Nucleotide sequences of genes encoding penicillin-binding proteins from Streptococcus pneumoniae and Streptococcus oralis with high homology to Escherichia coli penicillin-binding proteins 1a and 1b.

C Martin, T Briese and R Hakenbeck

Nucleotide Sequences of Genes Encoding Penicillin-Binding Proteins from *Streptococcus pneumoniae* and *Streptococcus oralis* with High Homology to *Escherichia coli* Penicillin-Binding Proteins 1A and 1B

CHRISTIANE MARTIN, THOMAS BRIESE,† AND REGINE HAKENBECK*

Max-Planck Institut für molekulare Genetik, Ihnestrasse 73, D-1000 Berlin 33, Germany

Received 18 December 1991/Accepted 17 April 1992

The nucleotide sequence of a 3,378-bp DNA fragment of *Streptococcus pneumoniae* that included the structural gene for penicillin-binding protein (PBP) 1a (*ponA*), which encodes 719 amino acids, was determined. Homologous DNA fragments from an *S. oralis* strain were amplified with *ponA*-specific oligonucleotides. The 2,524-bp *S. oralis* sequence contained the coding region for the first 636 amino acids of a PBP. The coding sequence differed by 437 nucleotides (27%) and one additional triplet, resulting in 87 amino acid substitutions (14%), from *S. pneumoniae* PBP 1a. Both PBPs are highly homologous to bifunctional high-Mr, *Escherichia coli* PBPs 1a and 1b.

Penicillin-binding proteins (PBPs) are penicillin-sensitive enzymes that catalyze late steps in murein biosynthesis. Together with β-lactamases, they form a superfamily of penicillin-interacting serine D,D-peptidases (for a review, see reference 16). In all PBPs, several homology boxes within the penicillin-binding domain can be identified: the SXXK box with the active-site serine, an SXN box, and the K(H)T(S)G triad. The three motifs constitute essential components of the active-site cavity as deduced from the known three-dimensional arrangement of several β-lactamases and the *Streptomyces* strain R61 low-Mr PBP (11, 23, 35, 37).

On the basis of their amino acid sequence relatedness (and presumably in respect to their enzymatic function), PBPs can be divided into three groups: low-Mr PBPs that act as D,D-carboxypeptidases, high-Mr PBPs related to the *Escherichia coli* transpeptidases PBP 2 and/or 3, and the two bifunctional high-Mr *E. coli* PBPs 1a and 1b, which are homologous to each other but not to any of the other known PBPs (16). *E. coli* PBPs 1a and 1b contain, in addition to the penicillin-sensitive transpeptidase domain (penicillin-binding domain), an unusually long N-terminal extension responsible for a penicillin-insensitive, moenomycin-sensitive transglycosylase activity (24, 36, 44, 45).

*Streptococcus pneumoniae* contains six PBPs: high-Mr PBPs 1a, 1b, 2x, 2a, and 2b and low-Mr, D,D-carboxypeptidase PBP 3, with Mr's ranging between 92 and 43 kDa as judged from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18). The genes that encode PBPs 2x (30) and 2b (13) and parts of low-Mr PBP 3 (42) are known. PBPs 2x and 2b are related to *E. coli* PBPs 2 and 3, whereas the deduced 310-amino-acid peptide of PBP 3 shares homology with *E. coli* D,D-carboxypeptidases PBPs 5 and 6. This suggests that *S. pneumoniae* contains the same principal set of PBPs as *E. coli*, and one might therefore expect that the largest PBP (PBP 1a) is related to *E. coli* PBPs 1a and 1b. A homologous PBP has apparently been conserved in various streptococcal species, since antibodies raised against pneumococcal PBP 1a cross-react with a PBP from *Streptococcus pyogenes* and different viridans group streptococci (5, 20, 21).

**Cloning strategy for *S. pneumoniae* PBP 1a.** Since PBP 1a does not participate in early steps of penicillin resistance development, it was not possible to screen for penicillin-resistant transformants by using donor DNA from a penicillin-resistant mutant and a susceptible acceptor strain, as in the case of PBP 2x or 2b (12, 30, 31). Instead, by taking advantage of a series of antibodies raised against highly purified PBP 1a, a gene bank was constructed in bacteriophage expression vector λgt11, which has also been used to identify a PBP 3 gene fragment (42). Chromosomal DNA of laboratory strain R6 (1), prepared as described previously (30), was digested with FnuDII-HaeIII and ligated to EcoRI linkers prior to being cloned into the single EcoRI site of the lacZ gene in λgt11 (47). Phages were screened with mouse antisera specific for PBP 1a using the procedure described in reference 42. Plaques (4 × 10³) were analyzed, and phages giving a positive signal were rescreened twice and further characterized with a series of anti-PBP 1a monoclonal antibodies (MAbs). Anti-PBP 1a and anti-PBP 3 sera (19) and MAbs directed against PBPs 1a and 2b (17) have already been described. Briefly, they were obtained with purified preparations of the respective PBPs as antigens. Antibodies were used in dilutions of 1:400 to 1:3,000. No cross-reactivity to other PBPs, or other proteins in the case of the MAbs, was observed.

Three of nine phages reacted with MAb 301 and contained one 0.9-kb DNA insert when restricted with EcoRI endonuclease. Lysogens were generated in *E. coli* Y1089 (40), and fusion proteins were analyzed on Western blots (immunoblots) (19) with PBP 1a-specific antibodies, confirming the reactivity of the λgt11 recombinants (data not shown). The 0.9-kb fragment was subcloned into M13mp18 (46) grown on *E. coli* JM103 (34) and sequenced by the dideoxynucleotide chain termination method (41) using [α-³²P]dATP (Amer sham-Buchler) and the T7 Sequencing kit (Pharmacia, Uppsala, Sweden). Ambiguous DNA sequences were resolved by using dITP and the Sequenase reaction (U.S. Biochemical Corp., Cleveland, Ohio), deaza analogs (Pharmacia),

* Corresponding author.
† Present address: Institut für Virologie der Freien Universität im Robert-Koch Institut, D-1000 Berlin 65, Germany.
E. coli cells

on the included

Dassel, Amersham-Buchler) (mmol; at done

Dassel, Amersham-Buchler) labeling of

described (40) (Fig. 1).

Hybridization (40) (Fig. 1). Plasmid

pJDC9 after SacI-HindIII restriction, and recombinant plasmids pCM1 and pCM2 were isolated by colony hybridization (40) (Fig. 1). Transformation of E. coli DH5α (22) was performed with CaCl2-treated cells (40) or by the method described by Chung et al. (7). For radioactive labeling of DNA fragments, the multiprime DNA-labeling system (Amersham-Buchler) and [α-32P]dATP (3,000 Ci/ mmol; Amersham-Buchler) were used. The labeled probe was purified by Elutip columns (Schleicher und Schüll, Dassel, Germany). Hybridization and washing steps were done at 65°C. The Rsal-SacI fragment of pCM2 was then used as a probe to isolate pCM3, a pSU2719 derivative (32) grown on E. coli DH5α (22) with the strategy described above, which contained a 1.5-kb HaeIII fragment covering the Y' region of the PBP 1a gene (Fig. 1). Plasmid DNA from E. coli cells was isolated by the method of Birnboim and Doly (2). The sequence of a 3,378-bp DNA fragment that included the gene that encodes PBP 1a (ponA) was determined from both strands in the various subclones by using universal and reverse primers and a set of oligonucleotides that primed along the DNA sequence. It mapped close to the PBP 2x gene on the chromosome (15).

For analysis of the DNA sequences and for amino acid comparisons, a VAX-VMS computer was used with the software previously described by Devereux et al. (10). The PBP 1a gene started at position A-946TG and terminated with T-3103AA (Fig. 2). Downstream of the TAA stop codon, multiple stop sites occurred in all three reading frames. Upstream of the PBP 1a gene, another open reading frame (A-353TG to T-947GA) was found. A Shine-Dalgarno sequence and a possible promoter site were identified by comparison with E. coli consensus sequences, as indicated in Fig. 3. The promoter site corresponded to a major E. coli RNA polymerase-binding site when analyzed by electron microscopy (data not shown). The sequence around the A-946TG start codon was verified with different sequencing methods, including chemical sequencing as described by Maxam and Gilbert (33). No evidence for multiple translation starts, as has been found for E. coli PBP 1b, was obtained (4, 26).

The open reading frame of the PBP 1a gene encodes a 719-amino-acid protein with a predicted molecular mass of 79.8 kDa. This is in contrast to the apparent molecular mass of 92 kDa estimated for PBP 1a after SDS-PAGE on a 7.5% acrylamide gel. The reason for this discrepancy is not known.

The deduced amino acid sequence contained all of the features typical of high-M
cBPB: a hydrophobic domain which most likely acts as a membrane anchor at the N-terminal end (Y-12 to V-36) and a penicillin-binding domain with the three homology boxes S-370TMK, S-428RN, and K-557TG. In addition to a long N-terminal domain, PBP 1a carried a hydrophilic, unusually structured C-terminal extension.
**FIG. 2.** Nucleotide sequence of a 3,378-bp DNA fragment that encodes *S. pneumoniae* PBP 1a and comparison to a homologous gene from *S. oralis*. The pneumococcal sequence and the deduced amino acids are numbered; *S. oralis* sequences are marked by an asterisk. The putative Shine-Dalgarno sequence, promoter, and terminator region are underlined. The three amino acid homology boxes are marked.

**Isolation and subcloning of homologous DNA from *Streptococcus oralis*...** Preliminary experiments detected PBPs that cross-react with antipneumococcal antibodies in various streptococcal species, including *S. pyogenes*, several viridans group streptococci, and *S. oralis* (5, 20, 21). PBPs from *S. oralis* 20066 (= *Streptococcus sanguis* II Kiel 25826), 20379 (= *Streptococcus viridans* II Kiel 59015), and 20395 (= *S. viridans* I Kiel 43364) (3), obtained from the Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany, were investigated further by using a set of antisera and MABs directed against *S. pneumoniae* PBPs 1a, 2b, and 3. *S. pyogenes* 7904 has already been described (20). Streptococci were grown in C medium (28) supplemented with 0.2% yeast extract (Difco, Detroit, Mich.). Pneumococci were lysed upon addition of Na-phosphate buffer (20 mM, pH 7.2) containing 0.1% Triton X-100. *S. oralis* and *S. pyogenes* cells were resuspended in the same buffer containing 0.2 mg of M1 muramidase (Sigma, Deisenhofen, Germany) per ml and 0.4 mg of lysozyme per ml. For radioactive labeling, resuspended cells (approximately 2 × 10^6 to 4 × 10^6 per sample) were incubated for 30 min at 37°C with [3H]propionyl-lactate as previously described (43). Details of SDS-PAGE and fluorography have already been described (29). Immunological detection of PBPs after Western blotting (immunoblotting) was done as previously described (19). Each of the strains contained a distinct PBP profile and a different antibody reactivity pattern. Examples are shown in Fig. 3. Of the strains reacted with both rabbit and mouse anti-PBP 1a sera and with at least one anti-PBP 1a MAb, whereas *S. pyogenes* reacted with polyclonal rabbit antiserum only. In none of the strains did the low-M₉ PBP react with anti-PBP 3 serum.

Different oligonucleotides specific for the *S. pneumoniae* PBP 1a gene were tested to amplify homologous regions from *S. oralis* chromosomal DNA. With three sets of primers, DNA fragments were obtained from *S. oralis* 20379 with sizes as expected from the locations of the oligonucleotides. No oligonucleotides were found that primed further down-
FIG. 3. Immunological relationship of pneumococcal PBPs and PBPs from *S. pyogenes* and *S. oralis*. Cell lysates were blotted onto nitrocellulose and immunostained with different antipeumococcal PBP antibodies. Aliquots were labeled with [125I]benzylpenicillin for preparation of a fluorogram after SDS-PAGE. (A) Western blot after immunostaining with anti-PBP 1a antibodies: a, rabbit antiserum 1220; b, mouse antiserum G13; c, MAb 221; d, MAb 301. The anti-PBP 2b antibodies were MAb 410 (c') and MAb 16 (d'). One anti-PBP 1a MAB (dark arrowheads) and one anti-PBP 2b MAB (light arrowheads) were used on the same blot strip. Lanes: R6, *S. pneumoniae* R6; S.p., *S. pyogenes* 7904; S.o.1, *S. oralis* 20066; S.o.2, *S. oralis* 20379; S.o.3, *S. oralis* 20395. (B) Fluorogram of PBPs. *S. pneumoniae* PBPs are indicated on the left. The molecular weights of PBPs 1a and 2b, as judged from the gels, are 92,000 and 78,000, respectively.

stream in the 3'-flanking region of the gene. Amplification trials using chromosomal DNAs of the other two *S. oralis* strains failed.

Amplification of *S. oralis* DNA with the polymerase chain reaction was carried out in a 100-μl reaction mixture containing 1 μg of chromosomal DNA, 1 mM synthetic oligonucleotide primers, 2 U of *Taq* DNA polymerase (Perkin Elmer, Norwalk, Conn.), deoxynucleoside triphosphates, and buffer as provided by the manufacturer. Thirty rounds of amplification with 6 min of extension at 72°C, 1.5 min of denaturation at 95°C, and 1.5 min of annealing at 52°C were carried out by using a Pharmacia LKB Gene ATQA Controller. The following pairs of oligonucleotide primers and cloning vectors were used for polymerase chain reaction amplification of three *S. oralis* DNA fragments: (i) pGC TCTAGA-924TTATATTAGGTGCTAAACCA-944 with a terminal XbaI site and pG-1598GTCATATTGTGTGGGC-1579 cloned into SmaI-digested pSU2719, (ii) pA-1469CTAGCTAATGGCAATAT-1488 and pT-2873CTC TGACGATCCCCCTCTGG-2854 cloned into M13mp19-digested with SmaI, and (iii) pA-274AACGCTAGCCATTTT TAA-293 and pG-1598GTCATATTGTGTGGGC-1579. This last fragment was not cloned but used directly for DNA sequencing. The ends of polymerase chain reaction fragments which were cloned were filled with Klenow polymerase and treated with kinase. Restriction fragments and polymerase chain reaction-amplified DNA fragments were isolated after separation from agarose gels by using the Biotrap electroseparation system (Schleicher und Schull). For direct sequencing, DNA fragments were further purified over miniprep spun columns (Pharmacia) as described by the manufacturer. The error introduced by *Taq* polymerase was less than 0.1%, as judged by multiple sequencing data obtained in the laboratory.

Comparison of *S. pneumoniae* and *S. oralis* sequences. The alignment of the *S. pneumoniae* and *S. oralis* sequences and their deduced amino acids are shown in Fig. 2. In the 1,908-bp coding region determined in the *S. oralis* PBP gene, 437 nucleotides (22.9%) differed from the *S. pneumoniae* sequence; the region upstream of the coding sequence differed by only 14%. Eighty-seven nucleotide changes were nonsynonymous (20% of all changes). One additional triplet, which encodes a glycine residue, was found in the N-terminal region of the PBP. The AT content of the *S. oralis* coding region (56.3%) was almost identical to that of the *S. pneumoniae* ponA gene, whereas upstream sequences had an AT content of 59% compared with the 62.5% of pneumococcus DNA. The codon usage of the *S. oralis* gene differed mainly by higher usage of the triplets GTG (Gly), GAT (Asp), GCT (Ala), AGC (Thr), and CGT (Arg).

The two streptococcal species are closely related, as indicated by the presence of ribitol teichoic acid and choline residues in their cell walls; they can be distinguished by their peptidoglycan, which contains different types of cross-linkages (27). Hybridization data obtained with chromosomal DNA confirmed a high degree of homology between the two streptococci (53% [27]). For an individual gene (amylomaltsase), only 4 to 6% divergence between the two species has been reported (14), which is less than the 14% divergence found in the 5'-flanking region of the PBP 1a gene or the 23% found within the PBP 1a coding region. These numbers are not necessarily in conflict, since individual genes may well evolve at different rates. A relatively high intraspecies variation was apparent in the amylomaltsase gene (3 to 8%) and was also evident in the variability of the PBP profiles and antibody reactivities of individual *S. oralis* PBPs (Fig. 3).

Comparison with *E. coli* PBPs 1a and 1b. The deduced amino acid sequence of *S. pneumoniae* PBP 1a showed no significant similarities to that of pneumococcal PBP 2x (30) or 2b (13), except to the regions around the homology boxes and the peptide stretch A-489FANGG, nor to that of any low-M₁ PBP or β-lactamase. However, a high degree of homology was made apparent by alignment with bifunctional *E. coli* PBPs 1a and 1b (4), except to the very N-terminal part of PBP 1b and the two insertions seen in *E. coli* PBP 1a compared with PBP 1b (4).

Regions of high similarity to both *E. coli* PBPs could be divided into an N-terminal part from L-62 to I-242 of *S. pneumoniae* PBP 1a and the penicillin-binding domain between residues A-330 and G-598. In the N-terminal part, we identified 10 highly homologous stretches in which *S. pneumoniae* PBP 1a has 41 and 37% of its amino acids identical to those of *E. coli* PBP 1a and 1b, respectively, i.e., homology as high as that of the two *E. coli* PBPs to each other (Fig. 4). In this alignment, the one amino acid gap in the *S. pneumo-
niae PBP 1a sequence at N-117 coincides with the position of the extra glycine residue of the S. oralis PBP.

The penicillin-binding domain contained five highly homologous stretches, including the three homology motifs N-338 to G-346, R-366 to P-374 with the active site serine, A-424 to V-434 with the SXN box, Y-487 to G-494, and A-555 to N-562 with the KTG triad. This part contained 25 and 27% identities, respectively, also as high as in the comparison between the two E. coli PBPs.

S. pneumoniae PBP 1a is the first high-Mr PBP of a gram-positive bacterium that shows extensive homology to bifunctional E. coli PBPs 1a and 1b. This confirms the structural relationship between S. pneumoniae and E. coli PBPs that has already been observed with pneumococcal PBPs 2x, 2b, and 3 and E. coli PBPs 3, 2, and 5/6, respectively (4, 12, 16, 30, 42), thus raising the question of functional similarities between PBPs of a gram-positive coccus and a gram-negative rod. The degree of homology between S. pneumoniae PBP 1a and E. coli PBPs 1a and 1b is much higher than that found for the other PBPs, suggesting pressure for maintenance of the structure (and thereby function) of this protein.

The transglycosylase domain of E. coli PBP 1b has been identified by deletion mutant analysis (36) and by assaying the impact on enzymatic activity of PBPs in the presence of various MAbs directed against different epitopes (9). The relatedness between the transglycosylase domain of E. coli PBPs 1a and 1b and the N-terminal extension of the pneumococcal PBP with 40% identical amino acids could mean that the latter protein also contains a transglycosylase activity. On the other hand, PBP-independent transglycosylase activity has been reported in S. pneumoniae and other gram-positive cocci as well, where no transglycosylase activity was found associated with the purified PBP fraction (38, 39), whereas the opposite was found in bacilli (25). It is possible that the substrate requirement of the presumed transglycosylase activity of S. pneumoniae PBP 1a differs from that of the E. coli enzymes. Determination of the actual enzymatic function of the S. pneumoniae PBP will require sufficient amounts of the protein. The identification of the gene described here provides the prerequisite for construction of an overexpressed derivative suitable for those studies.

Nucleotide sequence accession number. The sequences of the S. pneumoniae PBP 1a and S. oralis PBP 1 genes have been deposited in GenBank under accession numbers M90527 and M90528, respectively.

We thank Mike Hearne for synthesis of oligonucleotides. This work was supported by European Economic Community contract EEC SCI*0141-SC (AM) and the Fonds der Chemischen Industrie im Verband der Chemischen Industrie E.V.

REFERENCES
VOL. 29.


