent distinct genetic events. The high penicillin resistance of isolate 22861 (MIC 8 mg/L) is the consequence of the accumulation of mutations in the three PBPs, with notable changes around the C-terminus of PBP2B (Fig. 2).

In conclusion, these results show that the emergence of wild-type penicillin-resistant isolates of serotype 23F is the consequence of sequential alterations of the three major PBPs. These changes, including both point mutations and recombinations, reflect distinct, cumulative genetic events presumably selected by antibiotic pressure.
Fig. 2. Peptide alignments of PBP1A, PBP2B and PBP2X for nine strains of S. pneumoniae. A, Positions of active site components in the peptide sequence of each PBP: these residues converge in the folded protein. B, Peptide sequences of PBPs from eight different clinical strains of S. pneumoniae, compared with the susceptible R6 strain. Only sites where at least one amino acid differs from that of R6 are shown; the numbers of these sites are indicated.
The GenBank accession numbers of the sequence of pbp1a, pbp2b and pbp2x genes of the eight isolates of \textit{S. pneumoniae} were referred as AF210745–AF210768 (Table 1).

References