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Cloning of Genes Encoding a 15,000-Dalton Peptidoglycan-Associated Outer Membrane Lipoprotein and an Antigenically Related 15,000-Dalton Protein from *Haemophilus influenzae*

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We have cloned and expressed in *Escherichia coli* a gene encoding a 15,000-apparent-molecular-weight peptidoglycan-associated outer membrane lipoprotein (PAL) of *Haemophilus influenzae*. The nucleotide sequence of this gene encodes an open reading frame of 153 codons with a predicted mature protein of 134 amino acids. The amino acid composition and sequence of the predicted mature protein agree with the chemically determined composition and partial amino acid sequence of PAL purified from *H. influenzae* outer membranes. We have also identified a second gene from *H. influenzae* that encodes a second 15,000-apparent-molecular-weight protein which is recognized by antiserum against PAL. This protein has been shown to be a lipoprotein. The nucleotide sequence of this gene encodes an open reading frame of 154 codons with a predicted mature protein of 136 amino acids and has limited sequence homology with that of the gene encoding PAL. Southern hybridization analysis indicates that both genes exist as single copies in *H. influenzae* chromosomal DNA. Both genes encode polypeptides which have amino-terminal sequences similar to those of reported membrane signal peptides and are associated primarily with the outer membrane when expressed in *E. coli*.

The gram-negative bacterium *Haemophilus influenzae* is a major human pathogen. *H. influenzae* type b is the most frequent cause of meningitis in children aged 1 to 5 years and causes other invasive diseases in this group (11). Nontypable *H. influenzae* has also been implicated in a number of diseases, including pneumonia, bacteremia, meningitis, postpartum sepsis, and acute febrile tracheobronchitis in adults (35), and causes 20 to 40% of otitis media in children (15). A vaccine composed of purified type b capsular polysaccharide has recently been developed and has proven effective against *H. influenzae* type b disease in children aged 2 to 5 years (38). However, children under the age of 2 years, in whom 50 to 60% of the disease occurs (38), respond poorly to this vaccine. In addition, this vaccine is of no value against nontypable *H. influenzae* disease. Hence, other cell surface components, and in particular outer membrane proteins, have been looked at as potential vaccine candidates (for a recent review, see reference 13). One problem with this approach is that *H. influenzae* and *H. influenzae* type b isolates from independent sources are genetically diverse (36, 37). Specifically, outer membrane proteins from such isolates have been shown to vary electrophoretically (3, 4, 20, 21) and antigenically (10, 17, 33). However, recent studies have demonstrated that a peptidoglycan-associated outer membrane protein (OMP) of apparent molecular weight 15,000 can be found by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in all tested isolates of *H. influenzae* (types a to f and nontypable) (3, 20). This protein is highly conserved antigenically (14, 34, 35), and antibody raised against this protein activates complement-mediated killing of *H. influenzae* cells and is protective against *H. influenzae* type b infection in infant-rat protection studies (14, 32, 34). This protein has also been shown to be modified by fatty acylation (G. A. Weinberg, D. Towler, and R. S. Munson, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, K142, p. 226; G. W. Zlotnick et al., manuscript in prep.) and appears to be analogous to the low-molecular-weight peptidoglycan-associated lipoproteins (PAL) found in other gram-negative bacteria (28). Hence, we will refer to this protein as the *H. influenzae* PAL. Other groups have referred to a similar *H. influenzae* protein as OMP g (20) and P6 (33).

We have used polyclonal antibodies and monoclonal antibodies (MAbs) prepared against purified PAL to identify cloned fragments of *H. influenzae* DNA expressing the gene encoding this protein in *Escherichia coli*. We have identified and sequenced two *H. influenzae* genes encoding polypeptides of 153 and 154 amino acids which are recognized by these probes. These polypeptides contain potential lipoprotein signal peptide cleavage sites giving rise to predicted mature proteins of 134 and 136 amino acids. The predicted amino acid composition and sequence of the 134-amino-acid mature form agrees well with composition and partial sequence data for PAL purified from *H. influenzae* cells (Zlotnick et al., in preparation).

We have constructed a new plasmid cloning vector for these studies, pGD103. This plasmid is a composite of the low-copy-number vector pLG339 (44) and the *lac* α-polylinker region of pUC8 (46). This vector combines the advantages of low-copy-number plasmids for cloning genes whose expression on high-copy-number plasmids might be deleterious to the host cell with the rapid $Lac^{+/-}$ screening protocols for plasmid inserts and multiple cloning sites of the pUC plasmids.

MATERIALS AND METHODS

Bacterial strains and growth. *H. influenzae* strains used for this study were KW20(Rd) (2, 49), KW20b (31), b Eagan (48), and S2 (a spontaneous capsule-negative isolate derived from b Eagan [48]). *E. coli* strains used were HB101 (7), KH802 (23), JM83 (46), JM101 (27), and DS410 (1). *H. influenzae* strains were grown in brain heart infusion (Difco

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Laboratories, Detroit, Mich.) supplemented with 10 μg of heme (Sigma Chemical Co., St. Louis, Mo.) per ml and 2 μg of NAD (Sigma) per ml (BHI-XV medium). *E. coli* strains were grown in LB broth (22) or NZCYM broth (22).

Cloning vectors. Cloning vector systems used in this study were lambda Charon 4 (6), plasmid pLG339 (44), and a novel low-copy plasmid vector constructed for these studies, pGD103 (see results). M13mp18 and M13mp19 phages (27) (purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used for DNA sequencing.

Isolation of DNA. Chromosomal DNA from *H. influenzae* cells was isolated by the Marmur (25) procedure. Plasmid DNAs were isolated by alkaline SDS extraction followed by equilibrium banding in cesium chloride-ethidium bromide gradients (5). Bacteriophage lambda DNA was prepared by banding phage in cesium chloride followed by lysis with SDS-proteinase K (Bethesda Research Laboratories) and extraction with water-saturated phenol (22).

DNA sequencing. DNA sequencing was carried out by the dideoxy method of Sanger et al. (40). [α-^{35}S]dATP was purchased from Amersham Corp., Arlington Heights, Ill. Sequencing primers used were synthesized on model 380B DNA synthesizer (Applied Biosystems, Foster City, Calif.). DNA and protein sequence analysis was done on an IBM PC/AT computer (IBM, Boca Raton, Fla.) with Microgenie software (Beckman Instruments, Inc., Palo Alto, Calif.).

Restriction analysis. Restriction enzymes were purchased from Bethesda Research Laboratories or New England BioLabs, Inc., Beverly, Mass., and used as specified by the manufacturers. Restriction digests were analyzed by agarose (FMC Corp., Marine Colloids Div., Portland, Maine) gel electrophoresis with a 90 mM Tris-borate (pH 8.0)–2 mM EDTA buffer system (22). DNA bands were visualized by ethidium bromide fluorescence.

Protein blotting analysis (Western blotting). Protein immunoblotting was carried out by one of the following methods, depending on the application. For plaque lifts, a sterile nitrocellulose sheet (BA85; Schleicher & Schuell, Inc., Keene, N.H.) was gently laid upon a prechilled (4°C for 20 to 30 min) lambda titer plate and allowed to wet through. Positions were marked with a sterile needle, and the filter was lifted with forceps after 2 min. Filters were placed in a solution of 5% dehydrated milk in 50 mM Tris (pH 8.0)–150 mM NaCl (BLOTTO) (16) for >1 h at 37°C to block unbound sites on the filter. For SDS-PAGE anlaysis, gels were run under standard conditions (18) and proteins were transferred to nitrocellulose by horizontal electrophoresis (44) with a Transphor apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Filters were then blocked with BLOTTO as above. Prestained protein molecular weight standards were purchased from Bethesda Research Laboratories. For colony blots, colonies on agar plates were lifted onto nitrocellulose, placed on fresh plates, and allowed to grow for 2 to 3 h. Filters were then lifted, and the colonies were lysed by exposure to saturated chloroform vapor for 30 min. The filters were then soaked in BLOTTO containing about 50 μg of egg white lysozyme (Sigma) per ml for >3 h to digest and remove cell debris.

Antibody-binding reactions were carried out at 37°C in BLOTTO for 1 to 2 h. Primary antibody (polyclonal and monoclonal) was used at the appropriate dilution (determined by trial and error). Horseradish peroxidase-conjugated second antibody was purchased from Kirkegaard & Perry, Gaithersburg, Md., and used at 1 μg of conjugated antibody per ml. Positive horseradish peroxidase-conjugated antibody binding was detected by color development after treatment with 0.05% (wt/vol) 4-chloro-1-naphthol–0.01% H_2O_2 in 50 mM Tris (pH 7.0)–150 mM NaCl–20% (vol/vol) methanol at room temperature for 5 to 20 min. The color development was stopped by rinsing filters in deionized H_2O and air drying.

DNA hybridization analysis. DNA hybridization analysis was carried out by the Southern (43) procedure. Labeled probe was prepared by primer extension from appropriate M13 clones of the region of interest by using *E. coli* polymerase I (Klenow fragment) (Bethesda Research Laboratories). [α-^{32}P]dATP (>2,000 Ci/mmol) was purchased from Amersham.

Preparation of PAL antiserum. PAL was purified by differential extraction of *H. influenzae* outer membranes (14). Preparations were >95% pure by SDS-PAGE analysis with Coomassie blue or silver staining and by reverse-phase high-pressure liquid chromatography analysis (Zlotnick et al., in preparation). Approximately 20 μg of purified PAL in Freund incomplete adjuvant (Difco) was injected intramuscularly into New Zealand White rabbits. Rabbits were reimmunized 2 and 3 weeks following the initial immunization and bled 1 week following the final immunization. The activity of antisera was tested by SDS-PAGE Western blot against purified PAL and *H. influenzae* whole-cell lysates with pre- and postimmune serum. Immune serum recognized purified PAL and had very low background recognition of other *H. influenzae* proteins. Immune sera also recognized an approximately 21,000-molecular-weight OMP of *E. coli*, which has been identified as the *E. coli* PAL protein (see Results).

Preparation of MAbs. Female C57BL mice were injected intraperitoneally four times over a 2-month period with 10^6 *H. influenzae* S2 cells. At 3 months later, the mice were boosted with purified PAL. Spleen cells were fused with cells of mouse myeloma line X63.Ag8.6543 (12). Hybridoma cell culture supernatents were screened against *H. influenzae* total outer membrane by a standard enzyme-linked immunosorbent assay with 96-well polystyrene microtiter plates (Costar, Cambridge, Mass.). Bound antibody was detected with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G and immunoglobulin M (Kirkegaard & Perry) and *p*-dinitrophenol phosphate (Sigma phosphatase substrate). Positive supernatants were rescreened by dot blot against purified PAL, *E. coli* OMP, and purified S2 lipopolysaccharide (LPS; generously provided by Porter Anderson). Desired hybridomas were recloned by limiting dilution (26) and screened for anti-PAL activity by SDS-PAGE Western blot. Selected hybridomas were injected into BALB/c mice for growth as ascites (8). Five individual MAbs were used in this study. These MAbs recognized purified PAL, but no additional proteins in SDS-PAGE Western blot analysis of *H. influenzae* whole-cell lysates and failed to recognize purified *H. influenzae* LPS or any *E. coli* proteins.

Preparation of an *H. influenzae* chromosomal library in lambda Charon 4. The *H. influenzae* chromosomal library in lambda Charon 4 phage was prepared as previously described (23, 30). Total *H. influenzae* KW20b DNA was sheared to an average length of 15 kilobases by passage through a 25-gauge needle, blunt ended by treatment with T4 DNA polymerase (Bethesda Research Laboratories), modified with *Eco*RI DNA methylase (New England BioLabs), and ligated to *Eco*RI linkers (New England BioLabs). Linkers were cleaved with *Eco*RI, and DNA was ligated to the *Eco*RI arms of lambda Charon 4 DNA. A phage library of approximately 30,000 independent clones was generated and

plate amplified in *E. coli* KH802 on NZCYM plates to give an amplified library of 5×10^9 phage. The phage suspension was stored in NZCYM broth over chloroform. The phage library was screened by plating 1 to 2,000 PFU with 10^8 cells in 4 ml of NZCYM soft agarose (0.7%) on NZCYM plates and probing by plaque lifts as described above.

Preparation of an *H. influenzae* chromosomal library in plasmid pGD103. *H. influenzae* S2 chromosomal DNA was partially digested with *Sau*3A restriction endonuclease (Bethesda Research Laboratories) (about 10% complete digestion), and fragments were separated by size on 10 to 40% sucrose (1 M NaCl, 10 mM Tris [pH 8], 1 mM EDTA) velocity gradients (Beckman SW28 rotor, 25,000 rpm for 24 h). Fractions were collected dropwise and assayed by running portions on 0.8% agarose gels. Fractions containing 3- to 8-kb fragments were pooled, concentrated by ethanol precipitation, and suspended in 10 mM Tris (pH 8.0)–1 mM EDTA (TE buffer). Plasmid pGD103 (see Results) was digested with *Bam*HI and treated with calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). *Sau*3A-digested DNA and linearized plasmid were mixed at about a 1:1 molar ratio with approximately 100 μg of total DNA in 1 ml and ligated for 18 h at 15°C with T4 DNA ligase (25 U; Boehringer Mannheim). The ligation mixture was transformed into *E. coli* JM83 and plated on LB agar plates containing 50 μg of kanamycin (Sigma) per ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma). About 50% of the kanamycin-resistant colonies which developed after 24 h were Lac$^-$. Ten Lac$^-$ colonies were selected at random and were shown to contain plasmids with 4- to 8-kb DNA insertions into the *Bam*HI site of the pGD103 vector.

A total of 1,525 Lac$^-$ colonies were picked, amplified individually, and stored frozen at −70°C in LB broth containing 18% sterile glycerol in 96-well microtiter dishes. The library was screened for PAL by replica plating to LB-kanamycin plates, transfer to nitrocellulose, and colony blotting.

RESULTS

Construction of low-copy-number vector pGD103. Cloning of some genes encoding membrane proteins onto high-copy-number vectors such as pBR322 has proven difficult with *E. coli* (44), presumably owing to toxicity of these proteins to the host cell when produced at high levels. Hence, we decided to use a low-copy-number plasmid for these experiments. The plasmid pLG339 (carrying tetracycline and kanamycin resistance) is carried in 6 to 8 copies per cell in *E. coli* (44). We have modified this vector in the following manner to generate a new vector, pGD103. First, the 440-base-pair (bp) *Hae*II fragment of pUC8 encoding the *lac* α-peptide and polylinker region (46) was ligated into pLG339 at the *Eco*RI site (overhangs of both DNAs were removed). This plasmid (pGD101) was linearized with *Sph*I, which cleaves it once within the tetracycline resistance gene, and approximately 800 bp of DNA was removed by digestion with nuclease BAL 31 (Bethesda Research Laboratories). Plasmid digests were religated and transformed into *E. coli* JM83, and Kan^r Lac$^+$ colonies were selected by plating on LB-kanamycin–X-gal agar. Plasmid DNAs from several colonies were screened, and the smallest plasmid (approximately 5,600 bp [Fig. 1]) was saved as pGD102. Plasmid pGD102 was unexpectedly found to have a second *Eco*RI restriction site from BAL 31 digestion and religation. This site was removed by partially digesting the plasmid with *Eco*RI, isolating singly cut plasmid by preparative gel electrophoresis, and filling in the ends with DNA polymerase I (Klenow fragment). Digests were religated and transformed into *E. coli* JM83, and Kan^r Lac$^+$ colonies were selected. The plasmid from one such colony was isolated, and removal of the second *Eco*RI site was verified by restriction analysis. This plasmid was saved as pGD103 (Fig. 1). Plasmid pGD103 contains unique restriction sites within the polylinker region for *Eco*RI, *Bam*HI, *Pst*I, *Sal*I, and *Hin*cII; plasmids containing inserts into these sites can be rapidly identified by screening for the Lac$^-$ phenotype when transformed into an appropriate Lac$^-$ *E. coli* strain carrying the *lac* ZΔM15 gene, such as JM83 (46).

Screening of the lambda library. When the lambda Charon 4 library of the *H. influenzae* genome was probed with preabsorbed polyclonal anti-PAL antiserum, 0.4% of the plaques screened positive for PAL-binding activity, which was about the frequency expected for a single-copy gene. Positive plaques were lifted from the plate, suspended in 10 mM Tris (pH 8.0)–10 mM $MgCl_2$–0.1% gelatin buffer, and replated to verify PAL reactivity by plaque lift. *E. coli* KH802 lysates made from phage isolated from positive plaques were analyzed by SDS-PAGE Western blot and shown to contain a protein of apparent molecular weight 15,000 which was recognized by anti-PAL-antiserum and which was not present in control lysates. DNA was isolated from positive phage, digested with *Eco*RI, subcloned into pGD103, and transformed into *E. coli* JM83, and Lac$^-$ colonies were screened by colony blot. Positive colonies recognized by anti-PAL antiserum were found which contained a 5.8-kilobase *Eco*RI fragment. One such clone was saved as pAA130. SDS-PAGE Western blot analysis verified that *E. coli* cells carrying pAA130 expressed a 15-kilodalton (kDa) protein that was recognized by anti-PAL but was not present in cells carrying pGD103 (Fig. 2C). Minicell analysis (1) verified that the cloned DNA fragment encoded in the 15-kDa protein (Fig. 3A) which could be precipitated with anti-PAL antisera (data not shown). Sites for a variety of restriction enzymes were mapped within the cloned fragment (Fig. 4A), and the region of the plasmid encoding the

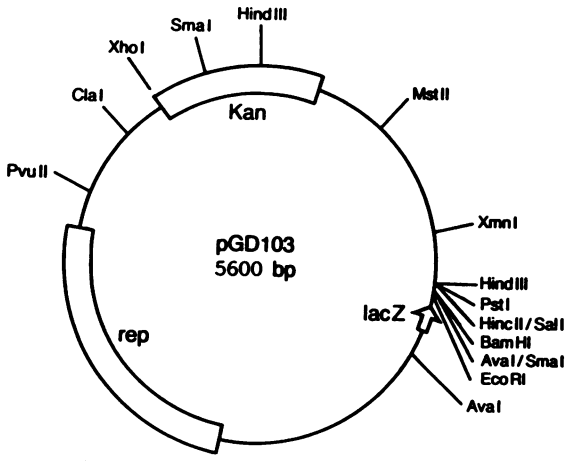


FIG. 1. Restriction map of plasmid pGD103. Kan, kanamycin resistance marker from plasmid pLG339; rep, replication region from plasmid pLG339 (43); lacZ, *lac* promoter and *lacZ* alpha-peptide translation initiation region of pUC8. Restriction endonucleases which fail to cleave pGD103 include *Bgl*II, *Eco*RV, *Pvu*I, *Sph*I, *Sst*I, *Sst*II, and *Xba*I.

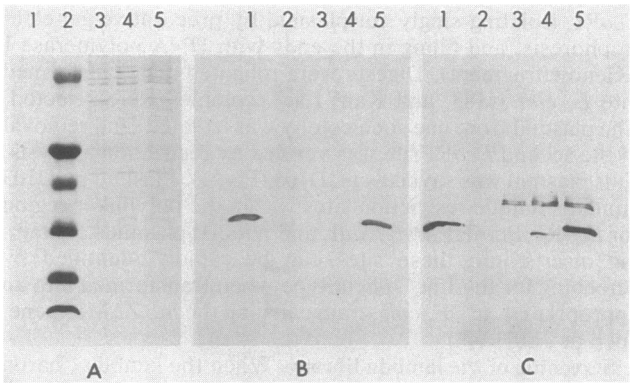


FIG. 2. Western blot analysis of proteins encoded by plasmids pAA130 and pAA152. Whole-cell lysates (50 μl of an overnight culture) were run on SDS-PAGE (15% acrylamide), transferred to nitrocellulose membrane filters, and probed with polyclonal antibodies or MAbs to *H. influenzae* PAL. Lanes 1 contain 100 ng of purified PAL. Lanes 2 contain prestained molecular weight standards (Bethesda Research Laboratories); sizes of standards are 43.0, 25.7, 18.4, 14.3, 6.2, and 3.0 kDa. Lanes 3 contain *E. coli* JM83(pGD103) lysate. Lanes 4 contain *E. coli* JM83(pAA130) lysate. Lanes 5 contain *E. coli* JM83(pAA152) lysate. (A) Coomassie blue-stained gel. (B) Western blot transfer probed with a mixture of five MAbs to PAL. (C) Western blot transfer probed with polyclonal anti-PAL antiserum.

15-kDa protein was localized to a 788-bp *Bst*EII-*Xmn*I fragment of the insert by a combination of deletion and subcloning analysis (Fig. 4B). This region was then subcloned into M13 vectors for DNA sequencing (see below). Clones of this fragment in M13 were also used to generate a labeled DNA probe for this region by primer extension. This probe was used for Southern analysis (43) of the *H. influenzae* genome and was found to recognize a single band in *Eco*RI- or *Bgl*II-cleaved *H. influenzae* chromosomal DNA from strains KW20 and b Eagan, but no sequences in *E. coli* DNA (Fig. 5).

Although the protein encoded by pAA130 was recognized by anti-PAL polyclonal antisera (antisera raised against several independent preparations of PAL were tested), it was not recognized by any anti-PAL MAbs tested (Fig. 2B). In addition, when the KW20b lambda gene bank was screened with anti-PAL MAbs, no positive plaques could be identified. We have directly verified by Western blot analysis that KW20b expresses a 15-kDa protein that was recognized by all five MAbs tested. A lambda library generated from strain S2 chromosomal DNA also failed to generate PAL-positive plaques when screened with anti-PAL MAbs. This raised the possibility that the 15-kDa protein encoded by pAA130 was not the *H. influenzae* PAL and that the true gene encoding PAL is not expressed in or is incompatible with the lambda cloning vector system used. We therefore decided to use a different system to screen the *H. influenzae* genome in *E. coli*.

Screening of the *H. influenzae* plasmid library. The 1,525 recombinant plasmids of the *H. influenzae* pGD103 library were plated onto LB-kanamycin plates and screened by colony blot with a mixture of five MAbs to PAL as described in Materials and Methods. Four colonies were identified which were recognized by the mixture of MAbs. Lysates from these cell lines were analyzed by SDS-PAGE Western blot, and three were found to encode a 15-kDa protein recognized by each individual MAb to PAL used in the original mixture and by polyclonal anti-PAL antiserum (Fig.

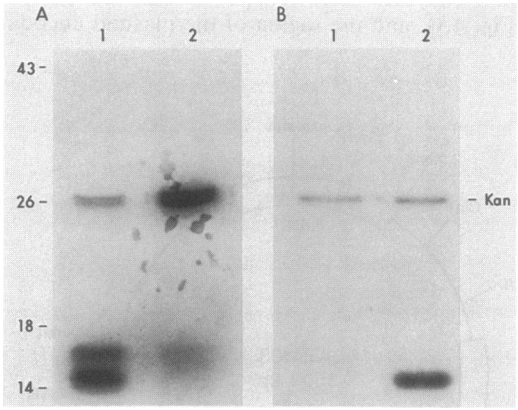
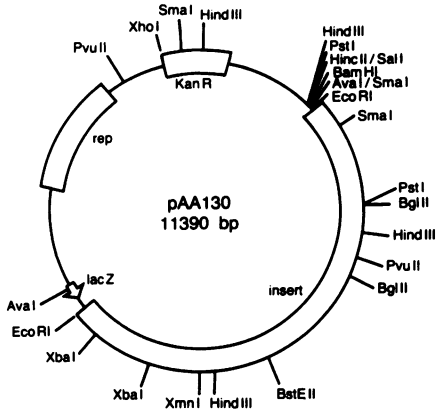


FIG. 3. Minicell analysis of plasmid-encoded proteins of pAA130 and pAA152. Minicells were prepared from *E. coli* DS410 carrying pGD103, pAA130, and pAA155 (a derivative of pAA152 generated by deletion of the *H. influenzae* DNA sequences between the *Bgl*II and *Sal*I restriction sites) and labeled with [^{14}C]leucine as described by Achtman et al. (1). Labeled minicells were run on SDS-PAGE (15% acrylamide) (25,000 to 30,000 cpm per lane). Gels were soaked in 1 M sodium salicylate (20 min), dried, and exposed to Kodak XAR-5 (Eastman Kodak Co., Rochester, N.Y.) film at −70°C. (A) Lanes: 1, pAA130 minicells; 2, pGD103 minicells. (B) Lanes: 1, pGD103 minicells; 2, pAA155 minicells. The position of molecular mass standards (in kilodaltons) is given. Kan marks the position of the 26-kDa product of the pGD103 kanamycin resistance gene.

A



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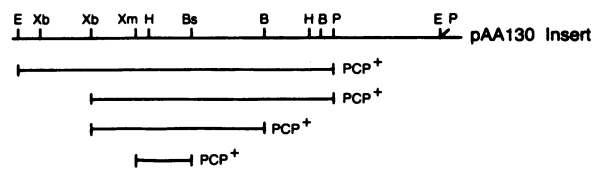

FIG. 4. (A) Restriction map of plasmid pAA130, encoding a 15,000-molecular-weight protein (PCP) recognized by anti-PAL antisera. The double-line labeled insert indicates the fragment of *H. influenzae* chromosomal DNA insert of pAA130 encoding PCP. (B) Identification of the region of the *H. influenzae* chromosomal insert of pAA130 encoding PCP. Abbreviations: E, *Eco*RI; Xb, *Xba*I; Xm, *Xmn*I; H, *Hin*dIII; Bs, *Bst*EII; B, *Bgl*II; P, *Pst*I.

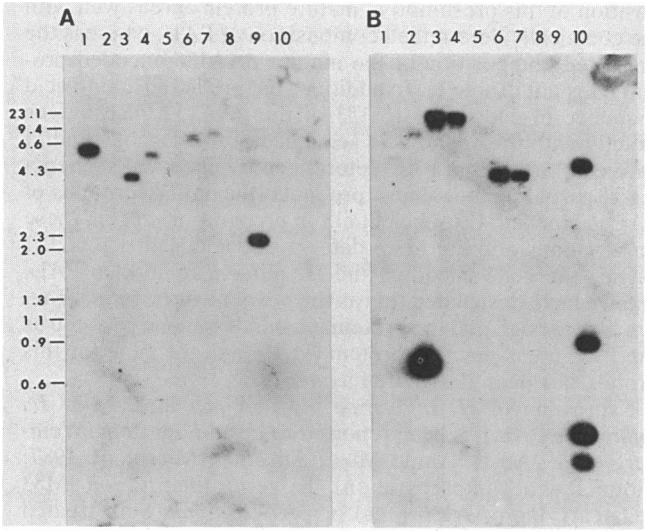

FIG. 5. Southern hybridization analysis of *H. influenzae* and *E. coli* chromosomal DNA with *pcp* and *pal* gene sequences. Lanes: 1, plasmid pAA130 DNA (*Eco*RI cut); 2, plasmid pAA152 DNA (*Bam*HI-*Bgl*II cut); 3, *H. influenzae* b (Eagan) DNA (*Eco*RI cut); 4, *H. influenzae* Rd (KW20) DNA (*Eco*RI cut); 5, *E. coli* HB101 DNA (*Eco*RI cut); 6, *H. influenzae* b (Eagan) DNA (*Bgl*II cut); 7, *H. influenzae* Rd (KW20) DNA (*Bgl*II cut); 8, *E. coli* HB101 DNA (*Bgl*II cut); 9, plasmid pAA130 (*Hin*dIII cut); 10, plasmid pAA152 (*Pst*I cut). (A) DNA probed with the 788-bp *Bst*EII-*Xmn*I fragment of pAA130 carrying the *pcp* gene (see Materials and Methods). (B) Same as panel A, except probed with the 737-bp *Bam*HI-*Bgl*II fragment of pAA152 carrying the *pal* gene. Positions of DNA size standards are given in kilobases.

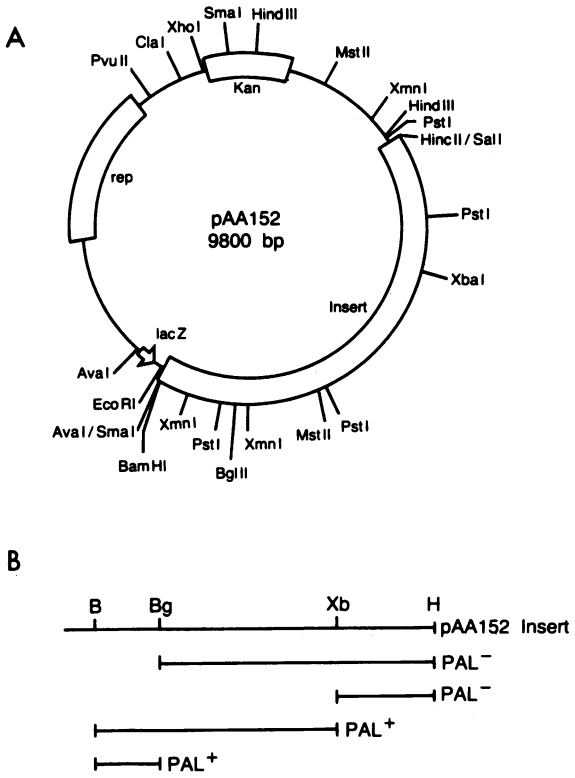

FIG. 6. (A) Restriction map of plasmid pAA152, encoding the second 15,000-molecular-weight protein (PAL) recognized by anti-PAL antiserum. Two additional closely spaced *Pst*I restriction sites were found between the *Bam*HI and *Bgl*II restriction sites by DNA sequence analysis (Fig. 8 and 10). The double line indicates the fragment of *H. influenzae* chromosomal DNA inserted into the pGD103 vector. (B) Identification of the region of the *H. influenzae* chromosomal insert of pAA152 encoding PAL. Abbreviations: H, *Hin*cII; B, *Bam*HI; Xb, *Xba*I; Bg, *Bgl*II.

2). Plasmids were prepared from the three PAL-positive clones and shown by preliminary restriction analysis and cross-hybridization to contain overlapping segments of *H. influenzae* chromosomal DNA. The plasmid with the smallest insert (4.2 kilobases) was saved as pAA152. Plasmid pAA152 was analyzed with a variety of restriction endonucleases (Fig. 6A), and the region coding for the 15-kDa protein was localized to a 737-bp *Bam*HI-*Bgl*II fragment by deletion analysis (Fig. 6B). The *Bam*HI site was shown to be the site within the pGD103 polylinker region that was fortuitously regenerated by the ligation. Minicell analysis (1) showed pAA152 codes for two proteins of 15,000 and 40,000 apparent molecular weight by SDS-PAGE, and the deletion containing only the *Bam*HI-*Bgl*II fragment encodes the 15,000-molecular-weight protein (Fig. 3B), which could be precipitated with anti-PAL antisera (data not shown). By Southern hybridization analysis (43), the *Bam*HI-*Bgl*II fragment recognized a single region in restriction digests of *H. influenzae* chromosomal DNA from either b Eagan or KW20, but no DNA sequences in *E. coli* (Fig. 5B). This probe also did not hybridize to pAA130 (Fig. 5).

Sequencing of the genes. The *Bam*HI-*Bgl*II fragment of pAA152 and the *Bst*EII-*Xmn*I fragment of pAA130 were cloned into M13mp18 and M13mp19, and DNA sequences were determined by the strategies shown in Fig. 7 and 8, respectively. The pAA130 *Bst*EII-*Xmn*I fragment of pAA130 contains 790 bp with a single open reading frame (ORF) encoding a polypeptide of 154 amino acids (Fig. 9). The gene also contains a second potential initiation codon (ATG) at codon 6 of this reading frame, which encodes a polypeptide of 149 amino acids. Only the 154-codon ORF has an upstream consensus ribosome-binding site (42). The 154-codon ORF also contains a consensus lipoprotein signal sequence-processing site (50) at codons 15 to 18, generating a predicted mature protein of 136 amino acids. The *Bam*HI-*Bgl*II fragment of pAA152 contains 737 nucleotides with a single ORF

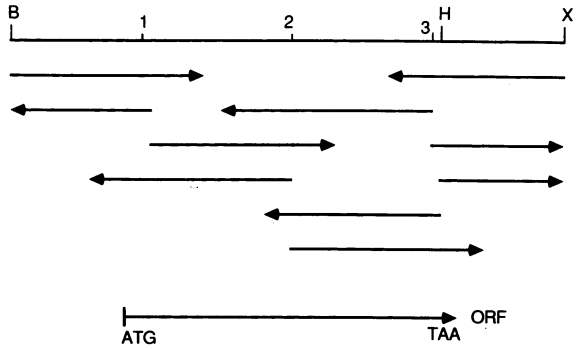

FIG. 7. Sequencing strategy for the pAA130 *Bst*EII-*Xmn*I fragment carrying *pcp*. Sequencing was done by the method of Sanger et al. (40) from the *Bst*EII *Xmn*I and *Hin*dIII restriction sites and from synthetic primers complementary to the following regions: 1, nucleotides 181 to 197 (from the *Bst*EII site); 2, nucleotides 398 to 414; 3, nucleotides 593 to 607. The location of the 154-codon ORF is shown. Abbreviations: B, *Bst*EII; X, *Xmn*I; H, *Hin*dIII.

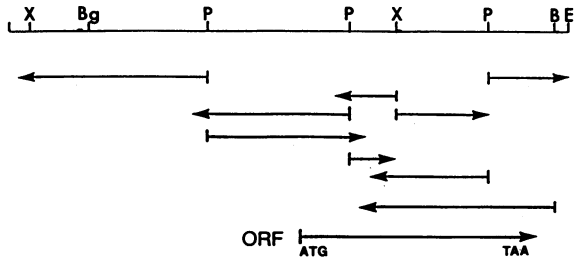

FIG. 8. Sequencing strategy for the pAA152 *Bam*HI-*Bgl*II fragment carrying *pal*. Restriction fragments were subcloned and sequenced by the method of Sanger et al. (40) as shown. The location of the 153-codon ORF is shown. Abbreviations: E, *Eco*RI; B, *Bam*HI; P, *Pst*I; Bg, *Bgl*II; X, *Xmn*I.

encoding a polypeptide of 153 amino acids (Fig. 10). This ORF contains a potential lipoprotein signal sequence-processing site at codons 16 to 19, generating a predicted mature protein of 134 amino acids. The predicted amino acid composition of this presumptive mature protein agrees well with the chemically determined composition of PAL, whereas the predicted composition of the mature pAA130-encoded protein does not (Table 1). In addition, the predicted amino acid sequence of codons 35 to 153 of the pAA152 ORF agrees exactly with the amino acid sequence of the carboxy end of native *H. influenzae* PAL determined by chemical sequencing of partial peptide digest products (the amino terminus of PAL is blocked) (Zlotnick et al., in preparation). From these observations and the MAb data, we conclude that pAA152 carries the gene encoding the *H. influenzae* 15-kDa PAL, and we have designated this as the *pal* gene of *H. influenzae*. We have designated the protein encoded by clone pAA130 as the PAL cross-reacting protein (PCP), and the gene for this protein has been designated as *pcp*.

Expression of *H. influenzae pal* and *pcp* in *E. coli*. *H. influenzae* PAL has been reported to be a lipoprotein (Weinberg et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1987; Zlotnick et al., in preparation). To verify this, *E. coli* JM83 cells carrying pAA130 (*pcp*) or pAA152 (*pal*) were treated with globomycin, a specific inhibitor of lipoprotein signal peptidase (SPase II). Western blot analysis verified the accumulation of precursors of PAL or PCP in globomycin-treated cells but not in untreated cells (Fig. 11). Globomycin

```
                                                                    61
GGTAACCAGCAGAAAGGATAGGAGGTTGTTATTGTGCATAAGTATGGTTCAACTTTAGTTG
BstEII

                                                                   121
TTGGTGCTTGTGTTTTAGTATCTGACAATGGTAATACTAAAAACATTTCAACTTTTTCTC

                                                                   181
GAAGAAACCCACTTTAATTCCTTCTAATATAGAGAATATTATATGAAAAAAACAAATATG
                                                 MetLysLysThrAsnMet

                                                                   241
GCATTAGCACTGTTAGTTGCTTTTAGTGTAACTGGTTGTGCAAATACTGATATTTTCAGC
AlaLeuAlaLeuLeuValAlaPheSerValThrGlyCysAlaAsnThrAspIlePheSer

                                                                   301
GGTGATGTTTATAGCGCATCTCAAGCAAAGGAAGCGCGTTCAATTACTTATGGTACGATT
GlyAspValTyrSerAlaSerGlnAlaLysGluAlaArgSerIleThrTyrGlyThrIle

                                                                   361
GTTTCTGTACGCCCTGTTAAAATTCAAGCTGATAATCAAGGTGTAGTTGGTACGCTTGGT
ValSerValArgProValLysIleGlnAlaAspAsnGlnGlyValValGlyThrLeuGly

                                                                   421
GGTGGAGCTTTAGGTGGTATTGCTGGTAGTACAATTGGCGGTGGTCGTGGTCAAGCTATT
GlyGlyAlaLeuGlyGlyIleAlaGlySerThrIleGlyGlyGlyArgGlyGlnAlaIle

                                                                   481
GCAGCAGTAGTTGGTGCAATTGGCGGTGCAATAGCTGGAAGTAAAATCGAAGAAAAAATG
AlaAlaValValGlyAlaIleGlyGlyAlaIleAlaGlySerLysIleGluGluLysMet

                                                                   541
AGTCAAGTAAACGGTGCTGAACTTGTAATTAAGAAAGATGATGGTCAAGAGATCGTTGTT
SerGlnValAsnGlyAlaGluLeuValIleLysLysAspAspGlyGlnGluIleValVal

                                                                   601
GTTCAAAAGGCTGACAGCAGTTTTGTAGCTGGTCGCCGAGTTCGTATTGTTGGTGGCGGC
ValGlnLysAlaAspSerSerPheValAlaGlyArgArgValArgIleValGlyGlyGly

                                                                   661
TCAAGCTTAAATGTTTCTGTGCTATAACCAATAGCATTAAAGTCTAATATGATTAATCAG
SerSerLeuAsnValSerValLeuEnd

                                                                   720
TGTCTTAACTTAGTAAGGCACTGATTTTTTTATAATTAAATTCATTTAAAATATATATTA

                                                                   781
TCGTCTATCTAAGATAAATTTAAAGGACTAAATTAGAATTTAGTCCTTTAGAAAACTTGG

AATTNNTTC
XmnI
```

FIG. 9. DNA sequence of the 790-bp *Bst*EII-*Xmn*I fragment of pAA130 carrying *pcp*. The 154-codon ORF and predicted amino acid seqeunce of the encoded polypeptide are shown.

```
                                                                    60
AGATCTCACAACGGGCGTTTCAGAGGTACTTAGTACATCTTTTCTAGGCGAAAGTCCAAG
BglII

                                                                   120
CCTCTCTCCAAATGGAATTATGATTATTTATAGTTCTACACAGGGCTTAGGAAAGGTGCT

                                                                   180
ACAATTGGTTTCTGCAGATGGTCGCTTTAAGGCGAGCCTTCCAGGAAGTGATGGTCAAGT

                                                                   240
TAAATTTCCAGCTTGGTCTCCATACTTAACTAAATAAAAAACTCATTTAGGAGAAATCTA

                                                                   300
ATGAACAAATTTGTTAAATCATTATTAGTTGCAGGTTCTGTAGCTGCATTAGCAGCTTGT
MetAsnLysPheValLysSerLeuLeuValAlaGlySerValAlaAlaLeuAlaAlaCys

                                                                   360
AGTTCATCTAACAACGATGCTGCAGGCAATGGTGCTGCTCAAACTTTTGGCGGTTACTCT
SerSerSerAsnAsnAspAlaAlaGlyAsnGlyAlaAlaGlnThrPheGlyGlyTyrSer

                                                                   420
GTTGCTGATCTTCAACAACGTTACAATACCGTTTATTTCGGTTTTGATAAATATGACATT
ValAlaAspLeuGlnGlnArgTyrAsnThrValTyrPheGlyPheAspLysTyrAspIle

                                                                   480
ACTGGTGAATACGTTCAAATCTTAGATGCGCACGCTGCATATTTAAATGCAACACCAGCT
ThrGlyGluTyrValGlnIleLeuAspAlaHisAlaAlaTyrLeuAsnAlaThrProAla

                                                                   540
GCTAAAGTATTAGTAGAAGGTAACACTGATGAACGTGGTACACCAGAATACAACATCGCA
AlaLysValLeuValGluGlyAsnThrAspGluArgGlyThrProGluTyrAsnIleAla

                                                                   600
TTAGGCCAACGTCGTGCAGATGCAGTTAAAGGTTATTTAGCTGGTAAAGGTGTTGATGCT
LeuGlyGlnArgArgAlaAspAlaValLysGlyTyrLeuAlaGlyLysGlyValAspAla

                                                                   660
GGTAAATTAGGCACAGTATCTTACGGTGAAGAAAAACCTGCAGTATTAGGTCATGATGAA
GlyLysLeuGlyThrValSerTyrGlyGluGluLysProAlaValLeuGlyHisAspGlu

                                                                   720
GCTGCATATTCTAAAAACCGTCGTGCAGTGTTAGCGTACTAATTCTTAGTATTTCTAATA
AlaAlaTyrSerLysAsnArgArgAlaValLeuAlaTyrEnd

CTTGAAAAACAGGATCC
          BamHI
```

FIG. 10. DNA sequence of the 737-bp *Bam*HI-*Bgl*II fragment of the pAA152 carrying *pal*. The 153-codon ORF and predicted amino acid sequence of the encoded polypeptide are shown.

also blocks the processing of PAL in *H. influenzae* cells (data not shown). In addition, both PAL and PCP could be labeled with [^{14}C]palmitic acid (data not shown). PAL and PCP are therefore both lipoproteins.

Fractionation of *E. coli* cells (39) expressing PAL or PCP demonstrated that both proteins are associated primarily with the outer membrane. Recombinant PCP has been prepared by electroelution of bands from SDS-PAGE of outer membranes of *E. coli* cells expressing PCP. Antisera raised against this recombinant PCP have been shown to recognize recombinant PAL expressed in *E. coli*, but not *E. coli* PAL (data not shown). This supports the conclusion that *H. influenzae* PAL and PCP are immunologically cross-reactive. In further support of this conclusion, we have found a MAb which recognizes both PAL and PCP expressed in *E. coli*. Further studies on the immunological properties of recombinant PAL and PCP are in progress.

Anti-*H. influenzae* PAL antisera recognize *E. coli* PAL. All polyclonal anti-PAL *H. influenzae* antisera tested also recognized a 21-kDa protein in *E. coli* (Fig. 2). *E. coli* has been reported to have a PAL of this mobility on SDS-PAGE (28). The sequence of the gene encoding *E. coli* PAL has recently been determined (9), and the predicted amino acid sequences of *E. coli* PAL and *H. influenzae* PAL show very strong homology. The predicted amino acid sequences of codons 66 to 153 of *H. influenzae pal* and codons 85 to 172 of *E. coli pal* are identical in 64 of 87 positions (74% homology). We have verified cross-reactivity of *H. influenzae* and *E. coli* PALs directly by purifying *E. coli* PAL by the procedure of Mizuno (28) and demonstrating binding with anti-*H. influenzae* PAL antisera (data not shown).

Alkaline phosphatase fusions. We have used the Tn*phoA* alkaline phosphatase gene fusion system of Manoil and Beckwith (24) to study the recognition of PCP by anti-PAL antisera. Fusions were generated by subcloning the *Eco*RI-*Pvu*II fragment of plasmid pAA130 containing *pcp* to the *Eco*RI-*Pvu*II fragment of plasmid pLG339 containing the replication origin and the tetracycline resistance gene and screening for *phoA*$^+$ transpositions into this plasmid (24). Three *phoA*$^+$ transpositions were isolated and found by restriction and sequence analysis to lie within the *pcp* gene. One fusion at codon 151 of the *pcp* ORF generated a 60-kDa hybrid protein which was recognized by PAL polyclonal antisera. Two other fusions at codons 111 and 87 generated hybrid proteins which were not detected by PAL antisera. Hence, the carboxy-terminal 33 amino acids of PCP are necessary (but not necessarily sufficient) for recognition by anti-PAL antisera. Fractionation of cells (39) carrying these fusions demonstrates that the alkaline phosphatase-PCP hybrid proteins are associated with the outer membrane fraction. This agrees with the previous observation that PCP is associated with the outer membrane when expressed in *E. coli* and indicates that the amino half of PCP contains all information required for directing the protein to the outer membrane.

TABLE 1. Comparison of the amino acid composition of *H. influenzae* PAL with the predicted compositions of the 15-kDa proteins encoded by pAA130 and pAA152

Amino acid	No. of residues in:		
	PAL[a]	pAA152[b] 15-kDa ORF	pAA130[c] 15-kDa ORF
Aspartic acid (Asp + Asn)	15	17	10
Threonine	6	7	5
Serine	7	6	13
Glutamic acid (Glu + Gln)	12	12	12
Proline	3	3	1
Glycine	18	16	24
Alanine	19	21	16
Cysteine	1	1	1
Valine	10	10	18
Methionine	0	0	1
Isoleucine	4	3	13
Leucine	11	9	5
Tyrosine	13	11	2
Phenylalanine	4	3	2
Lysine	8	7	7
Histidine	2	2	0
Arginine	7	6	6
Tryptophan	0	0	0

[a] Protein purified from *H. influenzae* cells and analyzed by acid hydrolysis (Zlotnick et al., in preparation).
[b] Predicted composition based on removal of the presumptive membrane signal peptide (amino-terminal residues 1 to 19).
[c] Predicted composition based on removal of the presumptive membrane signal peptide (amino-terminal residues 1 to 18).

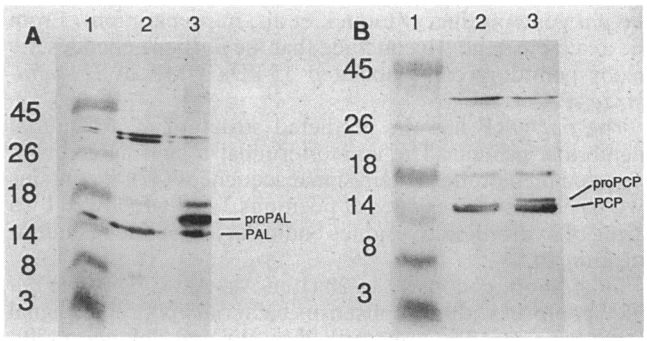

FIG. 11. Effect of globomycin on PAL and PCP expressed in *E. coli*. (A) Lanes: 1, molecular mass standards (sizes given in kilodaltons); 2, lysate of *E. coli* JM83 carrying pAA152 (*pal*); 3, lysate of *E. coli* JM83 carrying pAA152 grown in the presence of globomycin (25 μg/ml of 37°C for 1 h in LB broth). (B) Lanes: 1, Molecular mass standards; 2, Lysate of *E. coli* JM83 carrying pAA130 (*pcp*); 3, lysate of *E. coli* JM83 carrying pAA130 grown in the presence of globomycin as above. Lysates were run on SDS-PAGE (15% acrylamide), transferred to nitrocellulose, and probed with polyclonal anti-PAL antisera.

DISCUSSION

We have cloned and expressed in *E. coli* two *H. influenzae* genes encoding nonidentical 15-kDa proteins which are recognized by polyclonal antiserum to the PAL of *H. influenzae*. One of these genes, *pal*, has been shown to code for the *H. influenzae* PAL, since the amino acid composition (Table 1) and partial peptide sequence (Zlotnick et al., in preparation) of the predicted mature form of this gene product agree with those of purified PAL determined chemically (Table 1) and it is recognized by anti-PAL MAbs. The predicted amino acid composition of the second protein, PCP, does not agree well with the amino acid composition of purified PAL (Table 1), and this protein is not recognized by any tested anti-PAL MAbs. Determination of the N-terminal amino acid sequence of the purified PAL by Edman chemistry has proven impossible, since the amino terminus of the protein is blocked (Zlotnick et al., in preparation). However, amino acid sequence data from proteolytic fragments of PAL agree with predicted amino acid sequences occurring within

the *pal* gene product (Zlotnick et al., in preparation). From the available data, we conclude that the *pal* gene encodes the major peptidoglycan-associated 15-kDa OMP of *H. influenzae* (PAL).

The *pal* ORF has the predicted structure of a bacterial membrane protein. The amino-terminal region agrees with reported membrane protein signal sequences (47); it contains two basic (lysine) residues at positions 3 and 6 followed by a string of hydrophobic residues ending at a cysteine residue at position 20.

In addition, codons 17 to 20 (Leu-Ala-Ala-Cys) of the *pal* ORF conform with the consensus bacterial lipoprotein signal sequence cleavage site described by Wu and Tokunaga (50). Because the amino terminus of purified PAL is blocked to Edman sequencing (Zlotnick et al., in preparation), we have not been able to determine directly whether there is proteolytic removal of the amino-terminal signal sequence during PAL processing. Amino acid analysis of PAL indicates that the purified protein contains no methionine (Table 1), and minicell data for *E. coli* demonstrate that the cloned protein cannot be labeled with [^{35}S]methionine (data not shown), indicating that at least the single N-terminal methionine residue of PAL is removed during protein maturation. In addition, the *pal* gene product can be labeled with palmitic acid, and its processing is sensitive to globomycin (Fig. 11). Using MAbs specific for this protein, we have demonstrated that PAL is associated with the outer membrane fraction of *E. coli* cells expressing the *pal* gene.

The Cys-Ser-Ser tripeptide of the *pal* ORF (codons 20 to 22) presumably forms the amino terminus of the mature PAL protein of *H. influenzae*. This tripeptide has also been observed at the amino terminus of the 18-kDa *Proteus mirabilis* (29) and *E. coli* (9) PALs and at the amino termini of murein lipoproteins from a number of gram-negative bacteria, including *E. coli*, *Serratia marcescens*, *Erwinia amylovora*, *Morganella morganii*, and *P. mirabilis* (for a review, see reference 50), and appears to be a common structural feature of lipoproteins which are closely associated with the cell wall peptidoglycan in gram-negative bacteria.

The predicted amino acid sequence of the *H. influenzae* PAL also has a high degree of homology with that of the *E. coli* PAL (9). Antiserum against *H. influenzae* PAL has been shown to be cross-reactive with *E. coli* PAL, and Mizuno et al. (29) have reported that PALs of other gram-negative organisms are also immunologically cross-reactive. Although the biological function(s) of PAL is not known, this degree of structural homology between PALs from widely divergent organisms indicates a strong selective pressure on PAL conformation.

The amino-terminal 19 codons of the *pcp* ORF are consistant with reported membrane protein transport signal sequences (47), including two basic residues (lysine) at positions 2 and 3 followed by a hydrophobic region followed by a cysteine residue (position 19). Amino acids 16 to 19 of the *pcp* ORF (Val-Thr-Gly-Cys) have partial homology with the consensus bacterial lipoprotein signal sequence cleavage site (50). In particular, cleavage in other lipoproteins has been shown to occur at either glycine-cysteine or alanine-cysteine. In other reported bacterial prolipoprotein sequences, the only amino acid observed at the −3 position to cysteine is leucine, whereas alanine, serine, and valine have been observed at the −2 position (for a review, see reference 50). We have been unable to identify or isolate the *pcp* gene product from *H. influenzae* cells to determine how it is processed in *H. influenzae* cells. The product of the *pcp* gene expressed in *E. coli* can be labeled with palmitic acid, and its processing is sensitive to globomycin (Fig. 11), verifying that PCP is a lipoprotein.

The *pcp* gene was originally isolated from a type d × type b hybrid strain (KW20b [29]). Southern hybridization analysis indicates that both parental strains Rd Kw20 (2, 19, 49) and b Eagan (48) contain single copies of this gene (Fig. 5).

DNA sequence analysis of the two cloned genes indicates that they are of similar composition overall, being 58% A+T (*pal*) and 61% A+T (*pcp*). This is in general agreement with the reported 60% overall A+T content of *H. influenzae* DNA (41). The predicted amino acid compositions of the two gene products are also similar. Both proteins contain about 30% hydrophobic residues, are rich in glycine and alanine, and lack tryptophan. However, major differences in the amino acid compositions of the two proteins are observed for tyrosine (11 in PAL versus 2 in PCP), isoleucine (3 versus 13), histidine (2 versus 0), and internal methionine (0 versus 1) (results are summarized in Table 1). At the DNA and amino acid sequence level, the two genes are quite divergent. Interestingly, the only significant amino acid homology found between the two proteins lies in their presumed signal peptide regions. Amino acids 8 to 14 from the *pal* ORF (Leu-Leu-Val-Ala-Gly-Ser-Val) and 10 to 16 from the *pcp* ORF (Leu-Leu-Val-Ala-Phe-Ser-Val) form a sequence homology of six of seven residues. This region also contains a homology of 14 of 21 bp at the DNA sequence level. Homology in this region is somewhat surprising, since in general, membrane signal peptide sequences are not well conserved, except near the signal cleavage site (47, 50).

Since PAL and PCP show so little homology at the primary sequence level, the major question arises of why all polyclonal antisera raised to PAL recognize PCP. One possibility is that all PAL preparations are contaminated with small amounts of PCP. We have been unable to detect PCP in our PAL preparations, and amino acid analysis, particularly the absence of detectable methionine, indicates that PAL preparations are >90% homogeneous (Zlotnick et al., in preparation). Although this does not absolutely rule out the presence of very low levels of PCP in PAL preparations, the reactivity of recombinant PAL with antisera to recombinant PCP and the existence of a MAb which recognizes both proteins indicate that the proteins are truly immunologically cross-reactive. The nature of the cross-reactive epitope(s) of PAL and PCP is not clear, although Tn*phoA* fusion data suggest that the carboxy-terminal region of PCP is involved.

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