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Nucleotide sequences of the *pbpX* genes encoding the penicillin-binding proteins 2x from *Streptococcus pneumoniae* R6 and a cefotaxime-resistant mutant, C506

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Summary

Development of penicillin resistance in *Streptococcus pneumoniae* is due to successive mutations in penicillin-binding proteins (PBPs) which reduce their affinity for β-lactam antibiotics. PBP2x is one of the high-M_r PBPs which appears to be altered both in resistant clinical isolates, and in cefotaxime-resistant laboratory mutants. In this study, we have sequenced a 2564 base-pair chromosomal fragment from the penicillin-sensitive *S. pneumoniae* strain R6, which contains the PBP2x gene. Within this fragment, a 2250 base-pair open reading frame was found which coded for a protein having an M_r of 82.35 kD, a value which is in good agreement with the M_r of 80–85 kD measured by SDS-gel electrophoresis of the PBP2x protein itself. The *N*-terminal region resembled an unprocessed signal peptide and was followed by a hydrophobic sequence that may be responsible for membrane attachment of PBP2x. The corresponding nucleotide sequence of the PBP2x gene from C504, a cefotaxime-resistant laboratory mutant obtained after five selection steps, contained three nucleotide substitutions, causing three amino acid alterations within the β-lactam binding domain of the PBP2x protein. Alterations affecting similar regions of *Escherichia coli* PBP3 and *Neisseria gonorrhoeae* PBP2 from β-lactam-resistant strains are known. The penicillin-binding domain of PBP2x shows highest homology with these two PBPs and *S. pneumoniae* PBP2b. In contrast, the *N*-terminal extension of PBP2x has the highest homology with *E. coli* PBP2 and methicillin-resistant *Staphylococcus aureus* PBP2'. No significant homology was detected with PBP1a or PBP1b of *Escherichia coli*, or with the low-M_r PBPs.

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Introduction

Penicillin-binding proteins (PBPs), the target enzymes for β-lactam antibiotics, are minor components of the bacterial cytoplasmic membrane. They function in the late steps of murein biosynthesis, and therefore their active centre is on the outer surface of the cytoplasmic membrane. β-lactams inactivate the PBPs by acylating an essential serine residue in the active site of these proteins. Whereas inhibition of low-M_r PBPs, which are D-alanyl-D-alanine-carboxypeptidases, can be tolerated by the bacteria, inhibition of high-M_r PBPs, which are assumed to be transpeptidases, is lethal to the cells. Based on the sequences of their penicillin-binding domains, all PBPs are thought to be related to each other; they form, together with the penicillin-hydrolysing enzymes (β-lactamases), a superfamily of active-site serine, penicillin-recognizing proteins (Joris *et al.*, 1988). Much, however, remains to be learned about the evolutionary relationships among the PBPs, primarily because of the lack of information about the sequences and overall structures of the high-M_r PBPs of different species.

Streptococcus pneumoniae contains six PBPs (Hakenbeck *et al.*, 1986). The five high-M_r PBPs 1a, 1b, 2x, 2a and 2b, ranging from 92 to 78 kD, are of unknown function, whereas the 43 kD low-M_r PBP3 acts *in vitro* as a D,D-carboxypeptidase (Hakenbeck and Kohiyama, 1982). Penicillin resistance in pneumococci can be acquired by the stepwise accumulation of mutations in at least the three high-M_r PBPs 1a, 2x and 2b (Hakenbeck *et al.*, 1980; Zighelboim and Tomasz, 1980; Hakenbeck *et al.*, 1988). In earlier papers, only five PBPs were detected using different SDS-PAGE systems, and alterations were also shown to occur in a so-called PBP2a; however, we believe that this protein was actually PBP2x. Penicillin resistance can also be obtained by the stepwise transformation of sensitive strains using the DNA from resistant strains that have been isolated in clinics or produced in the laboratory

(Shockley and Hotchkiss, 1970). In this process, the transformants successively acquire the altered PBPs of the donor strain (Zighelboim and Tomasz, 1980). Since these PBP alterations lead to a decrease in affinity for β-lactams, characterization of the mutations involved will help to identify the protein regions responsible for the interaction with β-lactam antibiotics.

The problems of analysing these mutations in clinical isolates is at least two-fold: the strains are not isogenic, and the mechanism responsible for evolution of low-affinity PBPs is not known. Gene sequencing has revealed that PBPs from penicillin-resistant clinical isolates of *Neisseria gonorrhoeae* (PBP2) (Spratt, 1988) and *S. pneumoniae* (PBP2b) (Dowson *et al.*, 1989) have extensive alterations in their primary structure, thus aggravating the identification of those mutations that contribute to the decrease in penicillin affinity. We have therefore isolated a series of spontaneous, independent, laboratory mutants with increasing resistance to either piperacillin or cefotaxime (Laible and Hakenbeck, 1987). Cefotaxime resistance leads to affinity changes primarily of PBP2a and PBP2x. Using cefotaxime resistance as selective marker in transformation experiments, we have been able to identify the DNA coding for PBP2x. The gene and surrounding regions were sequenced, and the mutations responsible for cefotaxime resistance in the laboratory mutant C506 were identified. The deduced amino acid sequences of PBP2x were used for comparative analysis with PBPs of other bacterial species.

Results and Discussion

Isolation of the pbpX *gene of* S. pneumoniae

In the cefotaxime-resistant mutant, C506, PBP2x and PBP2a have reduced affinity for β-lactams (Laible and Hakenbeck, 1987). The strategy used for cloning the *pbpX* gene was based on identification of DNA fragments capable of transforming penicillin-sensitive strains 801 (which carries a *hex*$^-$ mutation) or R6 to cefotaxime resistance. No attempts were made to clone the gene directly into *E. coli* since unstable clones are frequently observed probably because of promoter-bearing regions in the AT-rich pneumococcal DNA (Stassi and Lacks, 1982).

After *Bam*HI-digestion of chromosomal C506 DNA, the 2.3–3.6 kb DNA fraction could transform strain 801 to cefotaxime resistance (0.06 μg ml^{-1}) and was cloned into plasmid pSP2. pSP2, carrying an erythromycin resistance marker, is stable in pneumococci only as a recombinant plasmid because of long inverted repeats (Prats *et al.*, 1985). After transformation of 801 with the recombinant plasmids, no transformants could be selected using

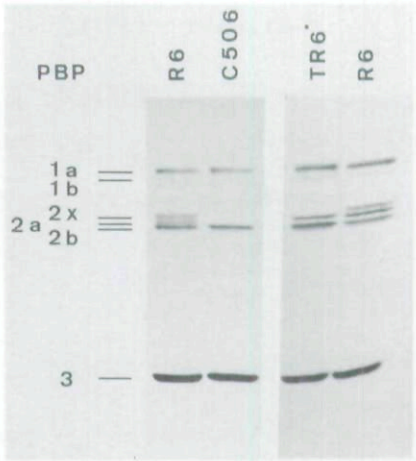

Fig. 1. PBPs in a cefotaxime-resistant transformant of *S. pneumoniae* R6. PBPs in cell lysates were labelled with [^{3}H]-propionyl ampicillin, separated on SDS-PAGE, and visualized after fluorography. For comparison, the PBP pattern of the parent strain R6 and the cefotaxime-resistant mutant C506 is shown in addition to the one found in a cefotaxime-resistant transformant TR6. TR6 has been obtained after transformation of R6 with the 0.8 kb *Eco*RI/*Bam*HI fragment (carrying the cefotaxime resistance marker of C506) and selection on 0.06 μg ml^{-1} cefotaxime.

erythromycin plus cefotaxime, indicating that cefotaxime resistance is not expressed. Therefore, erythromycin-resistant transformants were isolated and replated on plates containing cefotaxime alone. Clones containing recombinant pSP2 carrying the cefotaxime resistance marker should be able to produce cefotaxime-resistant colonies at low frequency because of homologous recombination with the chromosomal *pbpX* wild-type gene. Fifteen out of 200 erythromycin-resistant transformants produced cefotaxime-resistant colonies and contained the same pSP2 derivative, pPG1, with a 2.6 kb *Bam*HI insert. The cefotaxime-resistant transformants in which part of the genome had been replaced by recombination with the 2.6 kb fragment of C506 contained a low-affinity PBP2x (Fig. 1), demonstrating that part or all of the *pbpX* gene is located on pPG1.

Digestion of the 2.6 kb *Bam*HI insert with *Eco*RI produced two fragments. After transformation of the 801 recipient strain by either one of the fragments, only the smaller one (0.8 kb) yielded cefotaxime resistance. Sequencing of this 0.8 kb *Eco*RI-*Bam*HI fragment revealed an open reading frame which terminated 60 nucleotides upstream from the *Eco*RI site and continued through the *Bam*HI site, indicating that the *C*-terminal part of the PBP2x gene had been cloned.

The wild-type *pbpX* gene was then isolated in two steps using the 2.6 kb *Bam*HI fragment from C506. In the first step, the 1.8 kb *Eco*RI/*Bam*HI portion of this fragment, downstream from *pbpX*, was inserted into pR28, and this construct, pCG4, was inserted into the chromosome of strain 801 by homology-dependent integration (Fig. 2). A

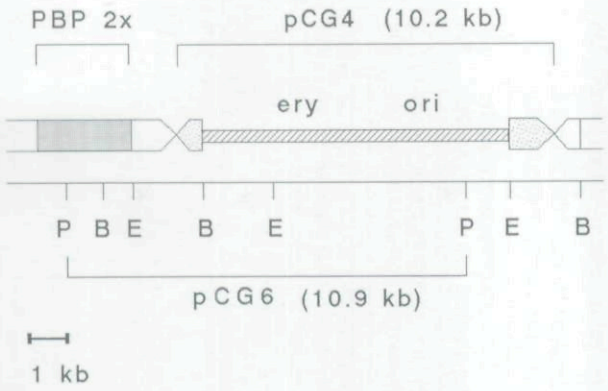

Fig. 2. Schematic illustration of the isolation of DNA fragments of the PBP2x gene by vector integration into the chromosome. Integration of plasmid pCG4 into the chromosome of strain 801 was forced by selecting erythromycin-resistant transformants. Recombination occurred between the *Eco*RI/*Bam*HI 1.8 kb C506 insert (dotted part) of pCG4 and the homologous chromosomal DNA (white) in strain 801. pCG6 was isolated after digestion with *Pst*I restriction enzyme, ligation, and selection of erythromycin-resistant *E. coli* transformants. Indicated are the positions of the origin (ori) and erythromycin resistance gene within the vector part of pCG4 (hatched region).

second recombinant plasmid, pCG6, was then isolated from the chromosome using *Pst*I restriction endonuclease. In the second step, the 0.4 kb *Eco*RV-*Pst*I fragment was removed from pCG6 and was used as a hybridization probe on R6 DNA digested with *Dra*I/*Eco*RV. A 2.4 kb fragment was found, which presumably carried the promoter region. It was cloned into plasmid pJDC9, in which strong terminators surrounding the cloning site should facilitate cloning of pneumococcal promoter sequences into *E. coli* (Chen and Morrison, 1987).

The restriction endonuclease cleavage map of the *pbpX* region is shown in Fig. 3. The 2250-nucleotide open reading frame of the *pbpX* gene encoded 750 amino acid residues (Fig. 4). It was preceded by an open reading frame, and another open reading frame started almost immediately after termination of the *pbpX* gene (Fig. 3). No other long open reading frames were found. Potential candidates for Shine-Dalgarno and promoter sequences were identified using *E. coli* consensus sequences. A putative rho-independent terminator region was found following the terminator codon, TAA (Fig. 4).

No differences between the nucleotide sequences of the *pbpX* genes of strains R6 and 801 were detected. The sequence obtained from mutant C506 differed in three nucleotides from R6 or 801, changing Met289 into Thr and the closely linked Gly597 and Gly601 into Val597 and Asp601, respectively (Fig. 4). Between the *N* terminus and Met289, no mutations were found even in more highly resistant mutants derived from C506.

The codon usage was very similar to that of the gene encoding the pneumococcal high-M_r PBP2b, which has been partly sequenced recently (Dowson *et al.*, 1989), but not to the highly expressed *lytA* gene coding for the major autolysin of *S. pneumoniae* (Garcia *et al.*, 1986) (not shown). Marked differences were found in the codon usages for Gly, Asp, Tyr, Phe, Ser and His, probably reflecting the more frequent use of modulator codons in weakly expressed genes, as has been suggested for *E. coli* PBPs (Asoh *et al.*, 1986).

The M_r of PBP2x, according to the derived amino acid sequence, is 82.35 kD, a value which is in agreement with an M_r of approximately 80–85 kD, as derived from SDS-gel electrophoresis.

Conserved boxes in the amino acid sequence of PBP2x

On the basis of homology searches and amino acid alignment, several conserved regions have been identified among the β-lactamases, low-M_r PBPs and penicillin-binding domains of the high-M_r PBPs (Joris *et al.*, 1988).

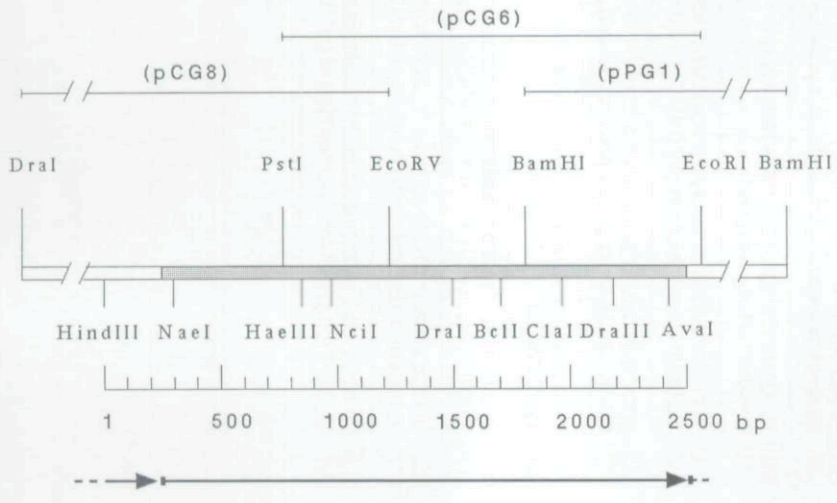

Fig. 3. Restriction map of the *pbpX* region. Shown here are the restriction sites used for cloning various parts of the PBP2x gene as indicated in pPG1, pCG6 and pCG8, and further unique restriction sites (lower part). The arrows indicate open reading frames between the *Hin*dIII and *Eco*RI sites. Dotted area: the region coding for PBP2x.


(-35)
(-10)
(SD)
1 MetLysTrpThrLysArgValIleArgTyrAlaThrLysAsnArgLys
SerProAlaGluAsnArgArgArgValGlyLysSerLeuSerLeuLeuSerValPheVal
PheAlaIlePheLeuValAsnPheAlaValIleIleGlyThrGlyThrArgPheGlyThr
AspLeuAlaLysGluAlaLysLysValHisGlnThrThrArgThrValProAlaLysArg
GlyThrIleTyrAspArgAsnGlyValProIleAlaGluAspAlaThrSerTyrAsnVal
TyrAlaValIleAspGluAsnTyrLysSerAlaThrGlyLysIleLeuTyrValGluLys
ThrGlnPheAsnLysValAlaGluValPheHisLysTyrLeuAspMetGluGluSerTyr
ValArgGluGlnLeuSerGlnProAsnLeuLysGlnValSerPheGlyAlaLysGlyAsn
GlyIleThrTyrAlaAsnMetMetSerIleLysLysGluLeuGluAlaAlaGluValLys
GlyIleAspPheThrThrSerProAsnArgSerTyrProAsnGlyGlnPheAlaSerSer
PheIleGlyLeuAlaGlnLeuHisGluAsnGluAspGlySerLysSerLeuLeuGlyThr
SerGlyMetGluSerSerLeuAsnSerIleLeuAlaGlyThrAspGlyIleIleThrTyr
GluLysAspArgLeuGlyAsnIleValProGlyThrGluGlnValSerGlnArgThrMet
AspGlyLysAspValTyrThrThrIleSerSerProLeuGlnSerPheMetGluThrGln
ACG
MetAspAlaPheGlnGluLysValLysGlyLysTyrMetThrAlaThrLeuValSerAla
Thr
LysThrGlyGluIleLeuAlaThrThrGlnArgProThrPheAspAlaAspThrLysGlu
GlyIleThrGluAspPheValTrpArgAspIleLeuTyrGlnSerAsnTyrGluProGly
SerThrMetLysValMetMetLeuAlaAlaAlaIleAspAsnAsnThrProGlyGly
GluValPheAsnSerSerGluLeuLysIleAlaAspAlaThrIleArgAspTrpAspVal
AsnGluGlyLeuThrGlyGlyArgMetMetThrPheSerGlnGlyPheAlaHisSerSer
AsnValGlyMetThrLeuLeuGluGlnLysMetGlyAspAlaThrTrpLeuAspTyrLeu

Fig. 4.

Fig. 4. (continued).

Fig. 4. Nucleotide sequence of the fragment from *Hin*dIII to *Eco*RI and deduced amino acid sequences of the PBPs 2x of the parent strain R6 and the cefotaxime-resistant mutant, C506. These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number X16367.

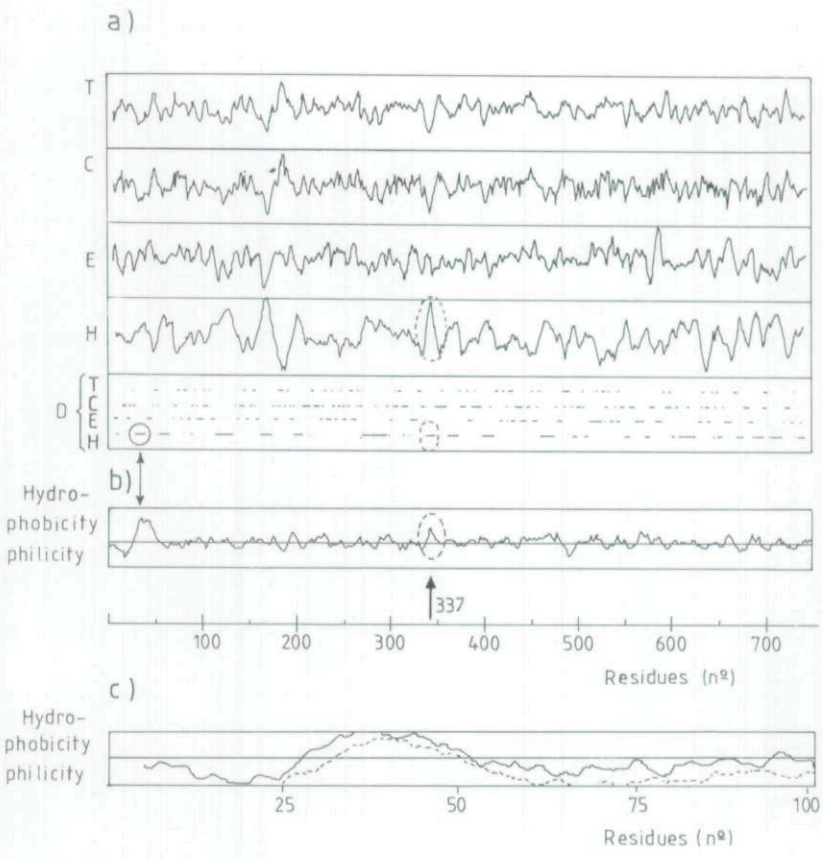

Fig. 5. Predicted secondary structures and hydrophobicity (a, b) and detection of a transmembrane segment at position 30–40 (c) in the *S. pneumoniae* PBP2x. T: turn; C: coil; E: extended; H: α-helix; D: decision. Programs used: (a) Garnier *et al.* (1978); (b): Kyte and Doolittle (1982); (c): Kyte and Doolittle (1982); Engelman *et al.* (1986). The position of the active-site serine 337 is indicated.

The active-site serine is always part of the sequence SXXK (box II) and, at a certain distance from the *C* terminus, one of the triad KTG, KSG or HTG (box VII) can be found. Recently, an SXN motif was identified on the carboxy-terminal side of the active-site serine (Spratt and Cromie, 1988).

In *S. pneumoniae* PBP2x, STMK 337–340 is thought to contain the active-site serine and KSG 547–549 is assumed to be the conserved triad HTG/KTG/KSG for the following reasons. (i) S337 is at the amino terminus of a hydrophobic stretch with a high α-helix potential (Fig. 5). The occurrence of the active-site serine at the end of an α-helix is expected from the three-dimensional data obtained from the crystal structure of *Streptomyces* R61 PBP (Kelly *et al.*, 1988). (ii) S395SN is 58 amino acids downstream of the STMK-sequence, corresponding to the reported 47–62 amino acid distance in other high-M_r PBPs (Spratt and Cromie, 1988). (iii) The size of the stretch S337TMK to K547SG (i.e. 210 amino acids) is that expected for a penicillin-binding domain (Table 1). In this table, and as proposed by Joris *et al.* (1988), the penicillin-binding domain of the PBPs is defined as a polypeptide stretch that starts 60 residues (or less) upstream of the active-site serine and terminates 60 residues (or less) downstream of the H or K of the conserved box VII. This pattern allows definition of an amino-terminal extension and a carboxy-terminal extension on both sides of the penicillin-binding domain. All the β-lactamases of classes A, C and D lack any *N*- or *C*-terminal extension (not shown). The low- and high-M_r PBPs have a *C*-terminal extension of varying length, except the *Streptomyces* R61 PBP, which is excreted during growth (Duez *et al.*, 1987) and, interestingly, the PBP2b of *S. pneumoniae* (Dowson *et al.*, 1989). Only the high-M_r PBPs have a long amino-terminal extension.

Membrane topology of PBP2x

Figure 5 reveals that one predominant region with a high hydrophobicity index occurs close to the amino terminus. The amino-terminal 100 amino acid region was re-examined using both the Kyte-Doolittle procedure (1982) and the Engelman-Steitz-Goldman program (1986) for identifying transmembrane segments. The two profiles (Fig. 5) strongly suggest that PBP2x is anchored into the membrane by amino acids L30–I48. It should be noted that the same stretch has a high α-helix potential according to the program of Garnier *et al.* (1978). This hydrophobic portion is preceded by a terminal hydrophilic as well as a basic region (known features of signal peptides)

(von Heijne, 1985). On the basis of the amino acid sequence, no cleavage site for a signal peptidase was found. We therefore assume that the mature form of PBP2x contains an unprocessed signal sequence followed by a membrane-anchoring segment, as has been shown for *E. coli* PBP1b (Spratt *et al.*, 1987) and PBP2 (Asoh *et al.*, 1986). In contrast, *E. coli* PBP3 is probably processed after synthesis (Nakamura *et al.*, 1983) and membrane binding might be mediated through modification of a cysteine residue with a lipid moiety (Hayashi *et al.*, 1988). If the membrane topology proposed for PBP2x is correct, then that portion of the amino-terminal extension which occurs upstream of the penicillin-binding domain and which might have some enzymatic function would be the 227 amino acid stretch, T50-M277.

Homology searches

The amino acid sequences of all the PBPs listed in Table 1 were compared pair-wise using the Goad and Kanehisa procedure (1982) and the SEQHP and SEQDP programs (for more details, see Joris *et al.*, 1988). For each pair of PBPs, the amino-terminal extensions (for the high-M_r PBPs) and the penicillin-binding domains, as defined in Table 1, were analysed separately. The procedure yields a score and a standard deviation unit (SDU). The more negative the score, the better the homology. An SDU value of five or higher indicates a statistically significant homology. The results of this analysis are shown in Fig. 6. It should be noted that part of the *N*-terminal sequence of pneumococcal PBP2b is absent.

The data show that PBP2x is related to all the other high-M_r PBPs, with the exception of *E. coli* PBPs 1a and 1b. These two proteins are highly homologous in their amino-terminal extensions, as has been reported previously (Broome-Smith *et al.*, 1985; Joris *et al.*, 1988), and both are known to perform transglycosylation *in vitro* (Tomioka *et al.*, 1982; Nakagawa *et al.*, 1984). No significant homology between high- and low-M_r PBPs was detected. Interestingly, the penicillin-binding domain of PBP2x is especially closely related to those of *E. coli* PBP3, *N. gonorrhoeae* PBP2 and *S. pneumoniae* PBP2b. In contrast, the amino-terminal extension of PBP2x is closely related to those of *E. coli* PBP2 and methicillin-resistant *S. aureus* PBP2'. It can be speculated that the *N*-terminal extension and the penicillin-binding domain of PBP2x have evolved independently from each other.

Comparison with E. coli *PBP3*

The two sequences of PBP2x and *E. coli* PBP3 have been aligned using the BESTFIT program (Fig. 7). In this alignment, the only short gap introduced in the sequence of PBP2x is N441. . .I442. The two amino-terminal extensions (PBP2x, 1→277) contain 48 identities (17%) and two highly homologous stretches. The two penicillin-binding domains (278→607) contain 80 identities (24%) and seven highly homologous stretches.

The homology found between these two proteins does not necessarily indicate any similarity in their respective enzymatic activity *in vivo*, or affinity for β-lactams. The high *in vivo* affinity of the *E. coli* PBP3 for aztreonam

Table 1. Amino-terminal extension, penicillin-binding domain and carboxy-terminal extension of PBPs.

		Amino-terminal extension		Penicillin-binding Domain			Carboxy-terminal extension		
	H_3^+N	←	→ ←	S*XXK		HTG KTG KSG	→ ←		COO
Low-M_r PBPs									
Streptomyces R61		0	1	62	(236)	298	349	0	349
B. subtilis 5		0	1	36	(191)	227	287	(125)	412
E. coli 5		0	1	44	(169)	213	273	(101)	374
High-M_r PBPs									
E. coli 1A		(404)	405	465	(251)	716	776	(74)	850
1B		(449)	450	510	(188)	698	758	(86)	844
2		(269)	270	330	(213)	544	604	(26)	630
3		(246)	247	307	(187)	494	554	(34)	588
S. aureus 2'		(344)	345	405	(194)	599	659	(111)	770
N. gonorrhoeae 2		(249)	250	310	(187)	497	557	(24)	581
S. pneumoniae 2x		(276)	277	337	(210)	547	607	(143)	750
S. pneumoniae 2b[a]		('131')	132	192	(229)	421	481	0	'481'
					average: 205				

References of sequences shown: *Streptomyces* R61 (Duez *et al.*, 1987); *B. subtilis* PBP5 (Todd *et al.*, 1986); *E. coli* PBP5 (Broome-Smith *et al.*, 1983); *E. coli* PBPs 1A and 1B (Broome-Smith *et al.*, 1985); *E. coli* PBP2 (Asoh *et al.*, 1986); *E. coli* PBP3 (Nakamura *et al.*, 1983); *S. aureus* PBP2' (Song *et al.*, 1987); *N. gonorrhoeae* PBP2 (Spratt, 1988); *S. pneumoniae* PBP2b (Dowson *et al.*, 1989).

a. Only 131 amino acid residues of the amino-terminal extension are known.

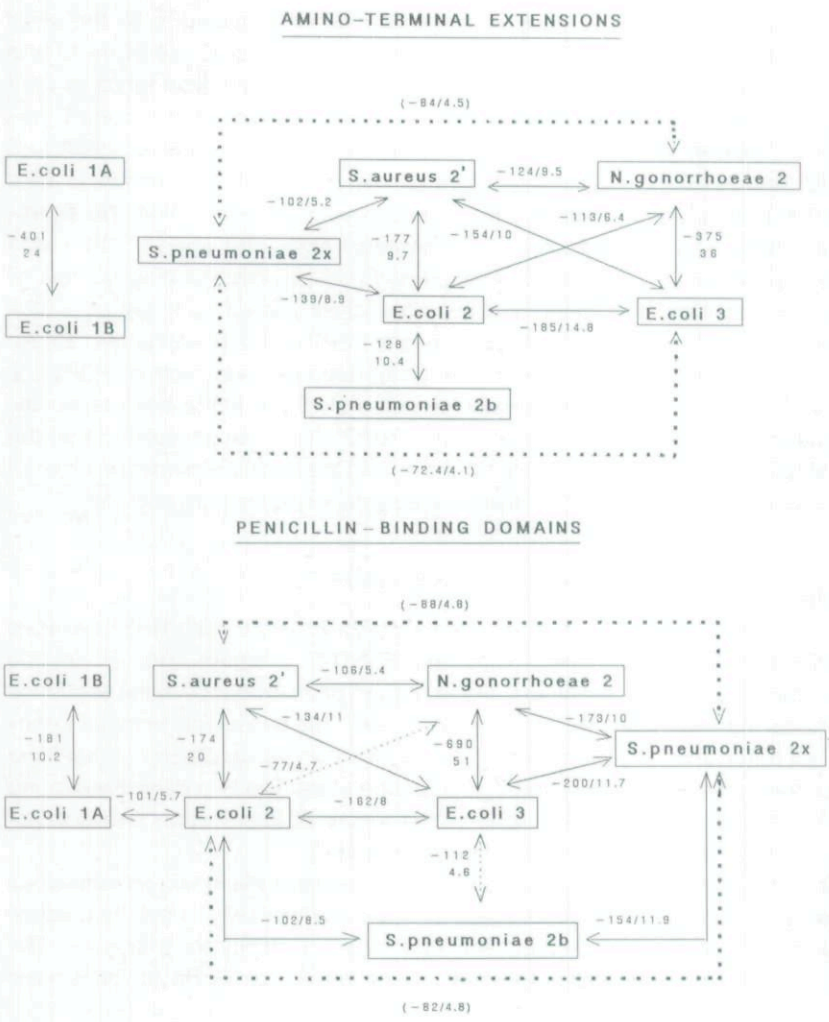

Fig. 6. Relationship between the *S. pneumoniae* PBP2x and other high-M_r PBPs of known primary structure. For each pair of PBPs, the amino-terminal and penicillin-binding domains were analysed separately using the Goad and Kanehisa procedure (1982). Dotted lines indicate a relationship of marginal significance.

(binding occurs at 0.01 μg ml^{-1}) (Briese *et al.*, 1988) was not found with the pneumococcal PBP2x (1 μg ml^{-1} and higher), and the related pneumococcal PBP2b does not bind aztreonam at all (Hakenbeck *et al.*, 1987).

Analysis of mutations in C506

The three point mutations found in PBP2x of the cefotaxime-resistant mutant, C506 (i.e., M289→T; G597→D; G601→V) are located at both ends of the penicillin-binding domain (Figs 4 and 7). We believe that the mutations, or some of them, are responsible for β-lactam resistance in that they decrease penicillin affinity or stabilize an already mutated PBP2x. The contribution of the individual mutations to β-lactam resistance is currently under investigation. Since PBP2x appears to be an essential protein, the mutations are unlikely to affect its actual enzymatic function.

The PBP2x mutations in C506 occur close to amino acid stretches that are highly homologous to other high-M_r PBPs (Fig. 8). The Val284 to Asp313 sequence of PBP2x shares homology even with the corresponding regions of the *E. coli* PBPs 1a and 1b, which in turn reveal high homology with that of the *E. coli* PBP2 (see also Fig. 6). It is striking that the mutated positions found in PBP2x are almost identical to those found in *E. coli* PBP3 of low-level cefalexin-resistant mutants isolated after mutagenesis (A257→V; V545→I), and that among the mutations inactivating PBP3 without impairing its ability to bind penicillin, substitutions in G542 as well as A547 were found (Hedge and Spratt, 1985a; 1985b) (Fig. 8). This region also corresponds to a peptide stretch that appeared highly mutated in the resistant gonococcal strains (Spratt, 1988). It is likely that the homologous peptide stretches belong to preserved three-dimensional structures that occupy important positions, and that the mutations fall into nearby variable loops important for access of β-lactam antibiotics.

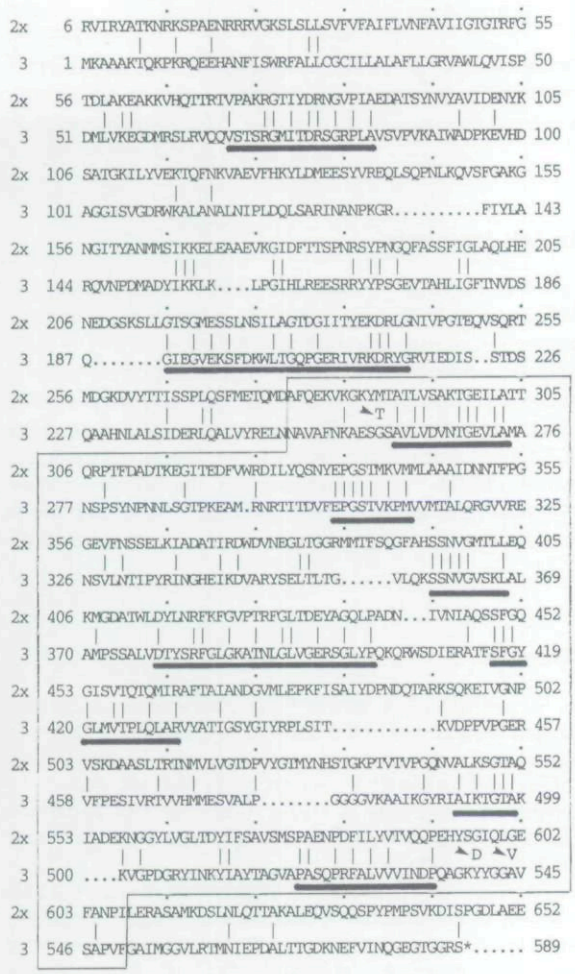

Fig. 7. Alignment of *S. pneumoniae* PBP2x with *E. coli* PBP3 using the BESTFIT program. Indentical amino acids are marked. Indicated are sequences of high homology (see text for details) and the penicillin-binding domain of the PBPs. Arrows: amino acid alterations of PBP2x from strain C506.

The PBP2b gene of one highly penicillin-resistant pneumococcal strain contains a vast number of nucleotide changes (74) coding for 17 amino acid substitutions (Dowson *et al.*, 1989). Similarly, a highly altered PBP gene occurs in resistant gonococci (Spratt, 1988). It would be of interest to search in the pneumococcal strains for alterations that would occur in PBP2x, and to compare the alterations found in laboratory mutants with those present in clinical isolates. It will also be important to localize the mutations in the PBP2x gene in cefotaxime-resistant mutants isolated independently from the one described here, and to determine whether there is a specific order of mutations that leads to decreased affinity for a given antibiotic.

Experimental procedures

Bacterial strains, growth conditions, plasmids and bacteriophages

S. pneumoniae R6 is a penicillin-sensitive, non-encapsulated laboratory strain (Ottolenghi and Hotchkiss, 1962) with an MIC value for cefotaxime of 0.02 μg ml^{-1}. The other pneumococcal strains used are derivatives of strain R6. C506 is a spontaneous cefotaxime-resistant mutant isolated after five selection steps on increasing concentrations of cefotaxime (MIC for cefotaxime: 1.28 μg ml^{-1}) (Laible and Hakenbeck, 1987). Strain 801 is an R6 derivative carrying a *hex*$^-$ mutation and is therefore defective in the mismatch repair process (Levèfre *et al.*, 1979). Cells were cultivated in C-medium supplemented with 0.2% yeast extract (Lacks and Hotchkiss, 1960) at 37°C without aeration. *E. coli* JM103 (Messing *et al.*, 1981) was used for growth of phagescript SK (Stratagene: San Diego, CA) and M13mp8/mp9 phages (Messing and Vieira, 1982), and *E. coli* DH5 was used for propagation of *E. coli* plasmids. Plasmids pJDC9 (Chen and Morrison, 1987), pSP2, which can be used for direct selection of recombinant plasmids (Prats *et al.*, 1985), and pR28, which cannot be maintained autonomously in pneumococci (Méjean *et al.*, 1981) have been described.

Transformation

Transformation of *S. pneumoniae* strains was carried out according to Tiraby and Fox (1974). Cefotaxime-resistant transformants

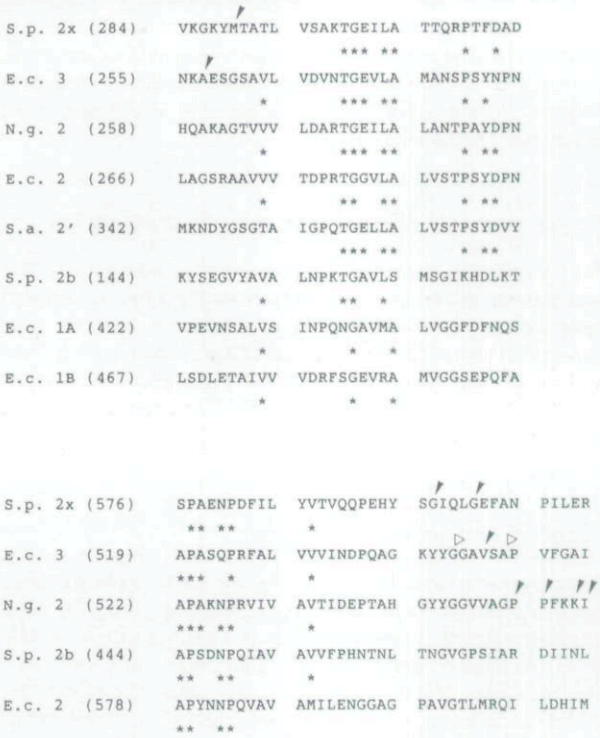

Fig. 8. Amino acid sequences in different PBPs with homology to the regions neighbouring the mutations in PBP2x of C506. Amino acids that appear conserved in most PBPs shown are marked by asterisks. Closed arrows point to the amino acids which are altered in PBPs of lowered β-lactam affinity. Open arrows indicate sites of mutations that inactivate PBP3 without interfering with β-lactam binding.

were screened on plates containing 0.06 μg ml^{-1} cefotaxime, and erythromycin-resistant colonies were isolated using 5 μg ml^{-1} erythromycin. Transformation of *E. coli* strains was performed using gradient-purified plasmid DNA and $CaCl_2$-treated cells (Brown *et al.*, 1979).

Preparation of pneumococcal chromosomal DNA

Pneumococci were harvested by centrifugation at late-exponential growth phase. Cells were resuspended in 0.1 M EDTA, 0.005% deoxycholate, 0.01% SDS, pH 8.0 and lysed during incubation at 37°C for 5 min. After ethanol-precipitation, DNA fibres were collected and resuspended in 0.03 M sodium citrate, pH 8.5. Following treatment with proteinase K (80 μg ml^{-1}) for 30 min at 37°C, phenol-extraction and ethanol-precipitation, DNA was resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, at about 200 μg ml^{-1}.

Isolation of plasmids from S. pneumoniae

Plasmids were prepared from pneumococci essentially as described by Birnboim and Doly (1979).

Southern blots and colony hybridization

Restriction endonucleases were obtained from commercial sources. Restricted DNA fragments were eluted from agarose gels by the method of Vogelstein and Gillespie (1979). For Southern blot hybridizations, ^{32}P nick-translated DNA was used. Colony hybridization was performed with random primed DNA fragments using a non-radioactive labelling and detection kit (Boehringer: Mannheim, FRG).

Sequencing of the PBP2x gene from S. pneumoniae

The 0.8 kb *Eco*RI-*Bam*HI insert of the recombinant plasmid pPG1 was subcloned into M13mp8/mp9. From the 1.8 kb *Eco*RI/*Pst*I insert of plasmid pCG6 (Fig. 3), unidirectional deletions were generated in the M13-derived vector phagescript SK using ExoIII and S1 nuclease (Guo *et al.*, 1983). The 2.4 kb *Eco*RV/*Dra*I insert of pCG8 (Fig. 3) was subcloned in pJDC9 and sequenced in the plasmid (Chen and Seeburg, 1985).

Reagents and general techniques

Restriction enzymes, T4 DNA ligase, and alkaline phosphatase were purchased from Boehringer (Mannheim, FRG), BRL (Gaithersburg, MD, USA) and New England Biolabs (Beverly, MA, USA) and used as described by the suppliers. Sequenase was from the United States Biochemical Corporation (Cleveland, OH, USA). All molecular manipulations were performed using standard procedures (Maniatis *et al.*, 1982) unless otherwise stated. Nucleotide sequencing was carried out by the chain-termination method (Sanger *et al.*, 1977) using [^{35}S]-dATP obtained from Amersham-Buchler (UK) in the sequenase reaction (Tabor and Richardson, 1987) and standard oligodeoxynucleotide primers.

Assay of PBPs

PBPs were visualized after incubation of cell lysates with [^{3}H]-propionylampicillin, followed by SDS-polyacrylamide gel electrophoresis and fluorography as described (Laible and Hakenbeck, 1987).

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