A Mutation in the d,D-Carboxypeptidase Penicillin-Binding Protein 3 of Streptococcus pneumoniae Contributes to Cefotaxime Resistance of the Laboratory Mutant C604

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Penicillin-binding proteins (PBPs) are the target enzymes for β-lactam antibiotics. They are minor membrane-associated proteins acting during the late steps of murein biosynthesis. PBPs are inhibited by β-lactams by enzymatically forming a covalent complex via an active-site serine. On the basis of their sizes and the homologies of their deduced amino acid sequences, they are grouped into high-molecular-mass (hmm) PBPs, some of which have been shown to be essential transpeptidases and transglycosylases, and low-molecular-mass (lmm) PBPs, which act as d,d-carboxypeptidases whose function may be dispensable for the cell (15, 16).

In Streptococcus pneumoniae, penicillin resistance involves the production of PBPs with a reduced affinity to β-lactams. In clinical isolates, low-affinity PBPs 2b, 2x, and 1a are encoded by mosaic genes that are the result of gene transfer and subsequent recombination events (14, 35, 37). These mosaic PBP genes contain multiple mutations compared to gene sequences of susceptible S. pneumoniae isolates, but most of the alterations in the mosaic genes are due to the different genetic origins of the DNA and are not necessarily relevant for resistance development.

The dissection of the development of β-lactam resistance into individual mutations was facilitated by the isolation of laboratory mutants (32). PBP 2x has been identified as a primary PBP target in the cefotaxime-resistant mutants, all of which contained mutations in PBP 2x that reduce the affinity for β-lactams and confer cefotaxime resistance when introduced into a sensitive strain (29, 33, 34), and PBP 2b has been identified as a primary PBP target for lytic β-lactams (17). Among the point mutations in PBPs selected in the laboratory, some were observed in clinical isolates as well and have thus helped to identify relevant alterations in the mosaic genes (10, 17, 29). It became clear, however, that single point mutations alone in PBP 2x or PBP 2b result only in a small susceptibility decrease and that the high-level β-lactam resistance of some of the laboratory mutants and of clinical isolates is the result of multiple mutations in multiple genes that represent highly engineered resistance determinants.

It has been assumed that the lmm PBP 3, the d,d-carboxypeptidase of this organism (22), plays no role in resistance development, mainly since in vivo inhibition of this PBP by a large number of β-lactams occurs far below the MIC of the respective antibiotic and no apparent effect on cellular growth has been observed under these conditions (57). Surprisingly, one cefotaxime-resistant laboratory mutant, C604, a derivative of the susceptible laboratory strain R6, expressed a low-affinity lmm PBP 3 (32). We describe here the isolation of the entire pbp3 gene and flanking regions and demonstrate that a PBP 3 mutation mediates decreased susceptibility to cefotaxime in this mutant.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. Penicillin-susceptible strain S. pneumoniae R6 is an uncapsulated derivative of Rockefeller University strain R36A (3). Cefotaxime-resistant mutant C604 is a derivative of the R6 strain and was isolated as a member of lineage 4 (last digit) after six successive selection steps (first digit) with increasing concentrations of the antibiotic (32). The S. pneumoniae clinical isolates of serotype 19A (Hu9) and 23F (456, F1 [47], and CS111 [38]) have been described (Table 1). For propagation of plasmid pID9 and its derivatives (8), Escherichia coli INVv0F (Invitrogen, Leek, The Netherlands) was used. S. pneumoniae was grown in C medium (31) supplemented with 0.2% yeast extract at 37°C without shaking. Transformants were selected in blood agar plates (3% sheep blood) containing the selective antibiotic (1 μg of erythromycin or cefotaxime per ml, as specified in Results and Discussion). MICs were determined on blood agar plates with narrow antibiotic concentrations; in addition, the E-test was used for cefotaxime MICs. E. coli was grown aerobically in Luria-Bertani medium.

Transformation. Transformation of S. pneumoniae was carried out essentially as described by Tiraby and Fox (55) but with a phenotypic expression period of 120 min in liquid medium. Cefotaxime-resistant, competence-defective mutant C504 could be used as recipient in transformation experiments in the presence of 10 ng of competence signalling peptide CSP per ml (25). Details of the isolation of cefotaxime-resistant transformants are given in the results section. The transformation of E. coli INVv0F with recombinant plasmid DNA was performed according to published procedures (9) or as described by Invitrogen.

Isolation of DNA. Details of the isolation of chromosomal DNA (34) and the preparation of phage and plasmid DNA have been described previously (28). PCR. PCRs were carried out in a Biometra thermocycler for 30 cycles consisting of denaturation for 30 s at 96°C, annealing for 1 min at 52°C, and extension for 1 min at 72°C, followed by a 5-min extension period at 72°C. A 100-μl reaction mixture contains 50 mM of each oligonucleotide primer, 200 μM deoxynucleoside triphosphates, 0.5 to 8 mM MgCl₂ (depending on the primers), 2.5 U of Taq polymerase (Perkin-Elmer, Norwalk, Conn.), and buffer as recommended by the manufacturer. Inverse PCRs (42, 56) were performed with the primers PCR1up (5’-GTATCCCTCAAGTTCTAGTGTA) and PCR1down (5’-TCACACTGATTCCCAAGAATCCC) and PCR2up (5’-CCTTAACACGAGTAACTCACATA) and PCR2down (5’-TCACACTGATTCCCAAGAATCCC). Purified DNA fragments were cloned into the PCRII vector (Invitrogen).

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TABLE 1. Susceptibilities to cefotaxime and oxacillin of \( \beta \)-lactam-resistant laboratory mutants and clinical isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (( \mu )g/ml)</th>
<th>Protein carrying mutation</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6</td>
<td>0.02-0.04</td>
<td>CiaH</td>
<td>3</td>
</tr>
<tr>
<td>C104</td>
<td>0.04-0.08</td>
<td>PBP 2x</td>
<td>33</td>
</tr>
<tr>
<td>C204</td>
<td>0.08-0.12</td>
<td>Unknown</td>
<td>33</td>
</tr>
<tr>
<td>C304</td>
<td>0.12</td>
<td>Unknown</td>
<td>33</td>
</tr>
<tr>
<td>C404</td>
<td>0.25</td>
<td>PBP 2x</td>
<td>33</td>
</tr>
<tr>
<td>C504.5</td>
<td>0.65-0.75</td>
<td>PBP 2x, 2b</td>
<td>33</td>
</tr>
<tr>
<td>C504</td>
<td>1–1.25</td>
<td>nd(^{*}) PBP 3</td>
<td>This study</td>
</tr>
<tr>
<td>C604</td>
<td>1.5–2</td>
<td>Unknown (PBP 2a)(^{\dagger})</td>
<td>32</td>
</tr>
<tr>
<td>F1</td>
<td>0.05</td>
<td>PBP 2x, 2b</td>
<td>47</td>
</tr>
<tr>
<td>456</td>
<td>2</td>
<td>PBP 1a, 2x, 2b</td>
<td>14, 35, 37</td>
</tr>
<tr>
<td>Hu9</td>
<td>1–1.5</td>
<td>PBP 1a, 2b</td>
<td>46</td>
</tr>
<tr>
<td>C5111</td>
<td>9</td>
<td>PBP 1a, 2x</td>
<td>10</td>
</tr>
</tbody>
</table>

\(^{*}\) Mutants C104 to C604 are first- to sixth-step mutants selected with increasing cefotaxime concentrations from parental strain R6 (32). Mutations in the mutants have been determined genetically (see references).

\(^{\dagger}\) MICs were re determined in our laboratory.

\(^{\dagger}\) PBP alterations of the clinical isolates were detected immunologically and/or by DNA sequencing.

\(\dagger\) Cefotaxime MICs were determined in at least two independent experiments.

\(\dagger\) nd, not determined.

\(\dagger\) PBP 2a not detectable on fluorograms.

DNA sequencing. DNA sequencing of cloned DNA fragments was performed by the dideoxy-nucleotide chain termination method (30) with the T7 sequencing kit (Pharmacia, Freiburg, Germany). For direct sequencing of PCR-amplified DNA, the Sequenase PCR product sequencing kit (Amersham, Braunschweig, Germany) was used. Nucleotides 1 to 500 of the sequence have been sequenced on one strand only and may contain errors.

**RESULTS AND DISCUSSION**

The \( \beta \)-lactam-resistant mutant C604. Laboratory mutant C604 is the sixth-step mutant of the lineage C004, the cefotaxime MIC for which is 1.5 \( \mu \)g/ml. Table 1 shows the increase in cefotaxime MICs that occurs at each of the selection steps mediated by the individual mutations at 37°C; similar results were obtained at 30°C or with the E-test (data not shown). A decrease in oxacillin susceptibility was also observed parallel to the \( \beta \)-lactam-resistant strain R6 had been isolated (51). The only two long open reading frames found on the 3,370-bp DNA fragment, \( \text{pbp3} \) and ORFU, were transcribed in opposite directions. Putative ribosome binding sites and extended 10 boxes known to direct transcription in the absence of 35 boxes (48) are indicated in Fig. 2. The two ATG start codons were separated by 103 bp. Both genes terminated with a TAA stop codon, which in the case of the \( \text{pbp3} \) gene was followed by a short inverted repeat. Downstream of \( \text{pbp3} \) and ORFU, palindromic sequence elements were found that have been described for a variety of intergenic regions of the pneumococcal chromosome. The sequence downstream of \( \text{pbp3} \) represents a so-called BOX element that consists of three sequence boxes, a, b, and c (36). BOX elements (with box b occurring with a variable number of copies) have been found in the vicinity of genes whose products were initially suspected of playing a role in the virulence and genetic competence of the pneumococcus (36) and have recently been associated with the phenomenon of phase variation in colony opacity (49). The other sequence located downstream of ORFU has also been found in intergenic regions close to genes encoding either a hyaluronidase, a neuraminidase, or enzymes involved in capsule biosynthesis (6, 7, 39). Similar to that of the BOX element, this sequence contains several inverted repeats that allow folding into prominent stem-loop structures according to computer predictions; therefore, it was named the STEM element. Thus, the common

FIG. 1. Restriction map of the \( \text{pbp3} \) gene of \( S. \) pneumoniae and flanking regions. The orientations of \( \text{pbp3} \) and ORFU are indicated by the arrows, and the positions of the DNA elements STEM and BOX ABC are shown as grey boxes. DNA fragments obtained in two inverse PCRs (inv PCR1 and inv PCR2) are shown as solid bars; the arrowheads indicate the oligonucleotide primers used in the reactions. Open bars indicate the sizes of DNA fragments cloned in pIDC9 for insertion duplication of ORFU and \( \text{pbp3} \). Restriction sites: E, EcoRI; H, HindIII; M, MfeI; S, Sau3AI.
denominator in all known cases of BOX and STEM elements is the proximity to genes encoding secreted or surface-associated proteins, including PBP 3 and the ORFU gene product.

The \textit{pbp3} gene product. PBP 3 encodes a putative 413-amino-acid (aa) protein that has all the characteristics of lmm PBPs acting as D,D-carboxypeptidases. Its molecular mass of 43.2 kDa is in good agreement with the 43 kDa calculated from the mobility in SDS-polyacrylamide gels (22). The deduced pI of 4.6 is close to the value of 4.35 estimated from isoelectric focusing gels (19). The three highly conserved motifs present in all penicillin-interacting enzymes are placed at positions with spacings similar to those of other lmm PBPs (16) and were designated S56ITK (with the active-site serine), S119AN, and K239TG. PBP 3 reveals a high degree of homology to lmm PBPs and D,D-carboxypeptidases throughout the sequenced region. The \textit{S. pneumoniae} PBP 3 showed the greatest similarity to one of the three lmm PBPs of \textit{Bacillus subtilis}, PBP 5, with 37% identical amino acids throughout the entire protein and 43% identical amino acids for the penicillin-binding domain (aa 1 to 299).

In contrast to hmm PBPs, lmm PBPs are processed and attached to the membrane via a C-terminal amphipathic helix as has been shown for \textit{E. coli} PBPs 5 and 6 (43, 44). In \textit{S. pneumoniae} PBP 3, the first 19 aa were predicted to represent a hydrophobic signal peptide and a cleavage site was positioned at Ala-20. An amphiphilic \alpha-helix could be predicted for the last C-terminal 30 aa.

The ORFU product. ORFU encoded a 369-aa protein. According to the hydrophobicity plot and the structural prediction, the protein is an integral membrane protein with 37% identical amino acids throughout the entire protein and 43% identical amino acids for the penicillin-binding domain (aa 1 to 299).
small hydrophobic amino acids and a pI of 10.3. It contained no known sequence motifs, and a computer-based homology search revealed several membrane proteins of unknown function without significant identity. It is remarkable that a similar gene arrangement—a lmm PBP gene transcribed in an orientation opposite to that of a transmembrane protein, which in both cases is an ATP binding cassette (ABC) transporter—is found in *Staphylococcus aureus* (13, 26) and in *Streptococcus pyogenes* (45).

A point mutation in p*pbp3* of C604. In order to identify the putative mutation in the *pbp3* of C604, the region between nucleotides nt 1732 and 3369 was amplified by PCR, cloned into the PCRII vector, and sequenced. One point mutation in the structural gene *pbp3* was detected, C to T at position 2648, resulting in a Thr-242-to-Ile substitution directly adjacent to the structural gene and the *pbp3* gene product, per ml (25). Transformants with an alteration in the *pbp3* gene and ORFU were obtained with low frequency at 1 μg of cefotaxime per ml. Four transformants that were further analyzed were indeed identical to the C604 mutant in terms of cefotaxime MIC and the presence of a low-affinity PBP 2a.

Attempts to introduce the *pbp3* mutation into the R6 strain via transformation and cefotaxime selection failed, suggesting that the MIC alteration mediated in this genetic background is very minor.

**Deletion analysis of p*pbp3* and ORFU.** Deletion of the C-terminal 51 aa of PBP 3 causes dramatic effects on cellular morphology and division septum formation (51). In these constructs, the penicillin-binding domain of PBP 3 was still intact and functional, and although the protein was secreted into the medium the experiments did not unambiguously document that PBP 3 is dispensable for the cell. In order to clarify this point, deletion derivatives of PBP 3 were constructed in which only the first 150 aa were expressed; thus, the derivatives terminated within the penicillin-binding domain.

Such derivatives were obtained by insertion-duplication mutagenesis with a *pbp3* gene fragment cloned into pJDC9 (8). One such transformant was analyzed in more detail; it contained no detectable PBP 3 (Fig. 4). It had a longer generation time than the parental strain, 65 to 70 min versus 35 to 40 min. Electron microscopy of cells from an exponentially growing culture revealed the same morphological distortions as those described previously (51): the cells grew in large lumps; the cell wall appeared thickened and irregular; shedding of cell wall material occurred; and septa were frequently completely misplaced, resulting in nonseparated cells (data not shown).

In *S. aureus*, disruption in the ABC transporter gene, *abcA*, has been shown to have an effect on methicillin and cefoxitin resistance, and a potential regulatory link between the PBP 4 gene and *abcA* was discussed (13). Therefore, a similar mutant with an alteration in the *S. pneumoniae* ORFU gene was con-
structed. An internal gene fragment of ORFU (nt 1139 to 1787) was cloned in pJDC9, and by using the same strategy outlined above, erythromycin-resistant transformants were isolated that contained an insertionally inactivated ORFU. Inactivation of ORFU had no apparent effect on cellular growth or morphology. The ORFU mutants contained the same amount of PBP 3 as the R6 strain, and the MIC of cefotaxime was also the same. This indicates that the ORFU product (or at least its last 136 aa) is not essential under laboratory conditions and that it has no detectable effect on PBP 3 (data not shown); these findings are similar to those in a subsequent report on S. aureus, in which no direct relationship between abcA and pbp4 regulation was found (27).

**PBP 3 in clinical isolates of S. pneumoniae.** Clinical isolates resistant to high levels of penicillin (penicillin MICs of 2 to 4 μg/ml) are generally cross-resistant to cefotaxime, with MICs ranging from around 0.5 to 1 μg/ml. In order to see whether PBP 3 is possibly involved in the β-lactam resistance of clinical isolates or is involved in gene transfer events as are the hmn PBPs of such strains, pbp3 was analyzed in three genetically distinct penicillin-resistant isolates with different cefotaxime MICs (Table 1). In order to increase the probability of detecting sequence alterations, representatives of distinct clones as determined by MLEE analysis were selected (47), including the multiply resistant serotype 23F clone first described in Spain (456) (40, 54), the Hungarian clone 19A clone (Hu9) (41), and a serotype 23F clone from France (F1) (47). Some clones of clinical isolates contain a variant form of PBP 3 with altered electrophoretic mobility (21, 24, 41, 54). Isolates F1 and Hu9 contained a PBP 3 of unusual electrophoretic mobility as shown in Fig. 4. For comparison, a member of the clonal group from the United States resistant to high levels of cefotaxime (CS111) with an unusually high cefotaxime MIC for which PBP 3 did not contribute to resistance was included (10). All isolates contained at least a PBP 2x with reduced penicillin affinity, and sequence analysis confirmed the mosaic nature of the genes (10, 21a, 35, 46).

The DNA sequence was determined between nt 1732 and 1787. Figure 5 summarizes the results. None of the pbp3 genes contained the mutation of the C604 mutant or had a mosaic structure. Thus, no indication was obtained that PBP 3 plays a role in resistance development of the clinical isolates, in agreement with the fact that none of the isolates contained a PBP 3 with low penicillin affinity (Fig. 4). Also, the transfer of penicillin resistance from clinical isolates with unusual PBP 3 into susceptible strain R6 was not accompanied by the acquisition of an altered PBP 3 (53, 59).

Differences from the R6 sequence that resulted in amino acid substitutions were found only in the C-terminal part of PBP 3. The greatest divergence was found between strains R6 and F1, with 8 bp changes and 4 aa alterations; this divergence is in the same range (<1%) as those documented for the pbp2b, pbp2x, and pbp1a genes of sensitive S. pneumoniae strains (14, 35, 37). The greatest similarity was found between the two isolates F1 and Hu9, which differed in only one nucleotide. Both isolates contained the unusual PBPs 3, and the only amino acid changes common to these two isolates were not present in the others and which therefore must include the changes responsible for the lower electrophoretic mobilities are the substitutions of two adjacent amino acids, Arg-333-Val to Gln-333-Ile, as a result of three consecutive nucleotide changes. The size of the peptide between the active-site serine and these mutations (Ser-56 to Gln-333) is predicted to be 30.2 kDa, which is in agreement with the estimate of at least 25 kDa determined by partial proteolysis of [3H]penicillin-labeled peptides of the two PBP 3 variants (21). It is remarkable that the pbp3 gene of F1 isolated in western Europe and that of Hu9 from eastern Europe differed by only one nucleotide. The two clones cluster in two distinct lineages, clearly separated from the majority of isolates included in the studies, and the sequence similarity of their pbp3 genes may indicate that gene transfer between these two clones has occurred (47).

In addition to alterations within the structural pbp3 gene, three of the isolates also contained a 32-bp insert within the downstream BOX element. This insert, which results in an extra loop in a potential stem-loop structure, has not yet been described in published BOX sequences. The insert is flanked by two 8-bp direct repeats (Fig. 5), an indication of site-specific recombination, which implies a potential role for BOX elements for the first time. Thus, although the pbp3 gene did not have a mosaic structure, it appears to be a target for recombination processes distinct from that assumed to take place during DNA transformation.

**Concluding remarks.** The present report is the first documentation that a point mutation in an lmm D,D-carboxypeptidase, the PBP 3 of S. pneumoniae, results in reduced affinity to penicillin and thereby contributes to β-lactam resistance. In S. aureus, overproduction of PBP 4 has been associated with a penicillin-resistant laboratory mutant (26). Both overproduction of a PBP and a lower affinity for penicillin of a PBP should have the same effect, i.e., should increase the availability of active PBP in the presence of the antibiotic compared to the wild-type situation. In agreement with this, the opposite phenotype, i.e., a reduced amount of S. pneumoniae PBP 3, has
been correlated with a reduction in β-lactam MIC (52), and hypersensitivity to β-lactams in response to the deletion of a lmm PBP has also been noted in several cases (11, 51, 58).

The location of the PBP 3 mutation in C604, directly adjacent to the homology box K(H)S(T)G, which is common to all PBPs, corresponds to the positions of mutations in hmm S. pneumoniae PBP 2x and PBP 2b in β-lactam-resistant mutants (23, 33). The importance of this region in PBPs in β-lactamases, especially the Thr residue following the KS/TG triad, for the binding of cefotaxime in particular has recently been explained at the atomic level (30), and our finding extends the general importance of this region to lmm PBPs. Although one of the amino acid alterations detected in the clinical isolates investigated here—Ser-291 to Pro in strain 456—was also located at the end of the penicillin-binding domain, a region where point mutations in PBP 2x and PBP 2b of S. pneumoniae 

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Penicillin resistance in clinical isolates of S. pneumoniae appears to be based on alterations in hmm PBPs only, including at least the hmm PBPs 1a, 2x, and 2b (4, 20), and clinical isolates of S. aureus have also evolved a resistance mechanism that is based on alterations of hmm PBPs rather than of lmm PBP 4 (2, 5). So far, no evidence has been obtained for either one of these organisms that changes in lmm PBP contribute to resistance in clinical isolates. The acquisition of accessible highly engineered hmm PBPs that can be transferred even between species outside the laboratory is probably more efficient than evolving resistance by the accumulation of point mutations within the pneumococcal itself. Still, the occurrence of point mutations unique for single isolates such as the Thr-550-to-Ala mutation in the PBP 2x of isolate CS111 (10) shows that such mutations can occur under appropriate conditions, e.g., cefotaxime treatment. The PBP 3 mutation has been selected in a mutant that already contained the PBP 2x mutation Ala-550. An increase in cefotaxime MIC of 0.25 to 0.5 μg/ml, as mediated by the PBP 3 mutation, still contributed to a selectable increase in MIC in that particular genetic background, and this increase may even be higher in a strain with a modified PBP 1a.

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