INFECTION AND IMMUNITY, Oct. 1990, p. 3388–3393
0019-9567/90/103388-06$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 58, No. 10

Antigenic Conservation of the 15,000-Dalton Outer Membrane Lipoprotein PCP of *Haemophilus influenzae* and Biologic Activity of Anti-PCP Antisera

ROBERT A. DEICH,* ALGIS ANILIONIS, JAMES FULGINITI, BENJAMIN J. METCALF, SALLY QUATAERT, TAMMY QUINN-DEY,† GARY W. ZLOTNICK, AND BRUCE A. GREEN

Praxis Biologics, 300 East River Road, Rochester, New York 14623

Received 19 April 1990/Accepted 2 August 1990

A gene from *Haemophilus influenzae* encoding an outer membrane lipoprotein of about 15,000 daltons and which comigrates with the peptidoglycan-associated lipoprotein (PAL) of *H. influenzae* on sodium dodecyl sulfate-polyacrylamide gel electrophoresis has been previously reported and designated *pcp* gene, and its product has been designated PCP. In order to obtain specific immunologic probes for the analysis of PCP expression, cellular location, and antigenic conservation in *H. influenzae*, *pcp* was fused to the *lac* polylinker region of plasmid pUC19 and the hybrid gene was expressed in *Escherichia coli*. PCP purified from these cells was used to generate rabbit and mouse polyclonal antisera and mouse monoclonal antibody against PCP. Western immunoblot analysis with anti-PCP monoclonal antibody demonstrated that PCP is present and antigenically conserved in 30 tested strains of *H. influenzae*, including 27 clinical nontypeable strains. Polyclonal antiserum against PCP killed 9 of 11 clinical *H. influenzae* strains in a complement-mediated bactericidal assay, and bactericidal activity was additive with bactericidal activity of antisera against PAL. These results indicate that PCP is a potentially valuable component for a subunit vaccine against nontypeable *H. influenzae* disease, especially in combination with PAL or other components.

The gram-negative bacterium *Haemophilus influenzae* is a major human pathogen. Type b encapsulated *H. influenzae* strains are the most frequent cause of meningitis in children <5 years old and cause other invasive diseases in this group (4). Nontypeable *H. influenzae* strains are responsible for 20 to 40% of otitis media in children (9) and have been implicated in a number of diseases in adults, including pneumonia, bacteremia, meningitis, postpartum sepsis, and acute febrile tracheobronchitis (18). Vaccines composed of purified type b polysaccharide coupled to an immunogenic carrier protein have proven effective against type b *H. influenzae* infections in children >18 months of age. However, these vaccines are of no value against nontypeable *H. influenzae* disease. Therefore, other cell surface components such as fimbrae and outer membrane proteins have been investigated as potential subunit vaccine candidates against nontypeable *H. influenzae* disease (for a review, see reference 6).

Previous work has demonstrated that an approximately 15,000-molecular-weight (MW) outer membrane peptidoglycan-associated lipoprotein (PAL or P6) of *H. influenzae* is a potential candidate as a component for such a vaccine (7, 16–18). This protein is highly conserved antigenically (7, 18, 27), and antisera raised to native and recombinant PAL (expressed in *Escherichia coli*) have been shown to be bactericidal against *H. influenzae* and nontypeable *H. influenzae* strains and protective against *H. influenzae* (6a, 7, 16).

During investigation of *H. influenzae* PAL, previous work in this laboratory (3) demonstrated that *H. influenzae* has a gene encoding a second potential lipoprotein of 15,000 MW, designated *pcp*. The *pcp* gene has been cloned, sequenced, and predicted to encode a protein of 136 amino acids (mature form). Southern blot analysis suggested that *pcp* is also conserved in diverse *H. influenzae* strains. The *pcp* gene product (PCP) was associated with the outer membrane when expressed in *E. coli* (3). However, further analysis of *H. influenzae* PCP was hindered by the low level of expression (<0.5%) of the protein in *H. influenzae* cells and lack of a specific probe to distinguish it from the comigrating *H. influenzae* PAL.

In order to overcome these problems, the *pcp* gene was fused to the *lac* polylinker region of plasmid pUC19 and the products of this hybrid *lac-pcp* gene were purified from *E. coli*. Polyclonal and monoclonal antisera were prepared and used to evaluate (i) the expression, distribution, and antigenic conservation of PCP in various laboratory and clinical *H. influenzae* strains, (ii) the biological activity of PCP antisera against *H. influenzae* cells, and (iii) the cooperativity of the activities of PCP and PAL antisera against *H. influenzae*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM103 was used for cloning and production of recombinant proteins. *H. influenzae* laboratory strains used were type b Eagan (24), S2 (24), and KW20 (25). The clinical nontypable *H. influenzae* strains used and their sources were HST31, HST32, HST33, HST34, HST35, HST36, HST37, HST41, HST44, and HST45 (P. Anderson, University of Rochester, Rochester, N.Y.); P86-0295, P86-1454, P81-0384, and P88-0859 (C. Bluestone, University of Pittsburg, Pittsburgh, Pa.); N1955, N83-0210M, DL322, N90, N10, N83-045E, N83-0133E, N1937, N82-0127E, N83-0100E, N83-026E, and N47 (E. Hansen, University of Texas Medical Center, Dallas); and 37567 (P. Rice, Boston City Hospital, Boston, Mass.). *Haemophilus parainfluenzae* C was obtained from H. O. Smith, Johns Hopkins University, Baltimore, Md. *E. coli* cells were grown in LB broth (14). *H. influenzae* cells were

* Corresponding author.
† Present address: Veterans Administration Medical Center, North Chicago, IL 60088.

grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with hemin (10 μg/ml) and NAD (2 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). *H. parainfluenzae* was grown in brain heart influsion broth supplemented with NAD (2 μg/ml) only.

Plasmids used for this study were pGD103 and pAA130 (3), pUC19 (26), and pPX140 and pPX163 (this study).

Genetic constructions. Recombinant plasmid pPX140 was constructed by isolating the 790-base-pair *Bst*EII-*Xmn*I fragment of pAA130 carrying *pcp* (3), blunt ending it with T4 DNA polymerase (Bethesda Research Laboratories [BRL], Gaithersburg, Md.), and ligating it into the *Hin*cII site of pGD103. Plasmid pPX163 was constructed as described in Results. Plasmid DNA was purified by alkaline lysis of cells and cesium chloride-ethidium bromide equilibrium gradient centrifugation (1). Restriction enzymes and T4 DNA ligase were purchased from BRL or Boehringer Mannheim (Indianapolis, Ind.) and used according to the directions of the manufacturer.

Purification of recombinant PCP. *E. coli* JM103 cells carrying pPX163 were grown to early log phase (optical density at 600 nm, ~0.1), induced with isopropylthio-β-D-galactopyranoside (2 mM; Sigma), and grown to stationary phase. Outer membranes from these cells were isolated (21) and separated on preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the 17,000- and 15,000-MW bands corresponding to recombinant PCP (see Results) were cut out and eluted by the procedure of Hager and Burgess (8). Protein yield was determined by the assay of Lowry et al. (13) as modified by Peterson et al. (20).

SDS-PAGE and Western immunoblot analysis. SDS-PAGE was performed by the Laemmli method (10). For visual analysis, gels were stained with Coomassie brilliant blue R-250 (Sigma). Western blot analysis was carried out by electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) (23), probing with specific polyclonal or monoclonal antisera, and detection with horseradish peroxidase-conjugated second antibody (Kirkegaard & Perry, Gaithersberg, Md.) as described by Deich et al. (3).

Protein sequencing. Protein sequencing was carried out by the microsequencing protocol of Matsudaira (15). Briefly, proteins were separated on SDS-PAGE, electrophoretically transferred to an Immobilon-P membrane (Millipore Corp., Bedford, Mass.), stained with Coomassie G-250 (Sigma), cut out, and sequenced directly.

Antisera. Antisera against gel-purified recombinant PCP (see Results) were prepared by intramuscular injection of 10 μg of protein in Freund incomplete adjuvant (Difco) in New Zealand White rabbits or Swiss Webster mice and boosting after 4 weeks. Mouse sera were also prepared by intramuscular injection of PCP absorbed to aluminum [$Al(OH)_3$]. Sera were collected prior to immunization (control), at the time of the second immunization, and 2 to 6 weeks following the second immunization.

Mouse monoclonal antibodies against PCP were prepared by fusing spleen cells from immunized mice to mouse myeloma line X63.Ag8.653 (5). Desired hybridomas were screened by Western blot against purified PCP and whole-cell lysates from *H. influenzae* S2 cells, *E. coli* JM103 cells expressing PCP from plasmid pPX163, and control *E. coli* JM103 cells carrying pUC19. One hybridoma, G61-1, was grown in ascitic fluid (2).

Preparation of polyclonal and monoclonal antisera against native and recombinant *H. influenzae* PAL is described elsewhere (3, 6a, 7).

Bactericidal assays. Bactericidal assays were carried out as described by Green et al. (7).

RESULTS

Expression of *pcp* in *E. coli*. Isolation of native PCP from *H. influenzae* cells proved difficult in that very little (<0.5% of cell protein, data not shown) is made in *H. influenzae* cells and the only available probes are polyclonal sera which also recognize the comigrating *H. influenzae* PAL (3). In order to overcome these difficulties, recombinant PCP was expressed in *E. coli*. The original *pcp* clone (pPX130) was expressed poorly (<1%) in *E. coli* (3). Attempts to clone the native gene and promoter onto high-copy-number vectors such as pBR322 or pUC plasmids were unsucccessful (3). Hence, a construction fusing *lac* polylinker of pUC19 to *pcp* was made to try to alleviate this limitation on the expression of native PCP. The plasmid was constructed by cleaving *pcp* clone pPX140 with the restriction enzyme *Ssp*I, which cut the plasmid at a position 5 bases 5′ to the *pcp* initiation codon, and *Eco*RI, which cut beyond the 3′ end of the gene in the pGD103 polylinker. This fragment was then ligated to pUC19 DNA doubly cut with *Sma*I and *Eco*RI, and the resulting plasmid was designated pPX163 (Fig. 1). Sequence analysis of pPX163 verified that the fusion of the *lac* and *pcp* genes at the *Sma*I-*Ssp*I junction generated a hybrid gene in the proper reading frame to express a Lac-PCP hybrid protein containing the 18 amino-terminal acids of pUC-Lac α, two new amino acids generated from the gene fusion, and the complete PCP protein (including signal peptide, Fig. 1). The predicted MW of the Lac-PCP hybrid protein is 17,599.

Analysis of pPX163 expression of *E. coli*. Plasmid pPX163 was transformed into *E. coli* JM103, and expression of recombinant PCP was tested. Cell lysates were analyzed by SDS-PAGE Western blot with polyclonal antiserum DM-1 directed against *H. influenzae* PAL which has been shown to contain activity against PCP also (3). Surprisingly, cells carrying pPX163 were found to express three polypeptides recognized by this antiserum; a 17,000-MW polypeptide and a doublet of 15,000 MW. None of these bands were recognized by any *H. influenzae* PAL-specific monoclonal antibodies (MAbs) tested (data not shown), and all three were recognized by PCP-specific MAbs (Fig. 2). Expression of all three forms was under *lac* regulation. Fractionation of cells expressing pPX163 by detergent extraction (21) demonstrated that all three forms were associated with the outer membrane fraction and that they composed 50 to 60% of the total outer membrane protein in these cells by SDS-PAGE analysis (Fig. 3). Fractionation of cells by isopycnic centrifugation (19) gave the same distribution of PCP (data not shown).

In order to determine whether any of these forms of PCP were lipidated, JM103 cells carrying pPX163 were induced with isopropylthio-β-D-galactopyranoside and labeled with ^{14}C-palmitic acid. Outer membranes isolated from labeled cells were separated by SDS-PAGE and stained, the individual PCP bands were cut out, and ^{14}C incorporation was determined. Results indicate that the lower band at 15,000 is preferentially labeled. This result has been confirmed by autoradiography of labeled cells separated on SDS-PAGE (data not shown). This band has gel mobility identical to that of PCP expressed from pPX140 and probably corresponds to the "native" fatty acylated form of PCP. The other two recombinant PCP forms were isolated and characterized by N-terminal amino acid sequence analysis (see Materials and Methods). The 17,000-MW band was found to correspond to

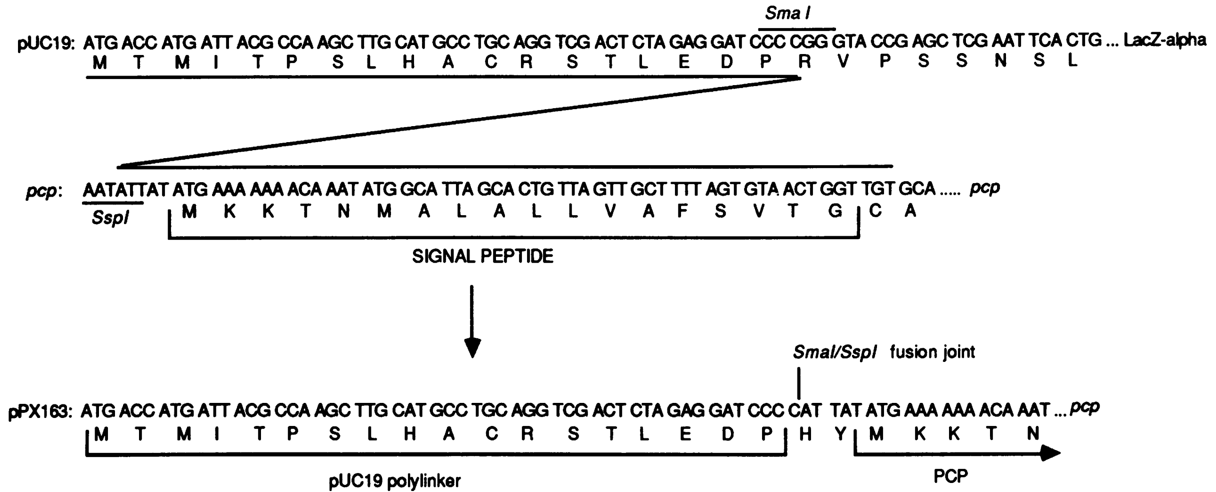

FIG. 1. Construction of the pUC19-*pcp* gene fusion. Plasmid pPX140 (*pcp*) was cleaved with restriction endonucleases *Ssp*I, which cleaves 5 bases upstream from the initiation codon of *pcp*, and *Eco*RI, which cleaves 3′ to *pcp* in the vector polylinker region. The *pcp* fragment was isolated and ligated into pUC19 cleaved with *Sma*I and *Eco*RI. The 5′-end DNA sequences and predicted amino acid sequences of pUC19-lacZα, *pcp*, and pPX163 (*lacZα-pcp* hybrid) are shown.

the predicted *lac-pcp* hybrid gene product. The upper band of the 15,000-MW doublet was found to start at the native initiation codon of the *pcp* gene and hence was a non-fatty-acylated form of PCP with the signal peptide unremoved (pro-PCP). This pro-PCP form is presumably due to secondary initiation of *pcp* translation at the native ATG codon. The pro-PCP protein which reaches the outer membrane appears to be quite stable in vivo and cannot be chased into the fatty acylated form if protein synthesis is blocked with chloramphenicol (data not shown). The lower 15,000-MW band of the doublet was blocked to N-terminal analysis, as predicted for a bacterial lipoprotein.

Isolation of recombinant PCP. Purification of recombinant PCP was carried out by separating isolated outer membranes (21) of cells expressing pPX163 on SDS-PAGE, cutting out the PCP bands, and diffusing the protein from the gel (in control gels, no *E. coli* outer membrane proteins were detectable at the mobilities of the PCPs). Because the 15,000-MW doublet forms were difficult to separate in large-scale gel purifications (Fig. 3), these two forms were pooled. This pooled preparation containing a mixture of native fatty acylated PCP and pro-PCP was used for immunological studies.

Specificity of anti-PCP antisera. Rabbit and mouse polyclonal antisera against PCP were tested by Western blot analysis and enzyme-linked immunosorbent assay (ELISA) to determine their specificities. At a 1:500 dilution, all sera detected a single 15,000-MW band in *H. influenzae* S2 whole-cell lysates. The sera also recognized all three forms of PCP expressed from pPX163 and the native form of PCP

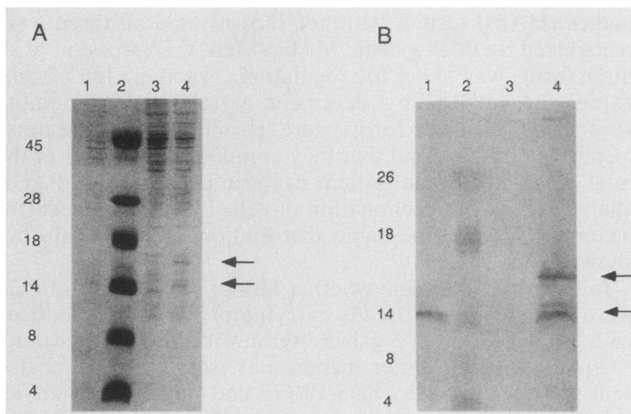

FIG. 2. Expression of recombinant PCP in *E. coli* JM103 from plasmid pPX163. Cells were grown as described in Materials and Methods in LB broth with 2 mM isopropylthio-β-D-galactopyranoside. Whole-cell lysates were separated on 15% SDS-PAGE and stained with Coomassie blue (A) or transferred to nitrocellulose and probed with anti-PCP MAb 61-1 (B). Lanes 1, *H. influenzae* KW20 lysate; lanes 2, molecular mass standards (BRL) (sizes given in kilodaltons); lanes 3, *E. coli* JM103 pUC19 lysate; lanes 4, *E. coli* JM103(pPX163) lysate. The mobilities of the 15- and 17-kilodalton forms of recombinant PCP are marked by arrows.

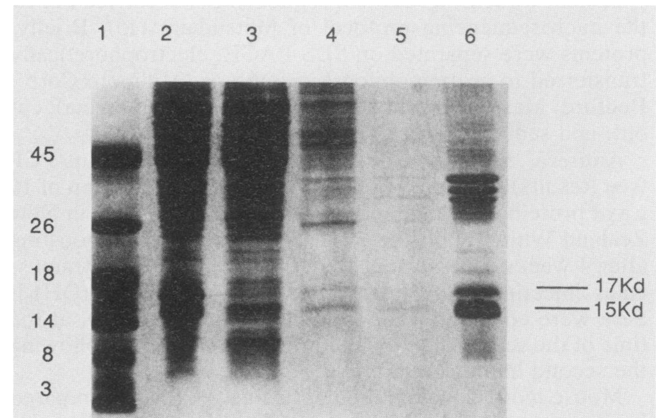

FIG. 3. Fractionation of *E. coli* JM103 cells expressing pPX163. Cells were grown, induced, and fractionated by detergent extraction (21). Fractions were separated on 15% SDS-PAGE and stained with Coomassie blue. Cell-equivalent amounts of protein were run in each lane. Lane 1, Molecular mass standards (BRL) (sizes given in kilodaltons); lane 2, *E. coli* JM103(pPX163) whole-cell lysate; lane 3, cytosol fraction; lane 4, first detergent extract (inner membrane); lane 5, second detergent extract; lane 6, outer membrane. The mobilities of the 15- and 17-kilodalton (Kd) forms of recombinant PCP are marked.

TABLE 1. ELISA response to recombinant PCP

Serum	Adjuvant	ELISA titer[a] against:			
		PCP		PAL[b]	
		Pre	Post	Pre	Post
Rabbit	IFA[c]	1/300	1/656,000	1/900	1/900
Mouse[d]	IFA	1/100	$>1/10^6$	1/900	1/2,700
Mouse[d]	Aluminum	1/100	$>1/10^6$	1/2,700	1/2,700

[a] Titers were assayed with antisera obtained 6 weeks after the second immunization.
[b] Recombinant *H. influenzae* PAL was expressed in and isolated from *E. coli* as described by Green et al. (6a).
[c] IFA, Freund incomplete adjuvant.
[d] Antisera from five mice were pooled.

expressed from pPX140 in *E. coli* cells. The sera did not detect the *H. influenzae* PAL protein expressed in *E. coli*. By ELISA, all sera showed a >1,000-fold increase in titer against PCP and no increased titer against *H. influenzae* PAL (Table 1).

PCP MAb 61-1 was tested by Western blot and found to react with a single 15,000-MW protein in *H. influenzae* S2 cells and all three forms of PCP expressed from pPX163 in *E. coli* (Fig. 2).

Localization of PCP in *H. influenzae* cells. *H. influenzae* S2 cells were fractionated as described by Loeb et al. (12). Individual fractions were tested by SDS-PAGE Western blot analysis with MAb 61-1 as a probe. More than 90% of the PCP in S2 cells was associated with the outer membrane fraction, verifying that PCP is an outer membrane protein in *H. influenzae* cells.

Antigenic conservation of PCP in *H. influenzae* strains. The distribution and antigenic conservation of PCP in *H. influenzae* isolates was tested by SDS-PAGE Western blot analysis of 27 clinical nontypeable *H. influenzae* strains by using MAb 61-1. *H. influenzae* b Eagan, S2, KW20, and 27 clinical nontypeable isolates were tested. In whole-cell lysates from all strains tested, MAb 61-1 recognized a single 15,000-MW protein (Fig. 4). Hence, PCP is expressed in a range of clinical nontypeable *H. influenzae* isolates and at least the epitope recognized by MAb 61-1 is antigentically conserved. MAb 61-1 also recognized a 15,000-MW protein in lysates of *H. parainfluenzae* C (Fig. 4).

Bactericidal activity of anti-PCP antisera. The bactericidal activity of rabbit anti-PCP antiserum was tested against *H. influenzae* S2, *H. influenzae* b Eagan, and nine clinical nontypeable *H. influenzae* strains. Anti-PCP antiserum killed S2 and seven nontypeable *H. influenzae* strains; no response over background was detected against the remaining two nontypeable *H. influenzae* strains or against *H. influenzae* b Eagan (Table 2). Mouse anti-PCP antiserum also showed equivalent bactericidal activity against *H. influenzae* S2 (data not shown). Anti-PCP antiserum or complement alone showed no bactericidal activity against *H. influenzae* cells.

Biologic activities of mixed anti-PCP and anti-PAL antisera. The biologic activities of mixtures of anti-PCP and anti-PAL antisera were evaluated by the bactericidal assay. Bactericidal activities of the individual and mixed sera at a variety of concentrations were tested (Table 3). Dilutions of anti-PCP antiserum of ≥1/80 and of anti-PAL antiserum of >1/320 were not bactericidal against S2. However, when antisera diluted two- to fourfold past their endpoints were mixed, bactericidal activity could be restored. Continued dilution of either antiserum abolished bactericidal activity (Table 3). The bactericidal activities of the two sera were complementary.

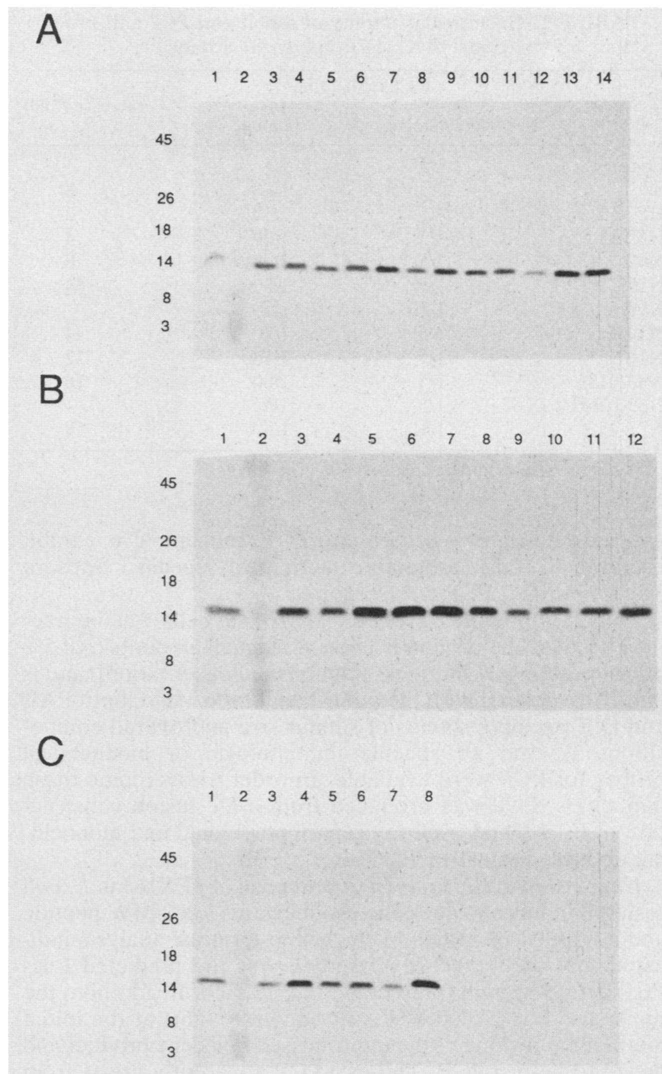

FIG. 4. Western blot analysis of clinical nontypeable *H. influenzae* strains with MAb 61-1. Whole-cell lysates were separated on 18% SDS-PAGE, transferred to nitrocellulose, and probed with MAb 61-1 ascitic fluid at 1:500 dilution. Sizes of molecular mass standards (BRL) are given on the left in kilodaltons. (A) Lane 1, Strain S2; lane 2, molecular mass standards; lanes 3 to 14, N1955, N83-0210E, DL322, N90, N10, N83-045E, N83-0133E, N1937, N82-0127E, N83-0100E, N83-026E, and N47, respectively. (B) Lane 1, Strain S2; lane 2, molecular mass standards; lanes 3 to 12, strains HST31, HST32, HST33, HST34, HST35, HST36, HST37, HST41, HST44, and HST45, respectively. (C) Lane 1, Strain S2; lane 2, molecular mass standards; lanes 3 to 7, strains P86-0295, P86-1454, P81-0384, P88-0859, and 37567, respectively; lane 8, *H. parainfluenzae* C.

DISCUSSION

Previous work has shown that some polyclonal antisera raised against the 15,000-MW *H. influenzae* peptidoglycan-associated lipoprotein (PAL or P6) also recognize the product of a second *H. influenzae* gene encoding a different 15,000-MW lipoprotein, PCP (3). In order to further characterize the immunological relationship between PAL and PCP

TABLE 2. Bactericidal activity of rabbit anti-PCP antiserum against diverse *H. influenzae* strains

Strain	Bactericidal titer[a]		Increase (fold)
	Preimmune	Immune	
Eagan	1/5	1/5	
S2	1/10	1/80	8
HST33	<1/5	1/10	2
HST35	1/5	1/20	4
N47	1/5	1/40	8
N1937	<1/5	1/320	≥64
N90	1/10	1/10	
N1955	<1/5	1/20	4
N10	1/5	1/160	32
N83-0133	1/5	1/80	16
N83-026E	<1/5	1/5	
N83-45E	1/10	1/80	8

[a] Greatest serum dilution to kill >50% of test cells.

and to determine whether anti-PCP antisera also exhibit bactericidal and protective activities, specific antisera against purified PCP were required.

Preparation of PCP from *H. influenzae* cells was impractical because the protein is present in small amounts (<0.5% of cell protein [G. Zlotnick, unpublished observation]) and is tightly associated with the outer membrane. Also, both PAL and PCP are lipoproteins of similar size and overall composition (3), and no specific immunologic or biochemical probes for PCP were available. In order to overcome these difficulties, PCP was produced from a *lac* fusion construction in *E. coli* and used to prepare polyclonal and monoclonal antisera against PCP.

Expression of the *lac-pcp* hybrid gene of pPX163 in *E. coli* resulted in three major gene products: a 17,000-MW peptide and a doublet of 15,000 MW. Amino-terminal analysis indicated that the 17,000-MW peptide was the predicted Lac-PCP hybrid protein (predicted MW, 17,599). In addition, the upper band at 15,000 MW was shown to start at the initial methionine residue of the unprocessed *pcp* gene product and hence to be pro-PCP. This pro-PCP apparently arises from secondary initiation of translation: the fusion gene sequence does provide a Shine-Dalgarno box 10 bases upstream from the initiation codon of *pcp* (Fig. 1). Finally, the lower 15,000-MW band has been shown to be lipid modified and blocked to N-terminal analysis and therefore is probably the mature PCP lipoprotein. It was not determined whether one or both pro-PCP and Lac-PCP were processed into mature PCP. All three forms of PCP expressed from pPX163 were transported to the outer membrane, demonstrating that lipoprotein processing and signal sequence cleavage are not required for localization of PCP in *E. coli*. Similarly, Lin et al. (11) have reported that processing of the *E. coli* murein lipoprotein is not required for its transport to the outer membrane.

TABLE 3. Bactericidal activities of mixtures of Anti-PCP and anti-PAL antisera against *H. influenzae* S2[a]

Anti-PCP serum dilution	Killing[b] at anti-PAL serum dilution of:					
	No antibody	1/320	1/640	1/1280	1/2560	1/5120
No antibody	−	+	−	−	−	−
1/40	+	+	+	+	+	+
1/80	−	+	+	+	+	+
1/160	−	+	+	+	+	−
1/320	−	+	+	−	−	−
1/640	−	+	−	−	ND	ND

[a] Rabbit antisera against recombinant PCP and recombinant PAL (Green et al., submitted) were used.
[b] +, >50% of test cells killed; −, <50% of test cells killed; ND, not done.

It was previously suggested (3) that recognition of PCP by anti-PAL antisera might be due to immunological cross-reactivity between the two proteins, despite the divergence of their primary sequences. In the current studies, no cross-reactivity between purified PCP and PAL could be detected by Western blot or ELISA (Table 1). Our results indicate that the anti-PCP antibody observed in anti-PAL antisera was due to the presence of trace amounts of PCP in the preparations used to produce these antisera. This has been supported by demonstrating that anti-PCP MAb detects low levels of PCP in those preparations. In further support of this conclusion, antisera raised against recombinant *H. influenzae* PAL prepared from *E. coli* cells (6a) raised no detectable antibody against recombinant PCP.

Anti-PCP antiserum was tested against *H. influenzae* S2, a nontypeable derivative of strain Eagan (24), and found to have bactericidal activity (Tables 2 and 3). In order to further evaluate PCP as a vaccine candidate, an anti-PCP MAb was used to probe nontypeable *H. influenzae* clinical isolates. The results demonstrated that PCP was expressed and antigenically conserved in 27 of 27 isolates tested (Fig. 4). In addition, polyclonal anti-PCP antiserum was shown to have bactericidal activity against 9 of 11 nontypeable *H. influenzae* tested. Anti-PCP antiserum was not bactericidal against *H. influenzae* b Eagan and hence was not tested in the infant rat protection model (22), which is valid only for type b *H. influenzae* infections.

Finally, antisera against PCP and recombinant *H. influenzae* PAL prepared from *E. coli* (6a) were tested to see whether their respective bactericidal activities against *H. influenzae* cells were complementary. The results (Table 3) demonstrated that mixtures of the two antisera were bactericidal at greater dilutions than either individual component; therefore, anti-PCP and anti-*H. influenzae* PAL activities were apparently additive.

The recombinant PCP protein used in these studies was subjected to severe denaturing conditions (boiling in SDS and 2-mercaptoethanol and separation on SDS-PAGE) yet still elicited a biologically active antiserum. It is possible the PCP prepared by milder methods would elicit antisera with stronger biologic activities. We currently have no data about the relative contributions of fatty acylated PCP and pro-PCP to the immunogenicity and antigenicity of the preparations used in this study.

These data suggest that the 15,000-MW PCP lipoprotein of *H. influenzae* is a potentially valuable component for a subunit vaccine against nontypeable *H. influenzae* disease, either alone or in combination with *H. influenzae* PAL or other components.

ACKNOWLEDGMENTS

We acknowledge the following for their assistance: Robert Seid for peptide sequencing, John Farley for DNA sequencing, Peter Paradiso and James Cowell for reading the manuscript and suggesting improvements, and Elaine Welch for assistance in preparing the manuscript.

LITERATURE CITED

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
2. **Brodeur, B. R., P. Tsang, and Y. Larose.** 1984. Parameters

affecting ascites tumor formation in mice and monoclonal antibody production. J. Immunol. Methods **71**:265–272.

3. **Deich, R. A., B. J. Metcalf, C. W. Finn, J. E. Farley, and B. A. Green.** 1988. Cloning of genes encoding a 15,000-dalton peptoglycan-associated outer membrane lipoprotein and an antigenically related 15,000-dalton protein from *Haemophilus influenzae*. J. Bacteriol. **170**:489–498.
4. **Fraser, D. W., C. C. Geil, and R. A. Feldman.** 1974. Bacterial meningitis in Bernalillo County, New Mexico: a comparison with three other American populations. Am. J. Epidemiol. **100**:29–34.
5. **Gefter, M. L., D. H. Margullies, and M. D. Scharff.** 1977. A simple method for polyethylene glycol promoted hybridization of mouse myeloma cells. Somatic Cell Technol. **3**:231–236.
6. **Granoff, D. M., and R. S. Munson.** 1986. Prospects for the prevention of *Haemophilus influenzae* type b disease by immunization. J. Infect. Dis. **153**:448–461.

6a. **Green, B. A., B. J. Metcalf, T. Quinn-Dey, D. H. Kirkley, S. A. Quataert, and R. A. Deich.** 1990. A recombinant non-fatty acylated form of the Hi-PAL (P6) protein of *Haemophilus influenzae* elicits biologically active antibody against both nontypeable and type b *H. influenzae*. Infect. Immun. **58**:3372–3378.

7. **Green, B. A., T. Quinn-Dey, and G. W. Zlotnick.** 1987. Biologic activities of antibody to a peptidoglycan-associated lipoprotein of *Haemophilus influenzae* against multiple clinical isolates of *H. influenzae* type b. Infect. Immun. **55**:2878–2883.
8. **Hagar, D. A., and R. R. Burgess.** 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal. Biochem. **109**:76–86.
9. **Howie, V. M., J. H. Ploussard, and R. L. Lester, Jr.** 1970. Otitis media: a clinical and bacteriological correlation. Pediatrics **45**:29–35.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Lin, J. J., H. Kanazawa, and H. C. Wu.** 1980. Assembly of outer membrane lipoprotein in an *Escherichia coli* mutant with a single amino acid replacement within the signal sequence of prolipoprotein. J. Bacteriol. **141**:550–557.
12. **Loeb, M. R., A. L. Zachary, and D. H. Smith.** 1981. Isolation and partial characterization of outer and inner membranes from encapsulated *Haemophilus influenzae* type b. J. Bacteriol. **145**:596–604.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Matsudaira, P.** 1987. Sequence from picomole quantities of protein electroblotted onto polyvinylidine diflouride membranes. J. Biol. Chem. **262**:10035–10038.
16. **Munson, R. S., and D. M. Granoff.** 1985. Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. Infect. Immun. **49**:544–549.
17. **Murphy, T. F., L. C. Bartos, A. M. Campagnari, M. B. Nelson, and M. A. Apicella.** 1986. Antigenic characterization of the P6 protein of nontypable *Haemophilus influenzae*. Infect. Immun. **54**:774–779.
18. **Murphy, T. F., M. B. Nelson, K. C. Dudas, J. M. Mylotti, and M. A. Apicella.** 1985. Identification of a specific epitope of *Haemophilus influenzae* on a 16,600 dalton outer membrane protein. J. Infect. Dis. **152**:1300–1307.
19. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
20. **Peterson, G. L.** 1977. A simplification of the protein method of Lowry *et al.* which is more generally applicable. Anal. Biochem. **83**:346–356.
21. **Phillip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115**:717–722.
22. **Smith, A. L., D. H. Smith, D. R. Averill, Jr., J. Marino, and E. R. Moxon.** 1973. Production of *Haemolphilus influenzae* type b meningitis in infant rats by intraperitoneal inoculation. Infect. Immun. **8**:278–290.
23. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4355.
24. **Weller, P. F., A. L. Smith, P. Anderson, and D. H. Smith.** 1977. The role of encapsulation and host age in the clearance of *Haemophilus influenzae* bacteremia. J. Infect. Dis. **135**:24–41.
25. **Wilcox, K. N., and H. O. Smith.** 1975. Isolation and characterization of mutants of *Haemophilus influenzae* deficient in an adenosine-5′-triphosphate-dependent deoxyribonuclease activity. J. Bacteriol. **122**:443–453.
26. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 cloning vectors and host strains: nucleotide sequences of the M13pmp18 and pUC19 vectors. Gene **33**:103–119.
27. **Zlotnick, G. W., V. T. Sanfilippo, J. A. Mattler, D. H. Kirkley, R. A. Boykins, and R. C. Seid, Jr.** 1988. Purification and characterization of a peptidoglycan-associated lipoprotein from *Haemophilus influenzae*. J. Biol. Chem. **263**:9790–9794.