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

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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-M_r *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-M_r 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-M_r PBPs that act as D,D-carboxypeptidases, high-M_r PBPs related to the *Escherichia coli* transpeptidases PBP 2 and/or 3, and the two bifunctional high-M_r *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-M_r PBPs 1a, 1b, 2x, 2a, and 2b and low-M_r D,D-carboxypeptidase PBP 3, with M_rs 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-M_r 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 pneu-

mococcal 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 antiserum specific for PBP 1a by using the procedure described in reference 42. Plaques (4×10^5) 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-^{35}S]dATP (Amersham-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),

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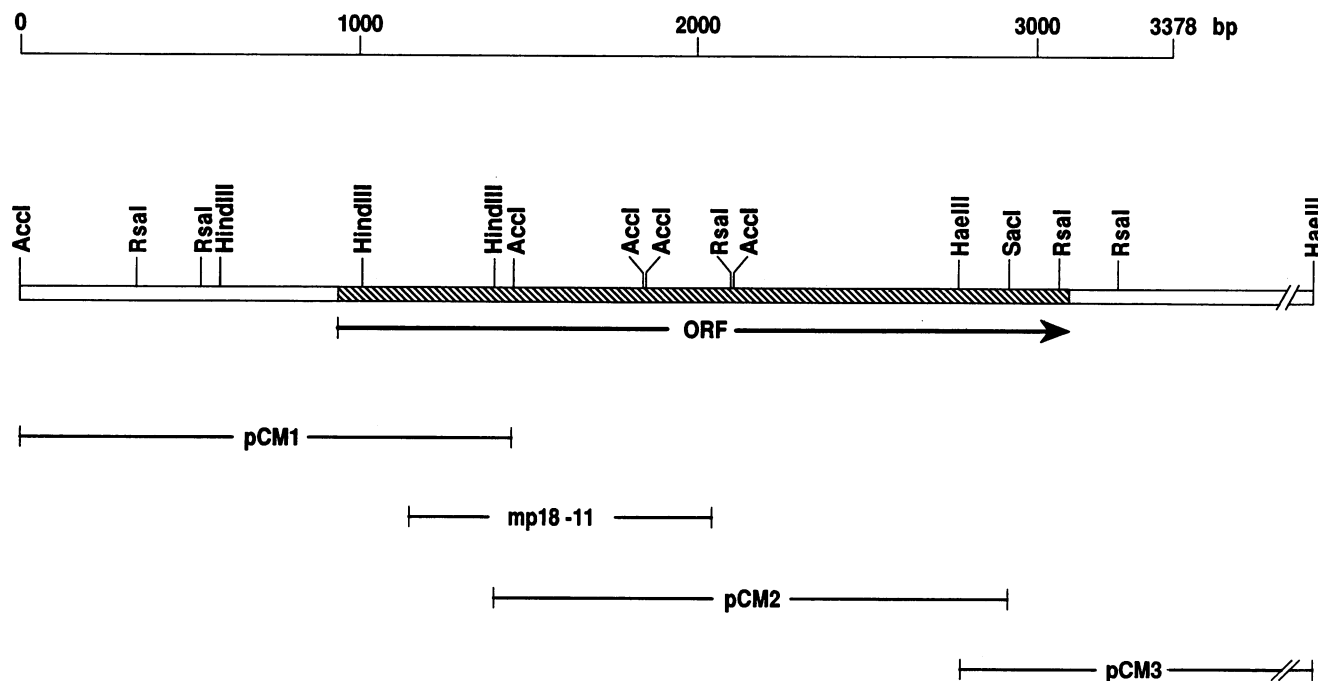
FIG. 1. Restriction map of the PBP 1a gene of *S. pneumoniae* and flanking regions. Restriction sites used for isolation of the different recombinant plasmids with overlapping inserts are shown. The shaded area indicates the coding region of PBP 1a. The DNA sequence was determined for the 3,378-bp region shown at the top. ORF, open reading frame.

and/or higher temperature for sequencing with the *Taq* Track Sequencing core system Deaza kit (Promega, Heidelberg, Germany). The 0.9-kb DNA fragment codes for a 300-amino-acid peptide which showed strong homology to the N-terminal parts of *E. coli* PBPs 1a and 1b, demonstrating that it represents an internal gene fragment.

By taking advantage of a single *Hin*dIII site located within the 0.9-kb fragment, we used the resulting 0.3- and 0.6-kb fragments as hybridization probes labeled with a nonradioactive labeling and detection kit (Boehringer, Mannheim, Germany) to identify 5'- and 3'-flanking regions in chromosomal DNA restricted with *Acc*I and *Sac*I-*Hin*dIII, respectively. Transfer of restriction fragments to a nylon membrane (Gene Screen Plus, NEN-DuPont, Boston, Mass.) was done by the method of Davis et al. (8). DNA fragments of the corresponding sizes were cloned into the *Sma*I site of pJDC9 (6) or into pJDC9 after *Sac*I-*Hin*dIII restriction, and recombinant plasmids pCM1 and pCM2 were isolated by colony hybridization (40) (Fig. 1). Transformation of *E. coli* DH5 (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 *Rsa*I-*Sac*I 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 *Hae*III fragment covering the 3' 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*ᵣ PBPs: 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.

1 GCGACTCAGTTTCATCTGATTGCCTTCATTCCAATTTTTCCCCATGGTTTCAAAAGCAAAAATGGTGGCCAACTGGAAACCGTATTCGGTTTTCATCTCCTGAGCAACCTCTAAAACCCAA

121 TATTCAAAACCCAAACTTCCTGTAAACACAAGCCAAGACACCCCATCTGCTGCCATAGCCTCTAAATCTTTACGTATCGCTTTTTTAATCAATTTAAGACGAGGATCCTTATCAGAAAAT

241 AAACCCAAATCAAAAGCAGAATAGCCTAAAACCAAAGCTGTAGCCATTT<u>TTAACC</u>CTTTCTGTGCAAATTATGG<u>TATAAT</u>AAAGTGATGTTATTGTACCAATAA<u>GGAG</u>AATTATGGTCAA

*   A-------------------------------

361 CTATCCACATAAAGTTTCATCACAAAAAAGACAAACATCTCTTTCTCAACCCAAAAATTTCGCAAATCGAGGAATGTCTTTTGAAAAGATGATCAATGCTACCAACGACTACTATTTGTC

*   -------------A----------G--G------G-TC-C-CG--A---A-T--------T--------G--------------------------G-----T--T--C-----

481 TCAGGGCTTGGCTGTTATACATAAGAAACCAACTCCTATTCAAATCGTACAAGTGGACTATCCACAACGAAGTCGTGCCAAGATTGTTGAAGCCTATTTTCGACAAGCTTCAACGACGGA

*   G--T--G--A--A--G--T--C--------G-----C--C----------GT--C--T-----C-----------A--------C-----------C--CA-------------C--A--

601 CTATTCTGGCGTTTATAATGGATATTACATCGACTTTGAAGTCAAGGAAACAAAACAAAAACGTGCGATTCCGATGAAAAATTTTCATCCACATCAGATTCAGCATATGGAACAAGTCCT

*   ------A--G------G---------------------CA--------T-GG-------A---------------G-----C----AC-------------------G--------

721 TGCCCAACAAGGAATCTGCTTTGTCCTTCTTCACTTTTCTTCTCAGCAAGAAACCTACTTATTGCCGGCATTCGATTTGATTCGCTTCTATCATCAAGATAAGGGACAAAAATCAATGCC

*   ------G-G----------------------------G----------------T--T-----------TG-T--CC-------T--------------------G--------

841 ACTTGAATATATTCGAGAATATGGATATGAAATCAAGGCTGGTGCCTTCCCTCAAATTCCTTATCTCAATGTTATCAAAGAACATTTATTAGGTGGTAAAACAAGATGAACAAACCAACG

*   -----G-------------A-C------AGG--TG--CTA--------T--C--G-----C------G--A----------------C---------------------A---T

                                                                                                                MetAsnLysProThr

*   ---------Gln---

961 ATTCTGCGCCTAATCAAGTATCTGAGCATTAGCTTCTTAAGCTTGGTTATCGCAGCCATTGTCTTAGGCGGAGGAGTTTTTTTCTACTACGTTAGCAAGGCTCCTAGCCTATCCGAGAGT

*   --C-----AA--GCT--A---A-C--T--CT---------CTG-AT----------T---A-GC-G--------TC-C---C-------T--------T-----AGC------T--A---

6 IleLeuArgLeuIleLysTyrLeuSerIleSerPheLeuSerLeuValIleAlaAlaIleValLeuGlyGlyGlyValPhePheTyrTyrValSerLysAlaProSerLeuSerGluSer

*   ---------IleAla------Ile------Cys------ThrValPhe-----------Met------------Leu---Leu------------Asn------Ala------------

1081 AAACTAGTTGCAACAACTTCTAGTAAAATCTACGACAATAAAAATCAACTCATTCTGACTTGGGTTCTGAACGCCGCGTCAATGCCCAAGCTAATGATATTCCCACAGATTTGGTTAAG

*   -----G--C--T-----G--C--C-----T--T--------CG--G-------C-----TC-T--A--A-----T--A--------G-----A--C--A--A--G--C--------C--C

46 LysLeuValAlaThrThrSerSerLysIleTyrAspAsnLysAsnGlnLeuIleAlaAspLeuGlySerGluArgArgValAsnAlaGlnAlaAsnAspIleProThrAspLeuValLys

*   ---------------------------------------AsnAspGlu---Glu-----------------Asn

1201 GCAATCGTTTCTATCGAAGACCATCGCTTCTTCGACCACAGGGGGATTGATACCATCCGTATCCTGGGAGCTTTCTTGCGCAATCTGCAAAGCAAT...TCCCTCCAAGGTGGATCAGCT

*   --T---T-----G--T-----------------TA-T---C-A--A--C-----T-----------A--T---AC-C-A--A-----T-GTG-TGGCGGAGG---T-----A-CT---A--

86 AlaIleValSerIleGluAspHisArgPhePheAspHisArgGlyIleAspThrIleArgIleLeuGlyAlaPheLeuArgAsnLeuGlnSerAsn...SerLeuGlnGlyGlySerAla

*   -------------------------------------Asn---------------------------------Thr------------ArgGlyGlyGlyGly---------Ala---Thr

1318 CTCACTCAACAGTTGATTAAGTTGACTTACTTTTCAACTTCGACTTCCGACCAGACTATTTCTCGTAAGGCTCAGGAAGCTTGGTTAGCGATTCAGTTAGAACAAAAAGCAACCAAGCAA

*   T-G--G---G--------C-----A--C--T--C--T--G--A-----T-----A--CC----------------------A---C-C--CG-C---C-------------G--T--A---

125 LeuThrGlnGlnLeuIleLysLeuThrTyrPheSerThrSerThrSerAspGlnThrIleSerArgLysAlaGlnGluAlaTrpLeuAlaIleGlnLeuGluGlnLysAlaThrLysGln

*   ---Leu-------------------------------Val-----------------------

1438 GAAATCTTGACCTACTATATAAATAAGGTCTACATGTCTAATGGGAACTATGGAATGCAGACAGCAGCTCAAAACTACTATGGTAAAGACCTCAATAATTTAAGTTTACCTCAGTTAGCC

*   -----------------C--C--C-----T--------A--C--------C--------A--G--T-------GT--------C-----------AG----G---A-C-----A--G---

165 GluIleLeuThrTyrTyrIleAsnLysValTyrMetSerAsnGlyAsnTyrGlyMetGlnThrAlaAlaGlnAsnTyrTyrGlyLysAspLeuAsnAsnLeuSerLeuProGlnLeuAla

*   ---Ser------------------LysAsp------Ile------------

1558 TTGCTGGCTGGAATGCCTCAGGCACCAAACCAATATGACCCCTATTCACATCCAGAAGCAGCCCAAGACCGCCGAAACTTGGTCTTATCTGAAATGAAAAATCAAGGCTACATCTCTGCT

*   C-C--T--C--------------T--------G-----T--A--C--G-----G-----C--------A-----T--TC-C---C-C-----G-----GGGG-----T-----TA-A--C

205 LeuLeuAlaGlyMetProGlnAlaProAsnGlnTyrAspProTyrSerHisProGluAlaAlaGlnAspArgArgAsnLeuValLeuSerGluMetLysAsnGlnGlyTyrIleSerAla

*   ---Glu---------------------------Gly------------Thr---

1678 GAACAGTATGAGAAAGCAGTCAATACACCAATTACTGATGGGCTACAAAGTCTCAAATCAGCAAGTAATTACCCTGCTTACATGGATAATTACCTCAAGGAAGTCATCAATCAAGTTGAA

*   -----A--C--A--------GA-T-----T--G-----A-----A--T-----C--A--------T-AC-G---T---C-A--------------------A--G--G--TG-C---------

245 GluGlnTyrGluLysAlaValAsnThrProIleThrAspGlyLeuGlnSerLeuLysSerAlaSerAsnTyrProAlaTyrMetAspAsnTyrLeuLysGluValIleAsnGlnValGlu

*   -----------------Ile---------------------------------------AsnSer------Pro-----------------------------Asp---------

1798 GAAGAAACAGGCTATAACCTACTCACAACTGGGATGGATGTCTACACAAATGTAGACCAAGAAGCTCAAAAACATCTGTGGGATATTTACAATACAGACGAATACGTTGCCTATCCAGAC

*   C-G--------T--C-----TT-A--T--G--T-----A-----------C--T--TTCTA-G-TC---C---G---C-----------------G-----G--T--CAA---------T

285 GluGluThrGlyTyrAsnLeuLeuThrThrGlyMetAspValTyrThrAsnValAspGlnGluAlaGlnLysHisLeuTrpAspIleTyrAsnThrAspGluTyrValAlaTyrProAsp

*   Gln-----------------------------Glu----------SerLysVal---GlnArg--------------------------------Asn---------

1918 GATGAATTGCAAGTCGCTTCTACCATTGTTGATGTTTCTAACGGTAAAGTCATTGCCCAGCTAGGAGCACGCCATCAGTCAAGTAATGTTTCCTTCGGAATTAACCAAGCAGTAGAAACA

*   -----G--A--G--A--C-----T--C--------CA-AG----A--------------AT----T--T-----C--A--------C--------T-----A-----G--T-------T

325 AspGluLeuGlnValAlaSerThrIleValAspValSerAsnGlyLysValIleAlaGlnLeuGlyAlaArgHisGlnSerSerAsnValSerPheGlyIleAsnGlnAlaValGluThr

*   -----------------------------------ThrAsp---

FIG. 2—*Continued on following page.*

2038 AACCGCGACTGGGGATCAACTATGAAACCGATCACAGACTATGCTCCTGCCTTGGAGTACGGTGTCTACGAGTCAACTGCCACTATCGTTCACGATGAGCCCTATAACTACCCTGGGACA

* -----T--------T--T--C-----G--T--T-----------------T-----A-----C--T--T--T--------TT-G--T---------AGT--T-----T-----C--A--C

365 AsnArgAspTrpGly__SerThrMetLys__ProIleThrAspTyrAlaProAlaLeuGluTyrGlyValTyrGluSerThrAlaThrIleValHisAspGluProTyrAsnTyrProGlyThr

* --Asp---------Ser------------Ser--------------------

2158 AATACCCCTGTTTATAACTGGGATAGGGGCTACTTTGGCAACATCACCTTGCAATACGCCCTGCAACAATCGCGAAACGTCCCAGCCGTGGAAACTCTAAACAAGGTCGGACTCAACCGC

* TC---------C-------------A-A-T--------A-----A--AC-C--G--T--T--C--G-----A--G--T--A--G--T--A-----G---G-G--A--A--G---G-T--T

405 AsnThrProValTyrAsnTrpAspArgGlyTyrPheGlyAsnIleThrLeuGlnTyrAlaLeuGlnGln__SerArgAsn__ValProAlaValGluThrLeuAsnLysValGlyLeuAsnArg

* Ser-------------------LysSer---Glu------------Asp---

2278 GCCAAGACTTTCCTAAATGGTCTCGGAATCGACTACCCAAGTATTCACTACTCAAATGCCATTTCAAGTAACACAACCGAATCAGACAAAAAATATGGAGCAAGTAGTGAAAAGATGGCT

* --------G--T--------A-----T--T-----------C--A-----TG-T-----T-----------T-----------T-----------G-----------C--C--G---------

445 AlaLysThrPheLeuAsnGlyLeuGlyIleAspTyrProSerIleHisTyrSerAsnAlaIleSerSerAsnThrThrGluSerAspLysLysTyrGlyAlaSerSerGluLysMetAla

* --Ala--

2398 GCTGCTTACGCTGCCTTTGCAAATGGTGGAACTTACTATAAACCAATGTATATCCATAAAGTCGTCTTTAGTGATGGGAGTGAAAAAGAGTTCTCTAATGTCGGAACTCGTGCCATGAAA

* --------T-----T-----C-----C--T-TC---CCG-----T---------A----GA-T--------------C---TC------A------G--TCT--T--A-----T-----

485 AlaAlaTyrAlaAlaPheAlaAsnGlyGlyThrTyrTyrLysProMetTyrIleHisLysValValPheSerAspGlySerGluLysGluPheSerAsnValGlyThrArgAlaMetLys

* ------------------------------Ile---Pro-----------Asn---Ile-----------------Ser------------AspSer-----------------

2518 GAAACGACAGCCTATATGATGACCGACATGATGAAAACAGTCTTGAGTTATGGAACTGGACGAAATGCCTATCTTGCTTGGCTCCCTCAGGCTGGTAAAACAGGAACCTCTAACTATACA

* --------------------A--T-----------C--T---GCA--C--------T---GGA-----C--CC-------A--A--A-----------T--T--G--A---------

525 GluThrThrAlaTyrMetMetThrAspMetMetLysThrValLeuSerTyrGlyThrGlyArgAsnAlaTyrLeuAlaTrpLeuProGlnAlaGly__LysThrGly__ThrSerAsnTyrThr

* ---Ala---------------Gly---------Pro--

2638 GACGAGGAAATTGAAAACCACATCAAGACCTCTCAATTTGTAGCACCTGATGAACTATTTGCTGGCTATACGCGTAAATATTCAATGGCTGTATGGACAGGCTATTCTAACCGTCTGACA

* --T--T--G---------T----T--A-ATA-GGGT-A------T--G--C---A-G--C-T---------T----------------C--T-----T--T--C--G-----C--T--T

565 AspGluGluIleGluAsnHisIleLysThrSerGlnPheValAlaProAspGluLeuPheAlaGlyTyrThrArgLysTyrSerMetAlaValTrpThrGlyTyrSerAsnArgLeuThr

* ---Asp------------Tyr------AsnThrGlyTyr--------------Met---Val--

2758 CCACTTGTAGGCAATGGCCTTACGGTCGCTGCCAAAGTTTACCGCTCTATGATGACCTACCTGTCTGAAGGAAGCAATCCAGAGGATTGGAATATACCAGAGGGGCTCTACAGAAATGGA

* --TA-C--T--TG-----T-CTAT--T-----T--G---------T--G---------T--T--A--------ATGATC----T-GT--C-----C---G

605 ProLeuValGlyAsnGlyLeuThrValAlaAlaLysValTyrArgSerMetMetThrTyrLeuSerGluGlySerAsnProGluAspTrpAsnIleProGluGlyLeuTyrArgAsnGly

* ---Ile------Asp---PheTyr---AspAspHis---Gly------ThrMet

2878 GAATTCGTATTTAAAAATGGTGCTCGTTCTACGTGGAGCTCACCTGCTCCACAACAACCCCCATCAACTGAAAGTTCAAGCTCATCATCAGATAGTTCAACTTCACAGTCTAGCTCAACC

645 GluPheValPheLysAsnGlyAlaArgSerThrTrpSerSerProAlaProGlnGlnProProSerThrGluSerSerSerSerSerSerAspSerSerThrSerGlnSerSerSerThr

2998 ACTCCAAGCACAAATAATAGTACGACTACCAATCCTAACAATAATACGCAACAATCAAATACAACCCCTGATCAACAAAATCAGAATCCTCAACCAGCACAACCATAACATTTATCATCC

685 ThrProSerThrAsnAsnSerThrThrThrAsnProAsnAsnAsnThrGlnGlnSerAsnThrThrProAspGlnGlnAsnGlnAsnProGlnProAlaGlnPro*

3118 AGATTTTTCTGGGTGATTTTTTCATAGCACTATTGTATTTATGTGCTTAAC__AAAAGCCTGAGAA__GACATC__TTCTCAGGCTTTT__TCTGTACTATAAACCTCATTGGAAAAGATAGAGACTA

3238 CTTCCGTAATCTTGTTAAGAGGTTTTACTAAATACTAGTCTTTCTTCGTTTTTACTACAAAGAGAGCAGATAGGGCTAGGCTAACACTTGCTAGGAAGAGGGCTGTGTCAAATTGACTCT

3358 CACCTGTTGCTGGCAAGATTT

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 2×10^8 to 4×10^8 per sample) were incubated for 30 min at 37°C with [^{3}H]propionylampicillin synthesized 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. All 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-M_r 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-
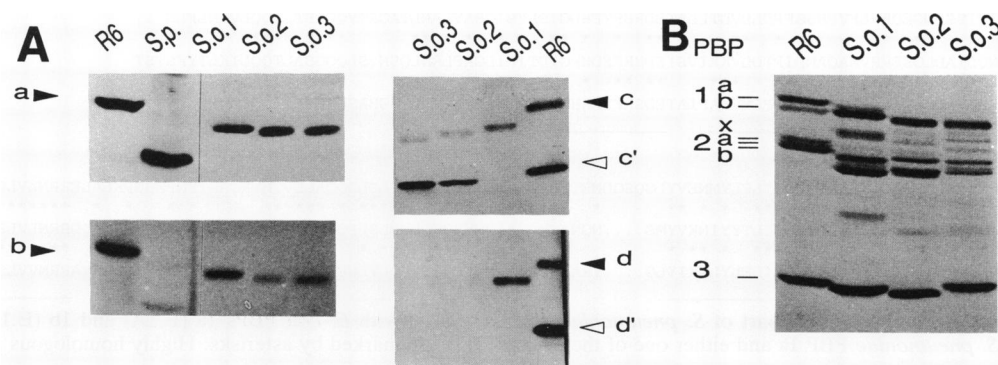
FIG. 3. Immunological relationship of pneumococcal PBPs and PBPs from *S. pyogenes* and *S. oralis*. Cell lysates were blotted onto nitrocellulose and immunostained with different antipneumococcal PBP antibodies. Aliquots were labeled with [^{35}S]benzylpenicillin for preparation of a fluorogram after 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 I6 (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 ATAQ 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-924TTTATTAGGTGGTAAAACAA-944 with a terminal *Xba*I site and pG-1598GGTCATATTGGTTTG GTGC-1579 cloned into *Sma*I-digested pSU2719, (ii) pA-1469CATGTCTAATGGGAACTAT-1488 and pT-2873TTC TGTAGAGCCCCTCTGG-2854 cloned into M13mp19 digested with *Sma*I, and (iii) pA-274AAGCTGTAGCCATTTT TAA-293 and pG-1598GGTCATATTGGTTTGGTGC-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 Schüll). 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-termi-

nal 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 GGT (Gly), GAT (Asp), GCT (Ala), ACG (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 (amylomaltase), 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 amylomaltase 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-M_r 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-*

```
E.1B  198  DPRLITMISSPNGEQR.LFVPRSGFPDLLVDTLLATEDRHFYEHDGISLYSIGRAVLANLTAGRTVQGASTLTQQLVKNLFLS...
             *  *      *  **    **  *  *  **    *  **  *  **     **  *  *****  *     *
S.1A   55  IYDNKNQLIADLGSERRVNAQANDIPTDLVKAIVSIEDHRFFDHRGIDTIRILGAFLRNLQSN.SLQGGSALTQQLIKLTYFSTST
             **   ***  *  **     **   ***    ** **  *  *  *    *  *    * *    **  *  ****      *
E.1A   50  IYSADGELIAQYGEKRRIPVTLDQIPPEMVKAFIATEDSRFYEHHGVDPVGIFRAASVALFSGHASQGASTITQQLARNFFLS...
```

```
E.1B       SERSYWRKANEAYMALIMDARYSKDRILELYMNEVYLGQSGDNEIRGFPLASLYYFGRPVEELSLDQQALLVGMVKGASIYNPWRNPKLALERRNLVLRLLQQQQII  386
             *   *** **  *        *  **  *  *  **     *  *  *   *  *     *** *  *** **     **  *   *  *  ******    *  *
S.1A       SDQTISRKAQEAWLAIQLEQKATKQEILTYYINKVYMS....NGNYGMQTAAQNYYGKDLNNLSLPQLALLAGMPQAPNQYDPYSHPEAAQDRRNLVLSEMKNQGYI  242
             *  **  *  ***   **  **  ***  *  *  *  **    *  ***  *  **    *  *   *  **  *  **    *     *  ***  ***  *   ***
E.1A       PERTLMRKIKEVFLAIRIEQLLTKDEILELYLNKIYLG....YRAYGVGAAAQVYFGKTVDQLTLNEMAVIAGLPKAPSTFNPLYSMDRAVARRNVVLSRMLDEGYI  235
```

FIG. 4. Alignment of the N-terminal part of *S. pneumoniae* PBP 1a (S.1A) with *E. coli* PBPs 1a (E.1A) and 1b (E.1B). Amino acids identical between *S. pneumoniae* PBP 1a and either one of the *E. coli* PBPs are marked by asterisks. Highly homologous peptide stretches are underlined.

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-M_r 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.

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