ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1997, p. 936–942 Vol. 41, No. 5
0066-4804/97/$04.00+0

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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Received 7 August 1996/Returned for modification 27 November 1996/Accepted 18 February 1997

Cefotaxime resistance in laboratory mutant C604 of *Streptococcus pneumoniae*, for which the MIC is 1.5 μg/ml, is independent of alterations in high-molecular-mass penicillin-binding protein (PBP) 1a. Instead, a point mutation in PBP 3, the D,D-carboxypeptidase of this organism, caused a reduced affinity for penicillin and contributed to the decreased susceptibility. The mutation Thr-242 to Ile was located directly adjacent to the triad Lys-239-Thr-Gly, a position known to be important for β-lactam interaction with high-molecular-mass PBPs and β-lactamases. This mutation was absent in the PBP 3's of four genetically distinct clinical isolates resistant to high levels of penicillin. None of the *pbp3* genes had a mosaic structure, but in three cases there was evidence for a site-specific recombination event within a BOX element immediately downstream of *pbp3*.

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 unencapsulated 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 pJDC9 and its derivatives (8), *Escherichia coli* INVαF′ (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. The 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* INVαF′ 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 Biomed 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 contained 50 pmol of each oligonucleotide primer, 200 μM deoxynucleoside triphosphates, 0.5 to 8 mM $MgCl_2$ (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′-GTTATCCCCTAGAGTTTCATTGTTA) and PCR1down (5′-TCACACCT GATTCCAAAGCAATCCC) and PCR2up (5′-GTCTTAAACAGCAGTTAAC TATTC) and PCR2down (5′-TCACACCTGATTCCAAAGACCTCCC). Purified DNA fragments were cloned into the PCRII vector (Invitrogen).

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TABLE 1. Susceptibilities to cefotaxime and oxacillin of β-lactam-resistant laboratory mutants and clinical isolates

Strain[a]	MIC[b] (μg/ml) of:		Protein carrying mutation[c]	Source or reference
	Cefotaxime[d]	Oxacillin		
R6	0.02–0.04	0.04	None (wild type)	3
C104	0.04–0.08	0.07	CiaH	14, 29
C204	0.08–0.12	0.1	PBP 2x	33
C304	0.12	0.1	Unknown	
C404	0.25	0.15	PBP 2x	33
C504	0.65–0.75	0.04	PBP 2x	33
$C504_{PBP3\text{-}C604}$	1–1.25	nd[e]	PBP 3	This study
C604	1.5–2	0.04	Unknown (PBP 2a)[f]	32
F1	0.05	2	PBP 2x, 2b	47
456	2	22	PBP 1a, 2x, 2b	14, 35, 37
Hu9	1–1.5	0.5	PBP 1a, 2x, 2b	46
CS111	9	1.5	PBP 1a, 2x	10

[a] 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).
[b] MICs were redetermined in our laboratory.
[c] PBP alterations of the clinical isolates were detected immunologically and/or by DNA sequencing.
[d] Cefotaxime MICs were determined in at least two independent experiments.
[e] nd, not determined.
[f] PBP 2a not detectable on fluorograms.

DNA sequencing. DNA sequencing of cloned DNA fragments was performed by the dideoxy-nucleotide chain termination method (50) 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.

PBP analysis. PBPs were routinely labeled in cell lysates (corresponding to approximately 5×10^7 cells per sample in 20 mM phosphate buffer [pH 7.2]–0.1% Triton X-100) in a 15-μl volume with [^{3}H]propionylampicillin during an incubation period of 20 min at 37°C (32). After sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, PBP profiles were analyzed by fluorography.

Computer-based sequence analysis. All sequence analysis programs are part of the Genetics Computer Group sequence analysis software package, version 8.0, of the University of Wisconsin Department of Genetics, including the programs Helicalwheel, Signalpeptide, Mfold, Pileup, GrowTree, and Distances (12). Sequence similarities were detected with the BLAST program (1).

Nucleotide sequence accession number. The sequence obtained in this study has been assigned EMBL accession number X99400.

RESULTS AND DISCUSSION

The cefotaxime-resistant mutant C604. Laboratory mutant C604 is the sixth-step mutant of the lineage C004, the cefotaxime MIC for which is 1.5 μg/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 decrease in cefotaxime susceptibility in mutants C104 to C404; in fifth-step mutant C504, the oxacillin MIC dropped again to the level of the parental R6 strain due to a special mutation in PBP 2x, Thr-550 to Ala (10, 29).

In contrast to what is found for clinical isolates, PBP 1a appeared not to be involved in cefotaxime resistance in any of the laboratory mutants isolated, although cefotaxime MICs of ≥1 μg/ml for some of the mutants are in the same range as that observed for clinical isolates resistant to high levels of penicillin. For the mutant lineage C004, the increase in cefotaxime MIC was due to the successive introduction of (i) a mutation in the histidine protein kinase CiaH in first-step mutant C104, (ii) mutations in PBP 2x at steps two, four, and five, and (iii) mutations in unknown genes in steps three and six (18, 33) (Table 1). C604 contains two alterations compared to C506: a low-affinity PBP 3 in addition to an apparent loss of penicillin affinity in PBP 2a (32), indicating that two separate mutations have incidentally occurred in this particular mutant. In order to investigate the role of PBP 3, the *pbp3* gene of C604 was isolated and compared to that of the parental R6 sequence.

Isolation and sequence analysis of the *pbp3* gene and flanking regions. Before this study, only an internal 630-bp *pbp3* (previously called *dacA*) gene fragment of penicillin-susceptible laboratory strain *S. pneumoniae* R6 had been isolated (51). In order to obtain flanking DNA regions, an inverse PCR with primers PCR1up and PCR1down and a partial *Sau*3AI digest of chromosomal R6 DNA was performed. Two fragments covering the 5′ and 3′ ends of the *pbp3* gene including the flanking regions were obtained (Fig. 1). In the upstream region, the 3′ end of a putative second open reading frame, designated ORFU, was found. The DNA sequence of the 1.8 kb upstream of *pbp3* that included the entire ORFU could be obtained after a second inverse PCR (PCR2up and PCR2down) with a complete *Mae*II digest of chromosomal DNA. Figure 1 illustrates the DNA fragments obtained by the inverse PCRs and shows a map of the entire 3,370-bp sequenced region.

The only two long open reading frames found on the 3,370-bp DNA fragment, *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 *pbp3* gene was followed by a short inverted repeat. Downstream of *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 *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

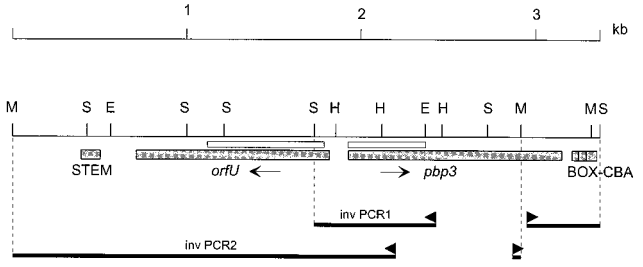


FIG. 1. Restriction map of the PBP 3 gene of *S. pneumoniae* and flanking regions. The orientations of *pbp3* and ORFU are indicated by the arrows, and the positions of the DNA elements STEM and BOX ABC are shown as grey boxes. DNA fragments obtained in two inverse PCRs (inv PCR1 and inv PCR2) are shown as solid bars; the arrowheads indicate the oligonucleotide primers used in the reactions. Open bars indicate the sizes of DNA fragments cloned in pJDC9 for insertion duplication of ORFU and *pbp3*. Restriction sites: E, *Eco*RI; H, *Hin*dIII; M, *Mae*II; S, *Sau*3AI.

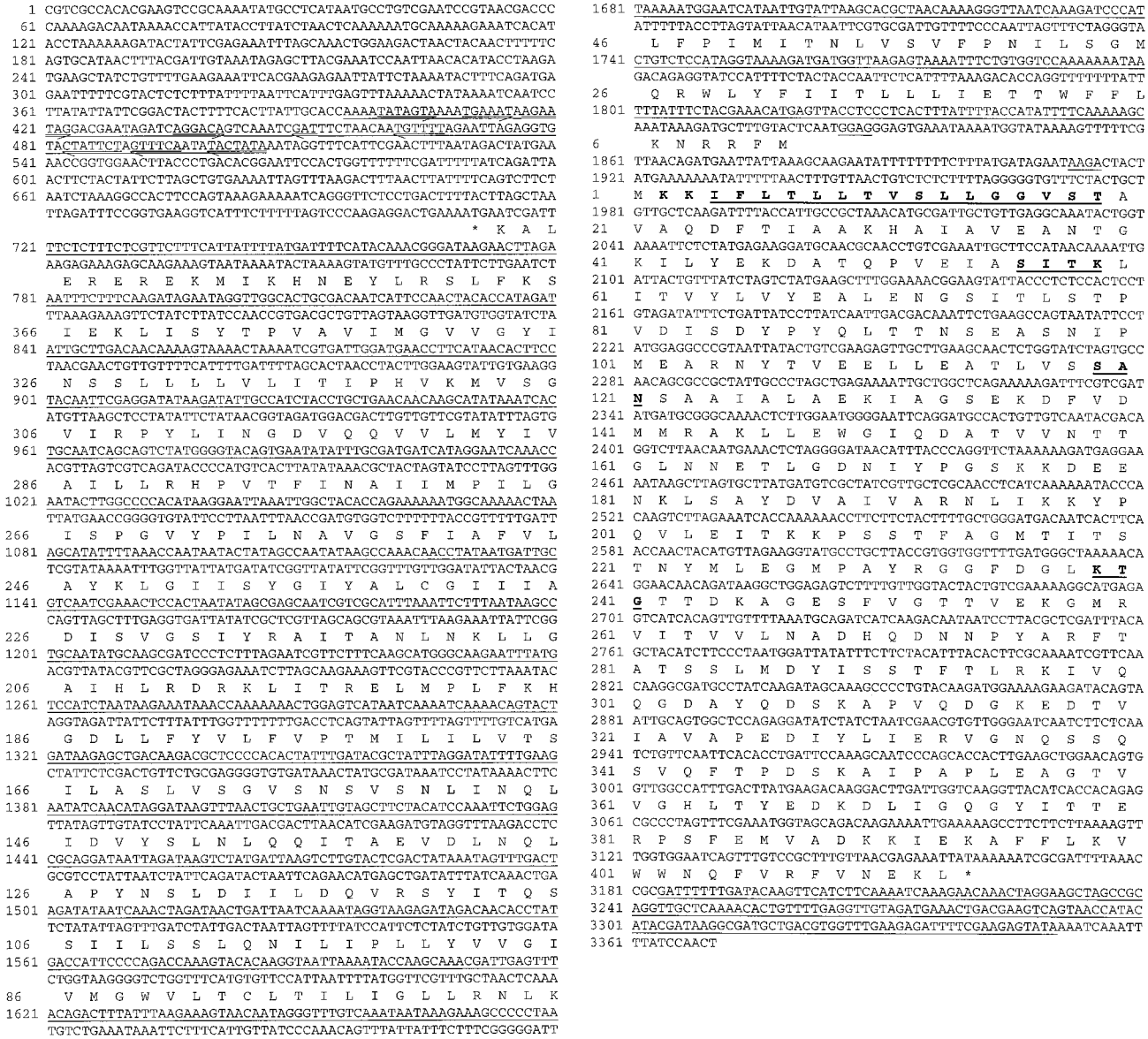


FIG. 2. Nucleotide sequence of a 3,370-bp DNA fragment encoding PBP 3 and a putative membrane protein of unknown function. Potential ribosome binding sites are underlined. The STEM element and the BOX element are also underlined. Inverted repeats that contribute to a stem-loop structure in the STEM element are marked by arrows. The proposed signal peptide and the three amino acid homology boxes are in boldface and underlined.

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 *pbp3* gene product. *pbp3* 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 isoelectrofocusing 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 *S. pneumoniae* PBP 3 showed the greatest similarity to one of the three lmm PBPs of *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 *E. coli* PBPs 5 and 6 (43, 44). In *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 α-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 43%

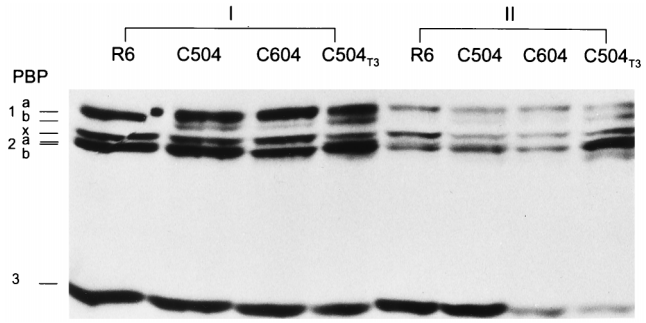


FIG. 3. The low-affinity PBP 3 in the cefotaxime-resistant mutant C604. Radioactivity used: I, 1 μCi; II, 0.05 μCi. After SDS-polyacrylamide gel electrophoresis, PBP profiles were analyzed by fluorography. Fluorograms of PBPs from cefotaxime-resistant mutant C504, C604, and a transformant of C504 with the *pbp3* of C604 ($C504_{T3}$) are shown, and the parental R6 strain is included for comparison. The PBPs are indicated on the left.

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 *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 K239TG box. The mutation was verified by direct sequencing of PCR-amplified C604 chromosomal DNA; mutant C504 did not contain this mutation. Mutations at homologous positions have been identified in the hmm PBPs of *S. pneumoniae* mutants: in PBP 2x, the Ala-550 mutation mentioned above and even more a Gly-550 mutation represent the most potent single-amino-acid changes, mediating increases in cefotaxime MICs of from 0.02 to 0.2 and 0.3 μg/ml, respectively (10, 17). In PBP 2b, the mutations Lys-Ser-Gly-617 to Ala (23) caused only a small, but nevertheless selectable, piperacillin MIC increase of from 0.04 to 0.05 μg/ml (17).

Cefotaxime resistance determinants in C604. In order to verify that the PBP 3 mutation in C604 indeed results in reduced penicillin affinity and thereby mediates reduced cefotaxime susceptibility, mutant C504 was transformed with the cloned *pbp3* of C604. Mutant C504 is not transformable, but a small number of competent cells could be obtained upon the addition of 10 ng of purified competence signalling peptide CSP, the *comC* gene product, per ml (25). Transformants could be selected at 0.72 μg of cefotaxime per ml, a concentration which was sufficiently above the MIC of C504 (in this particular experiment 0.65 μg of cefotaxime per ml) to allow for selection, but not at higher concentrations. Under these conditions, approximately 800 colonies were obtained without DNA being added and approximately 1,600 colonies were obtained with $pbp3_{C604}$ DNA being added. The cefotaxime MICs for four transformants were >0.6 μg/ml, and the C-to-T mutation at position 2648 was verified in two of them by direct sequencing of PCR-amplified chromosomal DNA.

Labeling of PBPs in cell lysates with radioactive penicillin demonstrated that the PBP 3 of the transformants had a reduced affinity to [^{3}H]propionylampicillin, similar to PBP 3 in C604, whereas PBP 2a could still be perfectly labeled, unlike the PBP 2a in C604 (Fig. 3). This shows that the mutated *pbp3* can transfer a selectable increase in cefotaxime MIC but that still another marker affecting PBP 2a contributed to the resistance level of C604.

In order to demonstrate the existence of the second resistance marker in C604, one transformant of C504 obtained with $pbp3_{C604}$ was used as an acceptor strain for chromosomal C604 donor DNA. Transformants 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 *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-

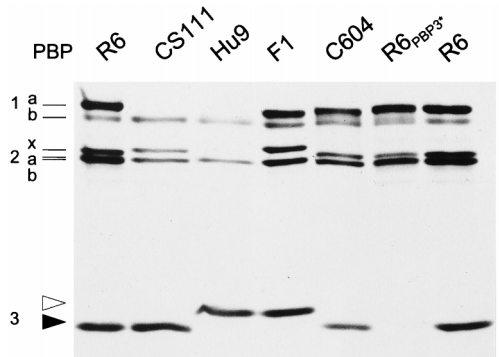
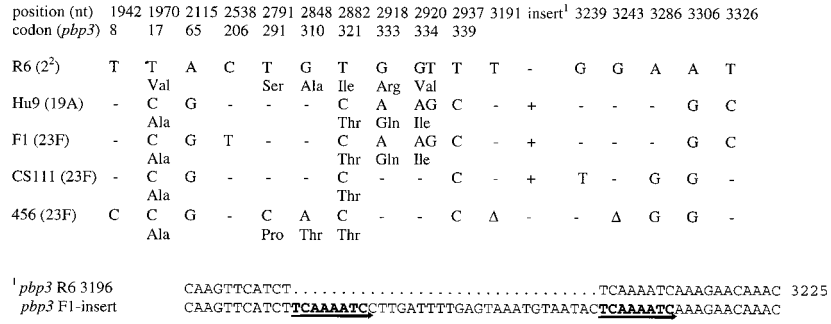


FIG. 4. PBP 3 in clinical isolates and laboratory mutants of *S. pneumoniae*. A fluorogram was prepared of [^{3}H]propionylampicillin-labeled cell lysates from strains CS111, F1, and Hu9. $R6_{PBP3*}$ is an R6 derivative with an insertionally inactivated PBP 3. Fluorograms for the mutant C604 and the R6 strains are also included. The PBPs are indicated on the left; the grey arrow indicates a PBP 3 with slower electrophoretic mobility.



position (nt)	1942	1970	2115	2538	2791	2848	2882	2918	2920	2937	3191	insert[1]	3239	3243	3286	3306	3326
codon (*pbp3*)	8	17	65	206	291	310	321	333	334	339							
R6 (2^2)	T	T Val	A	C	T Ser	G Ala	T Ile	G Arg	GT Val	T	T	-	G	G	A	A	T
Hu9 (19A)	-	C Ala	G	-	-	-	C Thr	A Gln	AG Ile	C	-	+	-	-	-	G	C
F1 (23F)	-	C Ala	G	T	-	-	C Thr	A Gln	AG Ile	C	-	+	-	-	-	G	C
CS111 (23F)	-	C Ala	G	-	-	-	C Thr	-	-	C	-	+	T	-	G	G	-
456 (23F)	C	C Ala	G	-	C Pro	A Thr	C Thr	-	-	C	Δ	-	-	Δ	G	G	-

[1] *pbp3* R6 3196 CAAGTTCATCT.....TCAAAATCAAAGAACAAAC 3225
pbp3 F1-insert CAAGTTCATCT**TCAAAATC**CTTGATTTTGAGTAAATGTAATAC**TCAAAATC**AAAGAACAAAC

FIG. 5. Nucleotide alterations and amino acid changes in *pbp3* genes of clinical isolates. Only those nucleotides and amino acids are shown that differ from the R6 consensus sequence, which is numbered according to Fig. 2. The superscript 2 indicates the capsular type of the progenitor strain (R6). △, deletion of the nucleotide. The presence of the insert in Hu9, F1, and CS111 (*pbp3* F1-insert) is indicated by the plus signs. Strains Hu9 from Hungary and F1 from France contain PBP 3's with lower electrophoretic mobilities. The two 8-bp direct repeats are in boldface and are underlined by arrows.

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 below those of laboratory mutants, 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 hmm 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 serotype 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 3369. 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 3s 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 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 PBP 3, and the only amino acid changes common to these two isolates which 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 [^{3}H]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* that reduce the affinity for β-lactams were described (23, 33), the lack of an apparent low-affinity PBP 3 in this and the other isolates investigated suggests that none of the PBP 3 mutations is relevant for the resistance phenotype.

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 pneumococcus 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.

ACKNOWLEDGMENT

We thank Don Morrison for a generous gift of purified CSP.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Archer, G. L., and D. M. Niemeyer.** 1994. Origin and evolution of DNA associated with resistance to methicillin in staphylococci. Trends Microbiol. **2:**343–347.
3. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1994. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. J. Exp. Med. **79:**137–158.
4. **Barcus, V. A., K. Ghanekar, M. Yeo, T. J. Coffey, and C. G. Dowson.** 1995. Genetics of high level penicillin resistance in clinical isolates of *Streptococcus pneumoniae*. FEMS Microbiol. Lett. **126:**299–303.
5. **Berger-Bächi, B.** 1994. Expression of resistance to methicillin. Trends Microbiol. **2:**389–393.
6. **Berry, A. M., R. A. Lock, S. M. Thomas, D. P. Rajan, D. Hansman, and J. C. Paton.** 1994. Cloning and nucleotide sequence of the *Streptococcus pneumoniae* hyaluronidase gene and purification of the enzyme from recombinant *Escherichia coli*. Infect. Immun. **62:**1101–1108.
7. **Cámara, M., G. J. Boulnois, P. W. Andrew, and T. J. Mitchell.** 1994. A neuraminidase from *Streptococcus pneumoniae* has the features of a surface protein. Infect. Immun. **62:**3688–3695.
8. **Chen, J.-D., and D. A. Morrison.** 1988. Construction and properties of a new insertion vector, pJDC9, that is protected by transcriptional terminators and useful for cloning of DNA from *Streptococcus pneumoniae*. Gene **64:**155–164.
9. **Chung, C. T., S. I. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86:**2172–2175.
10. **Coffey, T. J., M. Daniels, L. K. McDougal, C. G. Dowson, F. C. Tenover, and B. G. Spratt.** 1995. Genetic analysis of clinical isolates of *Streptococcus pneumoniae* with high-level resistance to expanded-spectrum cephalosporins. Antimicrob. Agents Chemother. **39:**1306–1313.
11. **Curtis, N. A. C., M. V. Hayes, A. W. Wyke, and B. J. Ward.** 1980. A mutant of *Staphylococcus aureus* H lacking penicillin-binding protein 4 and transpeptidase activity in vitro. FEMS Microbiol. Lett. **9:**263–266.
12. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
13. **Domanski, T. L., and K. W. Bayles.** 1995. Analysis of *Staphylococcus aureus* genes encoding penicillin-binding protein 4 and an ABC-type transporter. Gene **167:**111–113.
14. **Dowson, C. G., A. Hutchison, J. A. Brannigan, R. C. George, D. Hansman, J. Liñares, A. Tomasz, J. M. Smith, and B. G. Spratt.** 1989. Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **86:**8842–8846.
15. **Edwards, D. H., and W. D. Donachie.** 1993. Construction of a triple deletion of penicillin-binding proteins 4, 5, and 6 in *Escherichia coli*, p. 369–374. *In* M. A. de Pedro, J.-V. Höltje, and W. Löffelhardt (ed.), Bacterial growth and lysis. Plenum Press, New York, N.Y.
16. **Ghuysen, J.-M., and G. Dive.** 1994. Biochemistry of the penicilloyl-serine transferases, p. 103–129. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier Sciences BV, Amsterdam, The Netherlands.
17. **Grebe, T., and R. Hakenbeck.** 1996. Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of β-lactam antibiotics. Antimicrob. Agents Chemother. **40:**829–834.
18. **Guenzi, E., A. M. Gasc, M. A. Sicard, and R. Hakenbeck.** 1994. A two-component signal-transducing system is involved in competence and penicillin susceptibility in laboratory mutants of *Streptococcus pneumoniae*. Mol. Microbiol. **12:**505–515.
19. **Hakenbeck, R.** 1983. Purification of penicillin binding proteins from Streptococcus pneumoniae, p. 415–420. *In* R. Hakenbeck, J.-V. Höltje, and H. Labischinski (ed.), The target of penicillin. Walter de Gruyter, Berlin, Germany.
20. **Hakenbeck, R.** 1995. Target mediated resistance to β-lactam antibiotics. Biochem. Pharmacol. **50:**1121–1127.
21. **Hakenbeck, R., H. Ellerbrok, T. Briese, S. Handwerger, and A. Tomasz.** 1986. Penicillin-binding proteins of penicillin-susceptible and -resistant pneumococci: immunological relatedness of altered proteins and changes in peptides carrying the β-lactam binding site. Antimicrob. Agents Chemother. **30:**553–558.

21a. **Hakenbeck, P., and A. König.** Unpublished data.

22. **Hakenbeck, R., and M. Kohiyama.** 1982. Purification of penicillin-binding protein 3 from *Streptococcus pneumoniae*. Eur. J. Biochem. **127:**231–236.
23. **Hakenbeck, R., C. Martin, C. Dowson, and T. Grebe.** 1994. Penicillin-binding protein 2b of *Streptococcus pneumoniae* in piperacillin-resistant laboratory mutants. J. Bacteriol. **176:**5574–5577.
24. **Handwerger, S., and A. Tomasz.** 1986. Alterations in penicillin-binding proteins of clinical and laboratory isolates of pathogenic *Streptococcus pneumoniae* with low levels of penicillin resistance. J. Infect. Dis. **153:**83–89.
25. **Håvarstein, L. S., G. Coomaraswamy, and D. A. Morrison.** 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **92:**11140–11144.
26. **Henze, U. U., and B. Berger-Bächi.** 1995. *Staphylococcus aureus* penicillin-binding protein 4 and intrinsic β-lactam resistance. Antimicrob. Agents Chemother. **39:**2415–2422.
27. **Henze, U. U., and B. Berger-Bächi.** 1996. Penicillin-binding protein 4 overproduction increases β-lactam resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40:**2121–2125.
28. **Holmes, P. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114:**193–197.
29. **Krauss, J., M. van der Linden, T. Grebe, and R. Hakenbeck.** 1996. Penicillin-binding proteins 2x and 2b as primary PBP-targets in *Streptococcus pneumoniae*. Microb. Drug Resist. **2:**183–186.
30. **Kuzin, A. P., J. Liu, J. A. Kelly, and J. R. Knox.** 1995. Binding of cephalothin and cefotaxime to D-ala-D-ala-peptidase reveals a functional basis of a natural mutation in a low-affinity penicillin-binding protein and in extended-spectrum β-lactamases. Biochemistry **34:**9532–9540.
31. **Lacks, S. A., and R. D. Hotchkiss.** 1960. A study of the genetic material determining an enzyme activity in pneumococcus. Biochim. Biophys. Acta **39:**508–517.
32. **Laible, G., and R. Hakenbeck.** 1987. Penicillin-binding proteins in β-lactam-resistant laboratory mutants of *Streptococcus pneumoniae*. Mol. Microbiol. **1:**355–363.
33. **Laible, G., and R. Hakenbeck.** 1991. Five independent combinations of mutations can result in low-affinity penicillin-binding protein 2x of *Streptococcus pneumoniae*. J. Bacteriol. **173:**6986–6990.

34. **Laible, G., R. Hakenbeck, M. A. Sicard, B. Joris, and J.-M. Ghuysen.** 1989. Nucleotide sequences of the *pbpX* genes encoding the penicillin-binding protein 2x from *Streptococcus pneumoniae* R6 and a cefotaxime-resistant mutant, C506. Mol. Microbiol. **3:**1337–1348.
35. **Laible, G., B. G. Spratt, and R. Hakenbeck.** 1991. Inter-species recombinational events during the evolution of altered PBP 2x genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. Mol. Microbiol. **5:** 1993–2002.
36. **Martin, B., O. Humbert, M. Cámara, E. Guenzi, J. Walker, T. Mitchell, P. Andrew, M. Prudhomme, G. Alloing, R. Hakenbeck, D. A. Morrison, G. J. Boulnois, and J.-P. Claverys.** 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Res. **20:**3479–3483.
37. **Martin, C., C. Sibold, and R. Hakenbeck.** 1992. Relatedness of penicillin-binding protein 1a genes from different clones of penicillin-resistant *Streptococcus pneumoniae* isolated in South Africa and Spain. EMBO J. **11:**3831–3836.
38. **McDougal, L. K., J. K. Rasheed, J. W. Biddle, and F. C. Tenover.** 1995. Identification of multiple clones of extended-spectrum cephalosporin-resistant *Streptococcus pneumoniae* isolates in the United States. Antimicrob. Agents Chemother. **39:**2282–2288.
39. **Morona, J. K., A. Guidolin, R. Morona, D. Hansman, and J. C. Paton.** 1994. Isolation, characterization, and nucleotide sequence of IS*1202*, an insertion sequence of *Streptococcus pneumoniae*. J. Bacteriol. **176:**4437–4443.
40. **Muñóz, R., T. J. Coffey, M. Daniels, C. G. Dowson, G. Laible, J. Casal, R. Hakenbeck, M. Jacobs, J. M. Musser, B. G. Spratt, and A. Tomasz.** 1991. Intercontinental spread of a multiresistant clone of serotype 23F *Streptococcus pneumoniae*. J. Infect. Dis. **164:**302–306.
41. **Muñóz, R., J. M. Musser, M. Crain, D. E. Briles, A. Marton, A. J. Parkinson, U. Sorensen, and A. Tomasz.** 1992. Geographic distribution of penicillin-resistant clones of *Streptococcus pneumoniae*: characterization by penicillin-binding protein profile, surface protein A typing, and multilocus enzyme analysis. Clin. Infect. Dis. **15:**112–118.
42. **Ochman, H., A. S. Gerber, and D. L. Hartl.** 1988. Genetic application of an inverse polymerase chain reaction. Genetics **120:**621–623.
43. **Phoenix, D. A., S. E. Peters, M. A. Ramzan, and J. M. Pratt.** 1994. Analysis of the membrane-anchoring properties of the putative amphiphilic alpha-helical anchor at the C-terminus of *Escherichia coli* PBP 6. Microbiology **140:**73–77.
44. **Phoenix, D. A., and J. M. Pratt.** 1993. Membrane interaction of *Escherichia coli* penicillin-binding protein 5 is modulated by the ectomembraneous domain. FEBS Lett. **322:**215–218.
45. **Podbielski, A., B. Pohl, M. Woischnik, C. Körner, K.-H. Schmidt, E. Rozdzinski, and B. A. B. Leonard.** 1996. Molecular characterization of group A streptococcal (GAS) oligopeptide permease (Opp) and its effect on cysteine production. Mol. Microbiol. **21:**1087–1099.
46. **Reichmann, P., A. König, A. Marton, and R. Hakenbeck.** 1996. Penicillin-binding proteins as resistance determinants in clinical isolates of *Streptococcus pneumoniae*. Microb. Drug Resist. **2:**177–181.
47. **Reichmann, P., E. Varon, E. Günther, R. R. Reinert, R. Lütticken, A. Marton, P. Geslin, J. Wagner, and R. Hakenbeck.** 1995. Penicillin-resistant *Streptococcus pneumoniae* in Germany: genetic relationship to clones from other European countries. J. Med. Microbiol. **43:**377–385.
48. **Sabelnikov, A. G., B. Greenberg, and S. A. Lacks.** 1995. An extended −10 promoter alone directs transcription of the *Dpn*II operon of *Streptococcus pneumoniae*. J. Mol. Biol. **250:**144–155.
49. **Saluja, S. K., and J. N. Weiser.** 1995. The genetic basis of colony opacity in *Streptococcus pneumoniae*: evidence for the effect of box elements on the frequency of phenotypic variation. Mol. Microbiol. **16:**215–227.
50. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
51. **Schuster, C., B. Dobrinski, and R. Hakenbeck.** 1990. Unusual septum formation in *Streptococcus pneumoniae* mutants with an alteration in the D,D-carboxypeptidase penicillin-binding protein 3. J. Bacteriol. **172:**6499–6505.
52. **Selakovitch-Chenu, L., L. Seroude, and A. M. Sicard.** 1993. The role of penicillin-binding protein 3 (PBP 3) in cefotaxime resistance in *Streptococcus pneumoniae*. Mol. Gen. Genet. **239:**77–80.
53. **Severin, A., A. M. S. Figueiredo, and A. Tomasz.** 1996. Separation of abnormal cell wall composition from penicillin resistance through genetic transformation of *Streptococcus pneumoniae*. J. Bacteriol. **178:**1788–1792.
54. **Sibold, C., J. Wang, J. Henrichsen, and R. Hakenbeck.** 1992. Genetic relationship of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated on different continents. Infect. Immun. **60:**4119–4126.
55. **Tiraby, J.-G., and M. S. Fox.** 1974. Marker discrimination and mutagen-induced alterations in pneumococcal transformation. Genetics **77:**449–458.
56. **Triglia, T., M. G. Peterson, and D. J. Kemp.** 1988. A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res. **16:**8186.
57. **Williamson, R., R. Hakenbeck, and A. Tomasz.** 1980. In vivo interaction of β-lactam antibiotics with the penicillin-binding proteins of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **18:**629–637.
58. **Wyke, A. W., J. B. Ward, M. V. Hayes, and N. A. C. Curtis.** 1981. A role in vivo for penicillin-binding protein-4 of *Staphylococcus aureus*. Eur. J. Biochem. **119:**389–393.
59. **Zighelboim, S., and A. Tomasz.** 1980. Penicillin-binding proteins of multiply antibiotic-resistant South African strains of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **17:**434–442.