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Variability of Outer Membrane Protein P1 and Its Evaluation as a Vaccine Candidate against Experimental Otitis Media due to Nontypeable *Haemophilus influenzae*: an Unambiguous, Multifaceted Approach

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Candidate vaccine antigens for preventing otitis media caused by nontypeable *Haemophilus influenzae* (NTHI) should possess one or more conserved epitopes. We sought to evaluate the candidacy of P1, a surface-expressed outer membrane protein knowing that this antigen is subject to diversifying selection. Therefore, we selected NTHI strains from among >500 phylogenically variant isolates representative of the diversity found in natural populations of *H. influenzae*. Twenty-three variants of P1 (≤95% similarity) were identified among 42 strains. When chinchillas were immunized with recombinant P1 (rP1) obtained from one of these isolates (BCH-3), all animals developed antibodies specific for rP1. Immunized animals were protected against disease when challenged with BCH-3, but not with an *ompP1* mutant of BCH-3 or a strain (BCH-2) possessing a heterologous P1 (91% identity). We conclude that (i) while P1 induces protection against NTHI-mediated otitis media, development of a polyvalent vaccine reflecting the variability of P1 would be necessary to construct an efficacious vaccine and (ii) use of a phylogenically characterized collection of representative isolates in concert with gene sequencing, cloning, gene inactivation, and animal testing offers an efficient, rational, and rigorous strategy for evaluating the potential problems associated with variability of vaccine targets and specificity of related immune responses.

Nontypeable (unencapsulated) *Haemophilus influenzae* (NTHI) colonizes ~85% of humans (43, 44). NTHI is usually associated with noninvasive diseases, such as sinusitis, otitis media, and pneumonia (33) as well as persistent respiratory infection in patients with chronic bronchitis and cystic fibrosis (27, 64, 74). On occasion, NTHI may become invasive, causing bacteremia and sometimes crossing the blood-brain barrier to cause meningitis (33, 54). With the exception of the *H. influenzae* type b capsule, the multiple host and microbial factors differentiating commensal from pathogenic behavior are unknown for both typeable *H. influenzae* and NTHI (43, 44).

Unencapsulated NTHI strains having genes with no homology to capsule biosynthetic genes are found, while others have a partial or complete deletion of the IS*1016*-flanked capsule biosynthetic locus (35–37). While previous phylogenic studies suggest serotype-specific clustering of *H. influenzae* capsular serotypes a to f (31, 39, 51–53, 60), the precise evolutionary relationship between types a to f and NTHI remained obscure (50) until recently. Using a phylogenically organized collection of >500 *H. influenzae* and NTHI isolates (13; unpublished data), we examined the 329 NTHI strains using restriction fragment length polymorphism (RFLP) analysis to identify the serotype correlated with any strains encoding a capsule locus remnant (35–37). For each NTHI isolate in which an interpretable remnant was found, the RFLP profile correlated with the capsule type of the evolutionary lineage to which it was most closely related in the phylogenically organized collection (13).

Effective passive immunization in animals provides evidence that circulating antibodies can prevent both invasive and mucosal diseases caused by typeable *H. influenzae* (41) and NTHI (6). Further studies with both categories of *H. influenzae* demonstrated that antibodies specific to outer membrane proteins (OMPs) of homologous strains are associated with protection in animals (32, 69) and humans (7) and that the majority of bactericidal antibody activity present in serum from either a patient recovering from NTHI disease or from normal adult sera is directed to OMPs (24). However, virtually every major antigen on the surface of NTHI is known to be either variable or absent from some isolates (10, 18, 23, 24, 26, 38, 49, 62, 73), a likely consequence of diversifying selection in the absence of an outer capsule. This is the initial problem to be overcome when considering an OMP-based vaccine. The immunogenicity of one of these OMPs, P1, is the subject of the studies described in this report.

OMP P1 (47 kDa) accounts for ~10% of *H. influenzae* OMP content. Passive immunization with P1 induces protection against bacteremia in the infant rat model (48). The associated gene, *ompP1*, has been cloned and sequenced from *H. influenzae* serotype b (46) and d (19) strains. OMP P1 has eight potentially surface-exposed loops (14), four of which are immunogenic (57, 61, 62). The availability of several *ompP1* se-

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quences was particularly relevant, as it offered the information needed for a broader survey of the sequence conservation of the *ompP1* gene across a phylogenically classified collection of >500 typeable *H. influenzae* and NTHI isolates representing the natural population structure of the species (13; unpublished data). Based on this knowledge, we could evaluate the efficacy of P1 immunization in protecting against NTHI isolates with P1 antigens homologous and heterologous to the immunizing antigen. Doing so allowed us to test the proposition that such a phylogenic approach is a viable strategy for the rational development of vaccines by directly addressing the inherent problem of antigenic variation within a microbial species. For this purpose, infectious challenge was carried out with NTHI isolates known to express either a significantly variant OMP P1 on the one hand or an OMP P1 nearly identical to that of the P1 immunogen on the other, in order to determine whether a priori knowledge was predictive before actual animal testing. The *ompP1* DNA sequence information also allowed for (i) the cloning, expression, and purification of P1 immunogen and (ii) the construction of an otherwise isogenic *ompP1* mutant control strain used in these studies to directly test the specificity of the immunogen.

Results of these studies have direct implications in considering further development of OMP P1 as a vaccine candidate to protect against both NTHI-associated otitis media and other respiratory infections, while also providing basic information about the role of P1 in the virulence and viability of NTHI. Equally important, as utilized, the phylogenic strategy for vaccine development based on foreknowledge of the range of immunogen allele sequence variability within the natural population structure of *H. influenzae* is broadly applicable to other microbial pathogens.

MATERIALS AND METHODS

Bacterial strains. NTHI BCH-1 is a β-lactamase-producing *H. influenzae* strain isolated from the transtracheal aspirate of an adult with pneumonia (32). NTHI strain BCH-2 was isolated from the middle ear of a young child with acute otitis media (middle-ear infection) (32). NTHI strain BCH-3 is a non-β-lactamase-producing isolate from the nasopharyngeal culture of a young child. BCH-1, -2, and -3 each express a phylogenically variant wild-type (wt) *ompP1* allele characterized in this study. BCH-3 *ompP1* Km^r (kanamycin resistant) is an otherwise isogenic *ompP1* mutant of BCH-3, constructed as described below by insertional inactivation with a Km^r cassette. The subset of 42 *H. influenzae* isolates used for *ompP1* DNA sequence analysis represents the evolutionary diversity of a phylogenically organized collection of more than 500 *H. influenzae* and NTHI isolates (13; unpublished data) and are described in Table 1. *Escherichia coli* strains JM107 (79) and INVαF′ One Shot (Invitrogen) (28) cells were used for DNA cloning. *E. coli* strain BLR (Novagen) was used to express recombinant P1 antigen. *H. influenzae* and NTHI isolates were grown as previously described (24, 32) as were *E. coli* isolates (2–4).

Chromosomal DNA extraction. Ten milliliters of an overnight culture of *H. influenzae* was centrifuged at 4,300 × *g* for 10 min at 4°C, resuspended in 3 ml of 50 mM Tris–20 mM EDTA at pH 8.0, and recentrifuged. The cell pellet was resuspended in 4 ml of 50 mM Tris–2 mM EDTA (pH 8.0) containing 1 mg of lysozyme and incubated for 30 min at 4°C. Addition of 1 mg of proteinase K and 0.3 ml of 10% sodium dodecyl sulfate was followed by incubation of the lysate with agitation for 4 h at 56°C after which 1 ml of 10% lauroylsarcosine was also added. Chromosomal DNA was then purified and isolated by $CsCl_2$ equilibrium density gradient centrifugation (78) as previously described (2–4).

Plasmid DNA extraction. Plasmid DNA was extracted with an alkaline lysis procedure (65).

DNA sequencing and analysis. Forty *H. influenzae ompP1* DNA sequences were amplified by PCR from purified chromosomal DNA using primers P1-5 (5′-GCTCCTGCTAACTAGTCGTA-3′) and P1-6 (5′-AATCAAAAGCCGTC CGACTG-3′), derived from reported sequences flanking the *ompP1* open reading frame (109 bases upstream and 59 bases downstream, respectively) of type b *H. influenzae* and strain Rd (19, 46, 47). Primers P1-3 (5′-CAGGTGGCGTTT ATATTGATTCTAG-3′), P1-4B (5′-TGTGTCATTACAAGATAGAGCCGC T-3′), and P1-7 (5′-CTTCATTAAATTGATACATT-3′) were chosen based on nested sequences of the *ompP1* gene and used in combination with P1-5 and P1-6 to sequence the amplified products (see below). Parameters for PCR amplification involved initial denaturation at 94°C for 7 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and primer extension at 72°C for 2 min, followed by a final primer extension at 72°C for 4 min. PCR products were purified by electrophoresis through a 0.8% Tris-acetate-EDTA low-melting-point agarose gel and extracted with the phenol-chloroform method (65). PCR products were sequenced with an ABI 377 DNA sequencer using 0.25× Big Dye Terminator reaction mixtures.

Primers P1-5 and P1-6 were also used for PCR-based characterization of the putative, insertionally inactivated *ompP1* mutant of NTHI strain BCH-3 as described below.

Amino acid sequence alignment was performed with LASERGENE MegAlign software (DNASTAR) using the CLUSTAL multiple-alignment algorithm (30). In addition to the 40 OMP P1 sequences (this report), two reference OMP P1 sequences from strains Rd (19) and MinnA (46) (GenBank accession no. AAC22060 and AAA24990, respectively) were included in the OMP P1 protein alignment.

Nomenclature. In the genomically sequenced *H. influenzae* strain Rd (ATCC 51907) (19), the OMP P1-encoding gene (locus identifier, HI0401) was designated *fadL* for long-chain fatty acid transport protein (GenBank accession no. AAC22060; protein sequence identifier g1573372), based on the similarity of its product to that of *E. coli* strain K-12 *fadL* (GenBank accession no. M60607 and Y00552; SwissProt accession no. P10384; protein sequence identifiers 145910 and 41372). In *H. influenzae* Rd, *fadL* is transcribed from the plus DNA strand (coordinates 422144 to 423520), spanning 1,377 nucleotides. Its translated product is 459 amino acids (aa) in length with a predicted 49,477.13-Da molecular mass. This protein's cellular role is as a transport binding protein (carbohydrates, organic alcohols, and acids) as described in the *H. influenzae* genome database (http://www.tigr.org/tdb/CMR/ghi/htmls/SplashPage.html).

Marker-exchange construction of an *ompP1* mutant of NTHI strain BCH-3. Plasmid vector pFRG100 carries the 969-bp *Pst*I/*Hin*dIII *ompP1* DNA fragment of *H. influenzae* serotype b strain DL41 (25). *Pst*I/*Hin*dIII digestion allowed for isolation and subsequent ligation of *ompP1* into pUC18 (77). The resulting construct, pBJG1, was transformed (described below) into *E. coli* strain JM107 and plated onto Luria-Bertani (LB) broth (65) agar plates containing 50 μg of ampicillin/ml. Transformed cells were grown in 10 ml of LB broth with 50 μg of ampicillin/ml and incubated at 37°C overnight. In order to insertionally inactivate *ompP1* of pBJG1, plasmid pBJG1 was extracted and linearized through *Bgl*II digestion, disrupting *ompP1* 640 bp upstream from the *Pst*I site. A 1,264-bp kanamycin resistance cassette (Km^r) was then removed from pUC4K (56, 77) by digestion with *Bam*HI and ligated into the compatible, *Bgl*II-linearized pBJG1. The new Km^r construct, pBJG1-A, was transformed into *E. coli* JM107 cells, plated onto LB broth agar plates containing 65 μg of kanamycin/ml, and incubated overnight at 37°C. In order to carry out the marker exchange (20, 66), Km^r pBJG1-A, a circular plasmid incapable of replication in *H. influenzae*, was extracted and transformed into Km^s (kanamycin-sensitive) NTHI strain BCH-3. Transformed BCH-3 cells were selected by plating onto chocolate agar plates containing 65 μg of kanamycin/ml and incubated overnight in a candle extinction jar at 37°C.

In order to screen for absence of OMP P1, Km^r transformants were picked from the kanamycin-containing chocolate agar plates, suspended in 10 μl of Gey's balanced salt solution (Life Technologies), and dot blotted onto Immobilon P membranes (Millipore). wt BCH-3 that expressed P1 was used as a positive control. Membranes were washed twice in 100 ml of 0.1 M phosphate-buffered saline (PBS) containing 1% (wt/vol) nonfat dry milk powder for 10 min with agitation at room temperature, placed in 50 ml of monoclonal antibody (MAb) AD4 (described below) hybridoma cell supernatant (diluted 1:5 in 0.1 M PBS containing 1% bovine serum albumin plus 0.5 M NaCl), and incubated with agitation for 2 h at 20°C. The membranes were washed again as described above and incubated with agitation in 50 ml of 2-μg/ml anti-mouse immunoglobulin G conjugated to alkaline phosphatase (in 0.1 M PBS containing 1% bovine serum albumin) for 2 h at 20°C. A final wash was carried out as described above. The color reaction was developed for approximately 10 min at room temperature (12) and stopped by rinsing the membranes in 200 ml of double-distilled H_2O for 5 min.

Western blot analysis was also carried out to screen for the absence of OMP P1. Outer membrane preparations from wt BCH-1, wt BCH-3, and the putative, insertionally inactivated Km^r *ompP1* BCH-3 transformed cells were prepared as previously described (32). MAb AD4 (diluted 1:5 in 0.1 M PBS containing 1% bovine serum albumin plus 0.5 M NaCl) was used to probe the outer membranes to confirm the presence or absence of OMP P1. This was followed by incubation in buffer with 2 μg of staphylococcal protein A/ml conjugated to alkaline phosphatase (Sigma) in 0.1 M PBS with 1% bovine serum albumin. The color reaction was developed as described above.

Direct confirmation of the Km^r inactivated *ompP1* construct, described in Results, involved analysis of genomic DNA from both the putative Km^r *ompP1* mutant of BCH-3 and the wt BCH-3 strain by PCR amplification of *ompP1*. PCR products were then probed with an *ompP1* fragment isolated from pFRG100 and the Km^r cassette from pUC4K to confirm their identities.

Cloning and purification of recombinant P1. The gene encoding OMP P1 of BCH-3 (*ompP1*$_{BCH-3}$) was amplified by PCR using forward primer P1-1B (5′-C GGGATCCGGCAGCGTTTCAATTGGCG-3′) and reverse primer P1-2B (5′-GGAATTCTTAGAAACTATAATTTAAATTCAAACC-3′). The inclusion of the *Bam*HI and *Eco*RI sites (underlined) in P1-1B and P1-2B, respectively, was intended to ensure proper orientation within the open reading frame of the

TABLE 1. Alignment of four variable regions of OMP P1 from 42 phylogenically diverse typeable *H. influenzae* and NTHI isolates[a]

P1 type[b]	Strain	Sequence of variable region[c]: I (85–107)	II (198–232)	III (281–296)	IV (427–440)	S[d]	Loc[e]
1	BCH-1	DSYAIISDS---IKVTNDGSASA	LIADTVKDN-QVKNTLTVQQEPLKFIDKYLPSKDT	LEANVIKASKTGDLTL	KTIGDKRSLALSTT	NT	USA
	BCH-3	DSYAIISDS---MKVTNDGSASA	LIADTVKDN-QVKNTLTVQQEPLKFIDKYLPSKDT	LEANVIKASKTGDLTL	KTIGDKRSLALSTT	NT	USA
	3-H-1552	DSYAIISDS---IKVTNDGSASA	LIADTVKDN-QVKNTLTVQQEPLKYINKYLPSKDT	LEANVINASKTGDLTL	KTIGDKRSLALSTT	NT	USA
	17-H-783	DSYAIISNS---IKVTNDGSASA	LIADTVKDS-QVKTTLIVQQEPLKSIDKYLPSKDT	LEAEAIKAGKKGXLTL	KTIGDARSLALNTT	NT	USA
	21-H-1328	NSYAIISNS---IKVTNDGSASA	LIADTVKDS-QVKTTLIVQQEPLKSIDKYLPSKDT	LEAEAIKAGKKGDLTL	KTIGDARSLALNTT	NT	USA
	27-H-1433	DSYAIISNS---IKVTNDGSASA	LIADTVKDS-QVKNALIVQQEPLKSIDKYLPSKDT	LEAXAIKAGKKGDLTL	KTIGDARSLALNTT	NT	USA
2	200	NSDAIITSTSSGVKTIKHGSASQ	IIVDSLKDN-QVQTALSVQKEPLKYLHKYLPSKDT	VEADVIKAGKKGDLTL	KTIGDERSLALNTT	NT	Finland
	218	NSDAIITSTSSGVKTIKHGSASQ	IIVDSLKDN-QVQTALSVQKEPLKYLHKYLPSKDT	VEADVIKAGKKGDLTL	KTIGDERSLALNTT	NT	Finland
	667	NSDAIITSTNSGVKTIKHGSASQ	IIVDSLKDN-QVQTALSVQKEPLKYLHKYLPSKDT	VEADVIKAGKKGDLTL	KTIGDERSLALNTT	NT	Finland
	199	NSDAIITSTSNGVKTIKHGSASQ	IIVDSLKDN-QVQTALSVQKEPLKYLHKYLPSKDT	VEADVIKAGKKGDLTL	KTIGDERSLALNTT	NT	Finland
3	7416	DSSAIISNM---IKATKDGSASE	IIADSVKDN-QVKTALTVQQEPLKFLDKYLPSKDT	VEANVIKAGKKGDLTL	KTIGDERSLTLNTT	a	Kenya
	Rd	TSHATIITSSSGIKAIEGGSASA	IIADSVKDN-QVKTALTVQQEPLKFLDKYLPSKDT	VEANVIKAGKKGDLTL	KTIGDERSLTLNTT	d	USA
4	7004	T--ASIATTK--MNSAKYGSASE	IIANSVNDT-QVKTALSVLAPPLKGLDQNLPSKDK	VEANVIKEGKKGNLTL	KTIGDKRTLTLNTT	b	Holland
	6094	T--ASIATTK--MNSAKYGSASE	IIANSVNDT-QVKTALSVLAPPLKGLDQNLPSKDK	VEANVIKEGKKGNLTL	KTIGDKRTLTLNTT	b	UK
	6107	T--ASIATTK--MNSAKYGSASE	IIANSVNDT-QVKTALSVLAPPLKGLDQNLPSKDK	VEANVIKEGKKGNLTL	KTIGDKRTLTLNTT	b	UK
	7109	T--ASIATTK--MNSAKYGSASE	IIANSVNDT-QVKTALSVLAPPLKGLDQNLPSKDK	VEANVIKEGKKGNLTL	KTIGDKRTLTLNTT	NT	Sweden
	ATCC9795	T--ASIATTK--MNSAKYGSASE	IIANSVNDT-QVKTALSVLAPPLRGLDQNLPSKDK	VEANVIKEGKKGNLTL	KTIGDKRTLTLNTT	b	
	161	T--ASIATTR--MNSAKYGSASE	IIANSVNDT-QVQTALSVLAPPLKGLDQNLPSKDK	VEANVIKEGKKGNLTL	KTIGDKRALTLNTT	NT	Finland
	OT-9	T--ASIATTK--MNSAKYGSASE	IIANSVKDT-QVKTALSVLAPPRTALPQNLPSKDK	VEANVIKEGKKGNLTL	KTIGDKRALTLNTT	NT	Canada
5	1363	NSYATITSASSGVRTIKHGSASQ	IIVETI-DN-QVNTALSALQEPFRDLKKHLPSKDK	LEANVIKAGKKGDLTF	KTIGEPRALTFNTT	NT	Finland
	OT-81	NSYATITSASSGVRTIKHGSASQ	IIVETI-DN-QVNTALSALQEPFRDLKKHLPSKDK	LEANVIKAGKKGDLTF	KTIGEPRALTFNTT	NT	Canada
6	9-H-1194	TSHATIVTSSSGVRAIKDGSASA	ILAESVNDDDQVKGALLTLSEPFKNLNTHLPSKDK	LEANVIKAGKKADLTF	KTIGEQRSLTFDTT	NT	Canada
7	MinnA	TSYAQIITNQIGMKAIKDGSASQ	LIADSVKDN-QITSALSTQQEPFRDLKKYLPSKDK	LEANVIKEGKKGNLTF	KTIGDKRTLTLNTT	b	USA
8	1071	ASSVIISNM---VKATQDGSASA	TISDSVNDS-QLTGVLSTLAKPFKELNKHLPSKDK	LEAGVIGAGKTGNLTL	KTIADKSGLPLTST	NT	Finland
9	BCH-2	ASSAIVNSA---MKATKEGSASQ	IIVDTVNDD-QVKRGLTVLQPPYKTLDKYLTSKDK	LEAGVIGAGKTGNLTF	KTIGEKSGLPVTAT	NT	USA
10	LO-127	PSSVTTNST---MKTTMDGSASE	VIVDIVNDT-QIKXALAALSEPYKDFPNYLTSKDK	VEANVIKAGKKGDLTL	NTIGKESGLPLTTT	NT	Canada
11	5	TSSA--TPANSVMTAIRYGSASA	IIEGTVNDD-QIKRALTTQPKPYKDFPQHLSSKDK	LEAGVIGAGKKGDLTL	KAIGEKRSLTFDTT	NT	USA
12	1512A	SVSASITGAS--VKITRNGSASQ	LIADTVNDN-QIKQALSTQQTTLRDLPKHLTSKDK	LEAEAIKAGKKGDLTL	KAISQA-TLTLDTT	NT	USA
	49	DVRASITGVS--VKTTKNGSASQ	IIANTVNDN-QVKLPLSTQPETLRDLHKHLPSKDK	LEAEAIKAGKKGDLTL	KAITKS-VLTLDTT	NT	USA
13	1-H-1085	KCYASITGIS--VKTTKNGSASA	LIVDSV-EIARNGQAFTVLGEPLKTIGQYLTSKDK	LGAGVIKAGKKGDLTL	ETISQA-QLTLNTT	NT	USA
14	6181	E--AFIATLK--MKTTKNGSASE	IIADTV-KISQG--AFTVGSQEDKAIPKYLTSKDK	LEAGAIGAGKKGDLTL	ATIG--KSVILNTT	e	UK
15	22-H-1154	DSYAIISNS---IKVTNDGSASA	IIKESV-ELAKT--AFTVAQDDEKAIPTYLTSKNT	LEAEAIKAGKKGDLTL	KTIGDARSLALNTT	NT	USA
16	4-H-1094	K--SSTKNLQ--MLAAKYGSASQ	IIKESV-ELAKT--AFTVAQDDEKAIPTYLTSKNT	LEAGVIGAGKKGDLTL	KTTG--KTVILNAT	NT	USA
17	6255	D--SSITATT--MRTTKYGSASA	IIAESVKI-AQN--AIKTVNPKDK-ATDYLTSKDK	LEAEVIEAGKKGNLTL	QKAVGGF---ITTT	NT	UK
	ATCC9833	D--SSITATT--MRTTKYGSASA	IIAESVKI-AQN--AIKTVNPKDK-ATDYLTSKDK	LEAEVIEAGKKGNLTL	QKAVGGF---ITTT	f	
	C-105	N--SSITATT--MRTTKYGSASA	IIAESVKI-AQN--AIKTVNPKDK-ATDYLTSKDK	LEAEVIEAGKKGNLTL	QKAVGGF---IXTT	NT	Canada
18	2-H-1038	A--ASITGSS--IKVTNDDVASE	IIADSVKD-PQAKRALETVVPGTK-IPDYLTSKDK	LGKKGIEAGKTGNLTF	QKVADDF---ITAN	NT	USA
19	658	L--SSITSAS--MKTTKYGSASE	IIVRSVKD-SQAQTALKTVVPGTL-IPDLLTSKDK	LGQKSIEAGKTDNLTL	QKVADGL---ITTN	NT	Finland
20	13-H-1157	D--AYLQSGS--MKFTKYGSASQ	IIADSVQD-SQVKPALQAVDPQNK-ATGYLTSKDK	LEAGAIKAGKKGDLTL	QKAAGGH---IITT	NT	USA
21	9	N--ASIKAT---MNMTKYGSASQ	LIADTVQD-QQIQKALKAVDSKTV-IPDYVTSKDK	LEAGVIGAGKKGDLTL	QQV-GGH---IITN	NT	USA
22	7424	A--ASIKATA--MGRTKYGSASA	IIADSIQD-NQIQQALKAVDPQTK-LHNYLTSKDK	LGAKDIVAGKTGDLTF	QQA-GGH---IITT	NT	Kenya
23	4-H-1093	D--ASIKATA--MARTKYGSASQ	IIADSIQD-NQIQQALKAVDPQTK-IHEYLTSKDK	LGKKDIVAGKTGDLTL	KTI-GAH---IITN	NT	USA

[a] Alignment is based on data from references 47 and 61 and this study. Putative surface-exposed loops of OMP P1 correlating with variable regions I to IV are loops 2, 4, 5, and 8, respectively (13, 14, 47).
[b] Isolates with the same P1 type have OMP P1 amino acid sequences of ≥95% similarity.
[c] Numbers indicate amino acid positions at the beginning and end of a variable region. Dashes, gaps inserted to optimize alignment.
[d] S, *H. influenzae* serotype. NT, nontypeable.
[e] Loc, country of strain isolation. USA, United States; UK, United Kingdom.

IPTG (isopropylthio-β-D-galactoside)-inducible expression vector pTrcHisB (Invitrogen) (17). In order for the OMP $P1_{BCH-3}$ translation product to be isolated from cytoplasmic inclusion bodies, the primers involved in amplifying *ompP1*$_{BCH-3}$ did not include the leader sequence of the gene. pTrcHisB was designed by the manufacturer such that expression from it of an insert such as *ompP1*$_{BCH-3}$ generates a translated fusion product with 30 additional amino acids at the N terminus of recombinant $P1_{BCH-3}$ ($rP1_{BCH-3}$) that can be cleaved based on an enterokinase recognition sequence (Invitrogen). However, as described in the Results, this was not cleaved because these amino acids contain (i) six tandem histidine residues exploited for subsequent purification purposes, as they bind specifically to Invitrogen nickel-coated Pro-bond resin, and (ii) the epitope AspLeuTyrAspAspAspAspLys, recognized by the Invitrogen anti-Xpress MAb that was exploited for confirmatory purposes with $rP1_{BCH-3}$.

The PCR mixture and parameters used for amplification of *ompP1*$_{BCH-3}$ were similar to those described above for *ompP1* sequencing. PCR products were purified as described above. The purified DNA was cloned into pCR2.1 (original TA cloning kit; Invitrogen). The cloned *ompP1* DNA was removed from pCR2.1 using *Bam*HI and *Eco*RI. The *ompP1* DNA fragment was then inserted into the multicloning site of the *E. coli* expression vector pTrcHisB and transformed into *E. coli* strain BLR to form the new construct pBJG2. Transformed *E. coli* cells were grown overnight at 37°C on LB broth agar plates with 50 μg of ampicillin/ml. Expression of $rP1_{BCH-3}$ in a 500-ml culture was induced with 5 ml of 100 mM IPTG. The inclusion bodies that contained $rP1_{BCH-3}$ were harvested from the cells (65) and purified using Pro-bond resin under denaturing conditions as specified by the manufacturer (Invitrogen). The final sample volume was brought to 1.5 ml using a Centricon-30 concentrator (Amicon), and the protein concentration was estimated using Peterson's modification of the Lowry method (59). As described in the Results, the preparation was analyzed by Western blotting using MAbs AD4 and anti-Xpress (Invitrogen) (55), polyclonal chinchilla serum that contained antibodies to $P1_{BCH-1}$, and normal chinchilla serum. All antisera were diluted in 0.1 M PBS containing 1% bovine serum albumin plus 0.5 M NaCl.

Development of competence, and transformation of *H. influenzae*. Competence was achieved using the method of Barcak et al. (5). One-milliliter competent *H. influenzae* cell aliquots were added to 1 μg of DNA to be transformed and incubated for 30 min at 37°C. Five milliliters of brain heart infusion (BHI) broth supplemented with NADH and defibrinated horse blood (32) was added to the cells, and the mixture was incubated at 37°C for 90 min. Transformed cells were spread onto chocolate agar plates containing 65 μg of kanamycin/ml and incubated overnight at 37°C in a candle extinction jar.

Development of competence and transformation of *E. coli*. Competence was achieved using a modification of the $CaCl_2$ procedure (67). Aliquots (100 μl) of overnight cultures (*E. coli* strains JM107 and BLR, used for cloning and for P1 expression, respectively) were added to 100 ml of fresh LB broth, and the mixtures were incubated with agitation at 37°C until the *E. coli* reached mid-exponential phase. Cells were collected by centrifugation at 4,068 × *g* for 10 min at 4°C. The cell pellets were suspended in 20 ml of an ice-cold PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid] buffer (10 mM PIPES, 6.67% [vol/vol] glycerol, 60 mM $CaCl_2$) and incubated on ice for 30 min. Cells were harvested by centrifugation at 1,912 × *g* for 5 min at 4°C and gently resuspended in 4 ml of ice-cold PIPES buffer. One microgram of plasmid DNA (pBJG1 or pBJG1-A; described above) was added to 100 μl of competent *E. coli* JM107 cells, and the mixture was incubated for 20 min on ice. Similarly, 1 μg of pTrcHisB (17) containing *ompP1* DNA from NTHI strain BCH-3 (pBJG2) was added to 100 μl of competent *E. coli* strain BLR. Following the addition of 0.5 ml of LB broth, the cells were incubated at 37°C for 1 h. Aliquots transformed with pBJG1 or pBJG2 were spread onto LB broth agar plates with 50 μg of ampicillin/ml. Similarly, cells transformed with pBJG1-A were spread onto LB broth agar plates that contained 65 μg of kanamycin/ml. All plates were incubated for 18 h at 37°C.

P1-specific MAb AD4. MAb AD4, specific to OMP P1 of NTHI strain BCH-1, was produced using the method of Tsung et al. (75) as follows. BALB/cJ mice were immunized intraperitoneally once a week for 5 weeks with 12.5 μg of BCH-1 OMPs (24). Four days after the last immunization, the spleen cells were fused with P3-NS1/1-AG4-1 myeloma cells (34), with polyethylene glycol as the fusion agent (21). The fused-cell mixture was distributed into 96-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and hypoxanthine aminopterine thymidine (40). Supernatants (used at 1:5 dilution) from these actively growing cells were initially subjected to an enzyme-linked immunosorbent assay (ELISA) for antibody activity to outer membranes prepared from wt BCH-1 (24); positive supernatants were further screened by Western blotting using OMPs extracted from BCH-1 (24). One P1-specific MAb, AD4, was selected for additional characterization by demonstrating the binding specificity of AD4 for a P1-enriched preparation (46) and recombinant P1 (see above).

ELISA for BCH-3 P1 and rP1. Immulon I "U"-bottom microtiter plate (Dynatech Laboratories) wells were coated with 0.1 ml of a 5-mg/ml $P1_{BCH-1}$-enriched preparation or with $rP1_{BCH-3}$. The wells were washed and subsequently incubated with 0.1 ml of diluted chinchilla serum, 0.1 ml of a 2-mg/ml solution of staphylococcal protein A conjugated to alkaline phosphatase, and 0.1 ml of a 1-mg/ml alkaline phosphatase substrate (Sigma; 104 phosphatase). We have previously described these methods to measure antibodies against bacterial outer membrane antigens (13, 63).

Recombinant BCH-3 P1 immunization of chinchillas. $rP1_{BCH-3}$ was emulsified in RIBI adjuvant R-730 emulsion (RIBI Immunochem Research) to a final $rP1_{BCH-3}$ concentration of 125 μg/ml. Twenty-eight chinchillas were immunized with two 0.1-ml intramuscular (i.m.) injections in the hindquarters on days 0, 35, 57, 79, 99, 120, and 141. Eight control chinchillas received two 0.1-ml i.m. injections of RIBI adjuvant only on the same days. All chinchillas were bled by cardiac puncture using a 23-gauge 3-ml syringe on days 0 (before receiving the first immunization) and 148 (prior to challenge). Blood specimens were allowed to coagulate for 30 min at room temperature before centrifugation at 2,000 × *g* for 10 min at 4°C. The sera were used in ELISAs (to detect production of antibodies to $rP1_{BCH-3}$) and Western blots (to detect antibody specificity to $rP1_{BCH-3}$).

Transbullar middle-ear challenge and induction of otitis media. Seven days after the final immunization, four nonimmunized chinchillas were added to the experimental group as controls. All animals were examined by otoscopy and tympanometry (70) to document healthy, normal middle ears.

Loopfuls of wt strain BCH-3 and the isogenic BCH-3 *ompP1* mutant were inoculated separately into 1 ml of supplemented brain heart infusion medium and incubated without agitation for 16 to 18 h at 37°C. Overnight cultures were diluted by 2×10^{-3} in Gey's balanced salt solution (Sigma) (32). The chinchillas were divided into two groups. One group was challenged with wt BCH-3, and the other was challenged with the BCH-3 *ompP1* mutant by injecting 0.1 ml of the diluted cultures containing 50 to 60 CFU directly into the right middle-ear cavity through the superior bulla with a tuberculin syringe. Chinchillas were reexamined by otoscopy and tympanometry on days 2, 4, 6, 8, 10, 14, and 18. On these days, the middle-ear cavities were also examined through a small (4 mm in diameter) incision in the superior bulla, leaving the tympanic membrane intact. The right middle-ear cavities were cultured by swabbing the cavity with a calcium alginate swab (Calgiswab type 1; Hardwood Products Company LP) and streaking onto chocolate agar plates. The plates were incubated overnight at 37°C in a candle extinction jar. The outcome of challenge in animals that were immunized was compared with that in nonimmunized animals using Fisher's exact test (80).

Nucleotide sequence accession numbers. Forty *ompP1* nucleotide sequences have been deposited in the GenBank database under accession no. AF260337 through AF260376.

RESULTS

Analysis of *H. influenzae ompP1*. From the natural population structure of *H. influenzae* depicted in a phylogenically organized collection of >500 *H. influenzae* and NTHI (13; unpublished data) isolates we selected 42 phylogenically variant typeable *H. influenzae* and NTHI isolates representing the diversity of this population structure and performed PCR amplification and sequence analysis of *ompP1*. Alignment of the 42 *ompP1* open reading frames (not shown) and deduced amino acid sequences confirmed the presence of three variable regions described by Munson et al. (47) and identified an additional variable region (variable region III, loop 5) (Fig. 1 and Table 1). Each region is located within a putative surface-exposed loop (loops 2, 4, 5, and 8) (13). Regions I (loop 2), II (loop 4), and IV (loop 8) from the deduced P1 sequences vary in residue number, suggesting possible insertions or deletions at the DNA level within encoding genes. A comparison of the percent similarities among P1 sequences identified 23 variants (P1 type) with amino acid sequences of ≤95% similarity (Table 1).

NTHI strains BCH-1, BCH-2, and BCH-3 were included in the analysis and subsequently used to challenge chinchillas immunized with recombinant P1 prepared from BCH-3, i.e., $rP1_{BCH-3}$. Overall percentages of amino acid identity between $P1_{BCH-3}$ and $P1_{BCH-1}$ and between $P1_{BCH-3}$ and $P1_{BCH-2}$ are 99.6 and 91%, respectively. Table 1 displays the translated amino acid sequence alignment of the four potentially surface-exposed variable regions for the 42 *ompP1* gene sequences. Relevant to the P1 immunogen protection studies described below is the fact that $P1_{BCH-1}$ and $P1_{BCH-3}$ differ by only one of the 20 putative surface-exposed amino acid residues located in variable region I, while sequences of $P1_{BCH-2}$ and $P1_{BCH-3}$ differ considerably within all four variable regions (Fig. 1). Percentages of identity between individual variable regions in

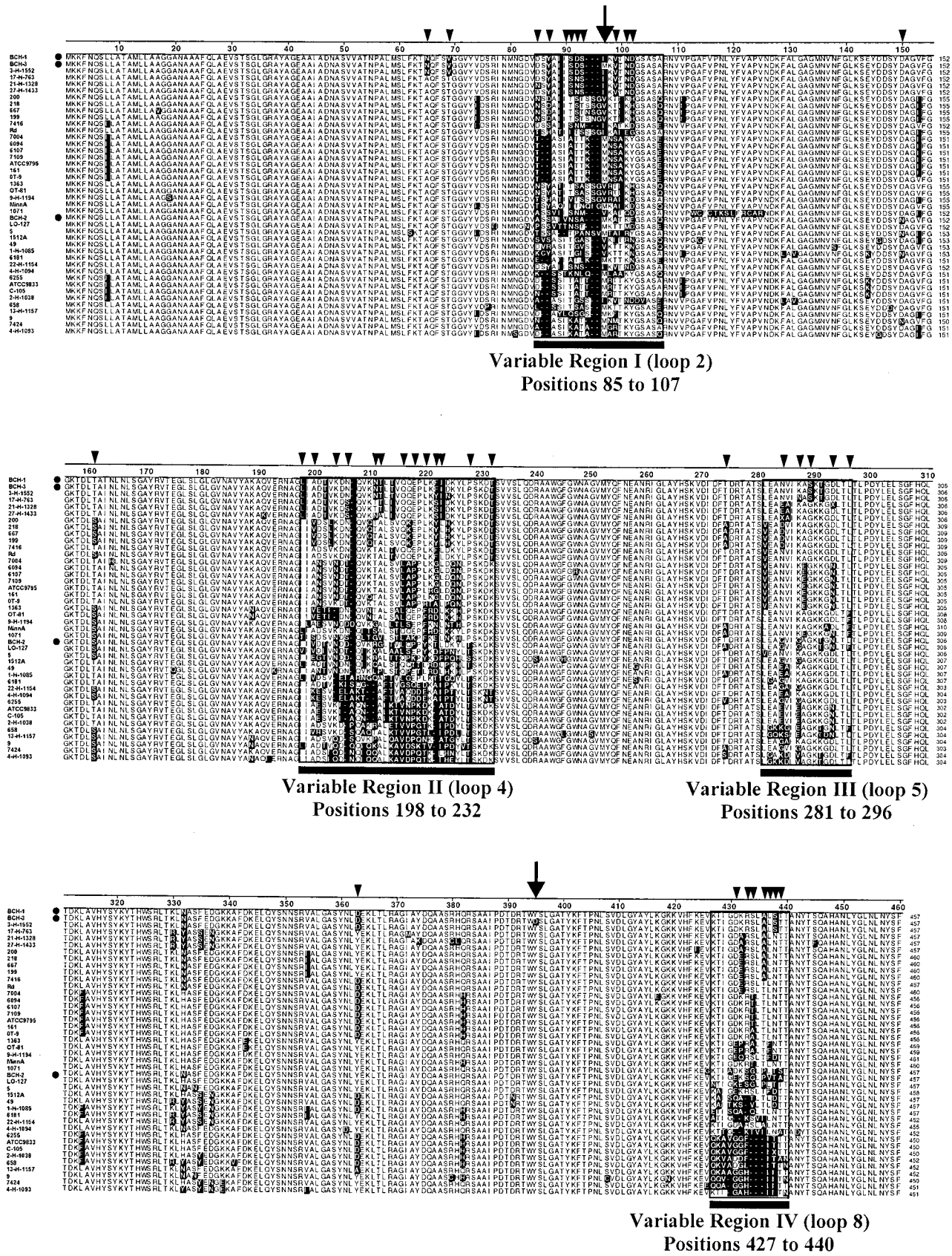

FIG. 1. Alignment of deduced amino acid sequences of *ompP1* alleles carried by the 42 phylogenically variant typeable *H. influenzae* and NTHI clinical isolates, listed as in Table 1. Dotted rows, isolates BCH-1, BCH-2, and BCH-3 used in the animal challenges; shaded residues, those that differ from the majority consensus sequence (not shown); black bars, variable regions. Numbers to the right of sequences indicate amino acid residue positions. Arrows above residues 97 (variable region I) and 395 (nonvariable region) identify the only two positions at which strains BCH-1 and BCH-3 differ. Smaller arrowheads indicate positions at which phylogenically variant strain BCH-2 differs from BCH-1, BCH-3, or both. Amino acid sequence alignment was performed with the LASERGENE software (DNASTAR), using the CLUSTAL multiple-alignment algorithm (30).

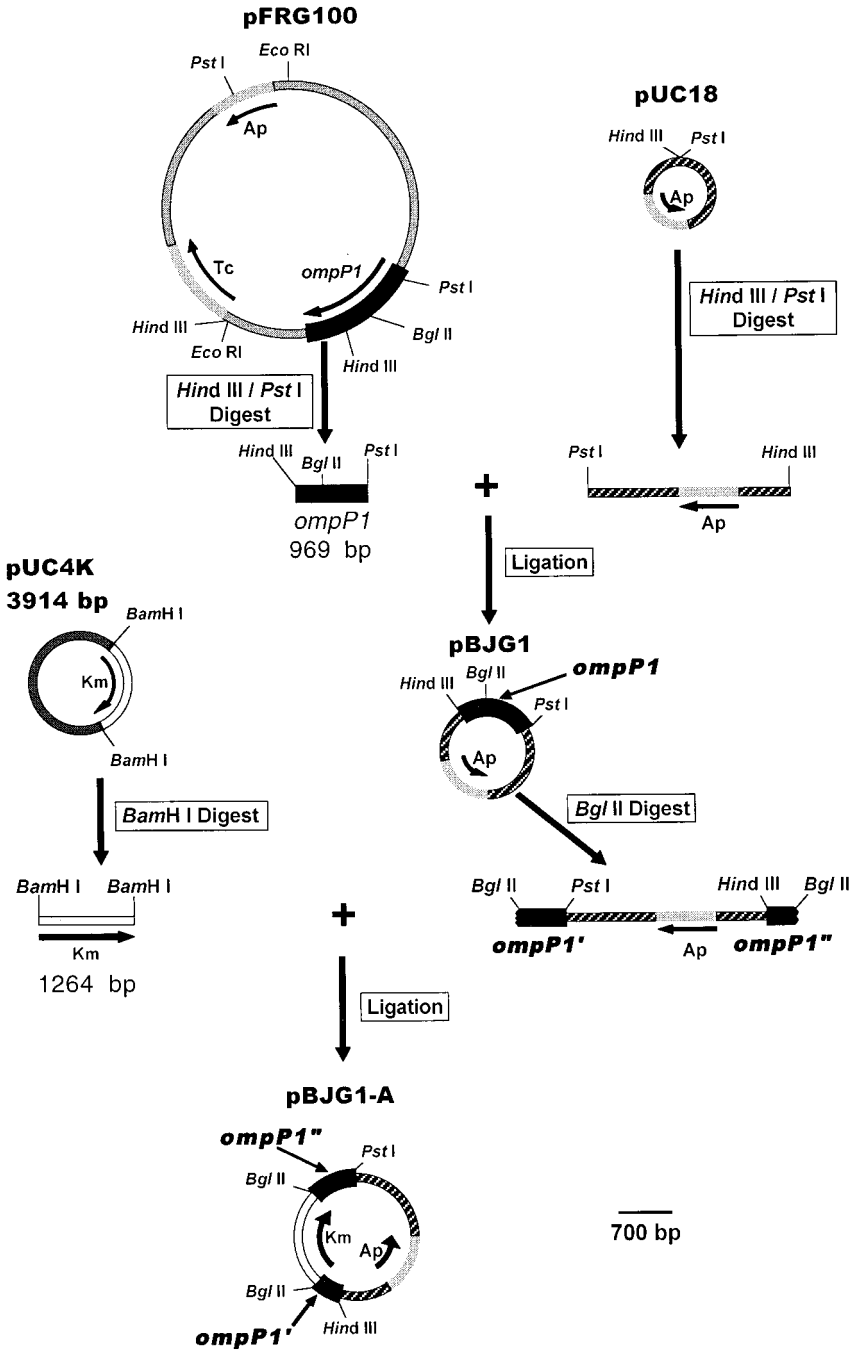

FIG. 2. Construction of pBJG1-A, a suicide plasmid vector carrying a 969-bp fragment of *ompP1* inactivated by insertional mutagenesis with a Km^r cassette. All depicted plasmids and constructs are to the scale noted at bottom right. Ap, ampicillin resistance; Tc, tetracycline resistance; Km, kanamycin resistance; *ompP1′* and *ompP1″*, *ompP1* fragments of the larger 969-bp *ompP1* fragment insertionally inactivated with a Km^r cassette at the indicated, unique *Bgl*II site. Note that products of two of the plasmid digests shown appear as base pair size markers in Fig. 4A, lanes 1 and 2. This includes (i) the *Hin*dIII/*Pst*I digest of pFRG100 yielding five fragments among which is the 969-bp *ompP1* fragment and (ii) the *Bam*HI digest of pUC4K yielding the 1,264-bp Km^r cassette as well as the 2,650-bp plasmid backbone.

the two sequences are 45 (region I), 38 (region II), 31 (region III), and 50% (region IV).

Construction of an otherwise isogenic *ompP1* mutant of NTHI strain BCH-3. A BCH-3 *ompP1*-deficient NTHI mutant was constructed to evaluate the specificity of immunization with $rP1_{BCH-3}$ against experimental otitis media (see Materials and Methods) (Fig. 2). Plasmid pFRG100 (25), carrying a 6.4-kb *Eco*RI DNA fragment that includes the *ompP1* gene of *H. influenzae* serotype b strain DL41, was digested with *Hin*dIII and *Pst*I. The isolated 969-bp *Pst*I/*Hin*dIII *ompP1* fragment was ligated into pUC18 and subsequently disrupted by insertion of a kanamycin resistance cassette within a unique *Bgl*II site. Unable to replicate in *H. influenzae*, the resultant Km^r suicide vector construct, pBJG1-A, was used to transform NTHI strain BCH-3. This was followed by growth in the presence of kanamycin to select for the desired homologous re-

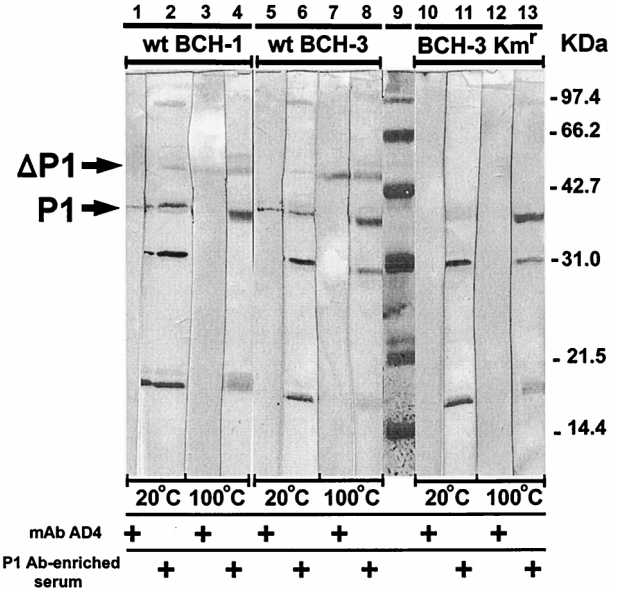

FIG. 3. Western blot analysis of outer membrane preparations from the *ompP1*$^+$ strains wt BCH-1 (lanes 1 to 4) and wt BCH-3 (lanes 5 to 8), and the putative mutant *ompP1* BCH-3 Km^r transformant (lanes 10 to 13). AD4 MAb and serum from a chinchilla immunized with a $P1_{BCH-1}$-enriched preparation were used to probe unheated (20°C) and heated (100°C) outer membrane preparations. P1 and ΔP1, non-heat-modified and heat-modified forms of OMP P1, respectively (25). Molecular mass standards appear in lane 9.

combination-based marker-exchange event substituting the Km^r inactivated *ompP1* gene in place of the wt chromosomal allele.

To screen for absence of OMP P1, 46 Km^r BCH-3 transformants were assayed by dot blot analysis using P1 MAb AD4 as a probe. One transformant that failed to bind to AD4 was further characterized by Western blotting (Fig. 3). Outer membrane preparations from NTHI strains wt BCH-1 and wt BCH-3 and the dot blot-negative BCH-3 Km^r transformant were electrophoresed and probed with MAb AD4 and also with serum from a chinchilla immunized with a native $P1_{BCH-1}$-enriched preparation. Western blots detected both the heat-modified (ΔP1) and nonmodified forms of P1 (76) in the outer membranes prepared from the P1$^+$ strains wt BCH-1 and wt BCH-3 (lanes 1 to 8). In contrast, P1 was not detected by the AD4 MAb (lanes 10 and 12) or by the immune chinchilla serum (lanes 11 and 13) in the outer membrane preparations from the Km^r BCH-3 transformant. Immune chinchilla serum did, however, detect other *H. influenzae* OMPs (lanes 11 and 13) with intensities similar to those found for wt BCH-1 and wt BCH-3 (lanes 2, 4, 6, and 8) when these outer membrane preparations were used.

Confirmation of the insertionally inactivated $ompP1_{BCH-3}$ gene was accomplished by PCR amplification of *ompP1* carried by the Km^r BCH-3 transformant. This yielded a DNA fragment that was 1,264 bp larger than the wt allele of strain BCH-3, precisely the size predicted for the Km^r gene cassette insertion (Fig. 4A, lanes 4 and 3, respectively). Southern blot hybridization analysis of this PCR product confirmed that the increase in size was due to the insertion of the Km^r cassette (Fig. 4B, lanes 3 and 4, and Fig. 4C, lanes 3 and 4). These results also indicate that a double-crossover event occurred to generate the insertionally inactivated $ompP1_{BCH-3}$ mutant by simple allelic exchange rather than cointegration of pBJG1-A.

As described below, this Km^r *ompP1* mutant of BCH-3 was used to evaluate the specificity of protection against otitis media following immunization with $rP1_{BCH-3}$.

Expression and purification of $rP1_{BCH-3}$. As described in detail in Materials and Methods, the complete *ompP1* DNA sequence of BCH-3, minus its leader sequence, was amplified and inserted into TA cloning vector pCR2.1 (Invitrogen). It was then subcloned into the inducible *E. coli* expression vector pTrcHisB and transformed into *E. coli* strain BLR to generate the new construct pBJG2. The induced $rP1_{BCH-3}$ protein formed insoluble cytoplasmic inclusion bodies that were harvested, purified, and concentrated.

Purified $rP1_{BCH-3}$ was analyzed by Western blotting with serum antibodies obtained from a chinchilla immunized with a native $P1_{BCH-1}$-enriched preparation. AD4 MAb and Invitrogen anti-Xpress MAbs (55) were also used as probes (Fig. 5). The anti-Xpress MAb is specific for the epitope AspLeuTyr AspAspAspAspLys, which had been fused to the N-terminal

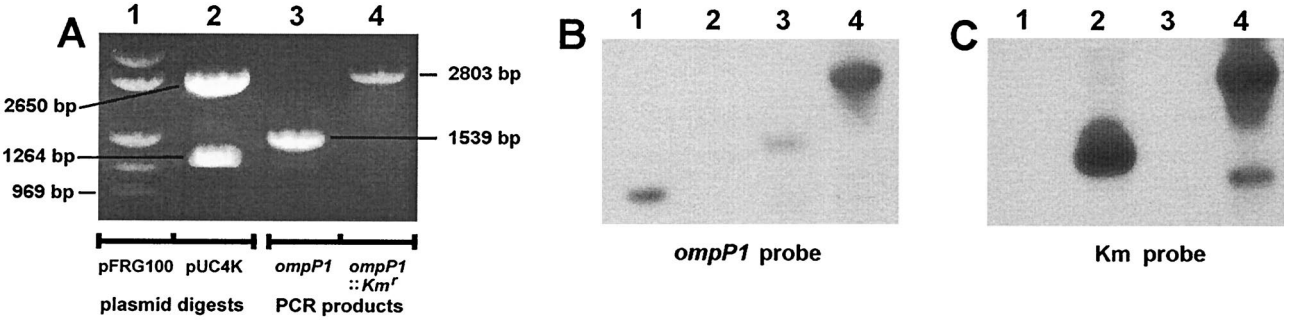

FIG. 4. Characterization of the putative P1-deficient isogenic mutant of NTHI BCH-3 by Southern blot analysis. (A) Agarose gel electrophoretic separation of digests and PCR products, stained with ethidium bromide. Lane 1, *Hin*dIII/*Pst*I double digest of the *ompP1*-carrying vector plasmid pFRG100; the bottom band is the predicted 969-bp *ompP1* fragment (see Fig. 2); lane 2, *Bam*HI digest of pUC4K; the bottom band is the predicted 1,264-bp Km^r cassette, and the upper band is the predicted 2,650-bp backbone of pUC4K (see Fig. 2); lane 3, PCR amplification of the *ompP1* gene (1,371 bp) plus flanks (109 bp upstream and 59 bp downstream) from wt BCH-3 (13; unpublished data) using flanking primers P1-5 and P1-6 (see Materials and Methods) to generate a 1,539-bp product; lane 4, PCR amplification of *ompP1* from the putative, insertionally inactivated *ompP1* BCH-3 Km^r transformant using flanking primers P1-5 and P1-6 (Materials and Methods); when this product is compared with the *ompP1* PCR product in the adjacent lane 3, the size increase is found to correlate with that predicted for insertion of the 1,264-bp Km^r cassette as indicated in Fig. 2. (B) Southern blot transfer of the panel A gel electrophoretic separation to a Zeta Probe nylon membrane (Bio-Rad), followed by hybridization with a ^{32}P-labeled (2) *ompP1* 969-bp fragment probe (the bottom *Hin*dIII/*Pst*I fragment shown in panel A, lane 1, as isolated from an equivalent digest and electrophoretic separation). (C) Duplicate electrophoretic separation of the identical digests and PCR products in panel A, transferred by Southern blotting to a Zeta Probe nylon membrane and hybridized with a ^{32}P-labeled (2) Km^r cassette probe (the smaller, 1,264-bp *Bam*HI fragment shown in panel A, lane 2, as isolated from an equivalent digest and electrophoretic separation). The lower band in lane 4 of panel C is likely a PCR breakdown product.

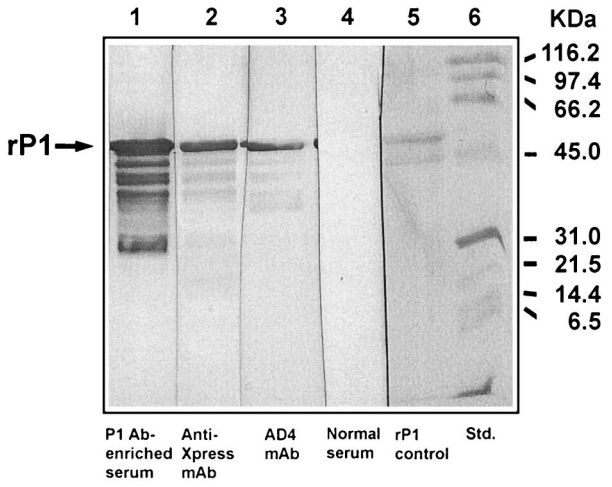

FIG. 5. Western blot analysis of recombinant *H. influenzae* OMP $P1_{BCH-3}$ (rP1) purified from cytoplasmic inclusion bodies of an *E. coli* cloning host. The rP1 was probed with serum from a chinchilla immunized with a $P1_{BCH-1}$-enriched preparation (lane 1), the Invitrogen anti-Xpress MAb (lane 2), the AD4 MAb (lane 3), and normal preimmune chinchilla serum (lane 4). Coomassie blue-stained rP1 and molecular mass standards are shown in lane 5 and lane 6, respectively. As described in Materials and Methods, the rP1 generated from expression vector pTrcHisB is a translated fusion product with 30 additional amino acids at the peptide N terminus containing the epitope AspLeuTyrAsp AspAspAspLys recognized by the Invitrogen anti-Xpress MAb (lane 2).

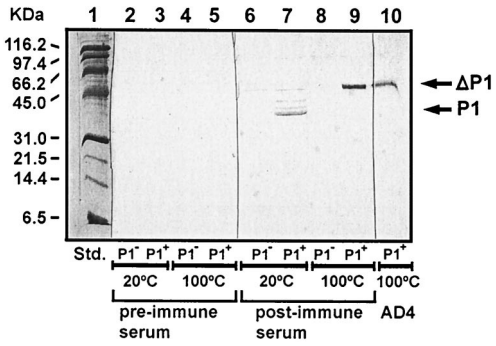

FIG. 6. Western blot analysis of outer membrane preparations with $rP1_{BCH-3}$ immune serum. Outer membrane preparations from NTHI wt BCH-3 ($P1^+$) and the otherwise isogenic BCH-3 *ompP1* mutant ($P1^-$) were probed with preimmune chinchilla serum (lanes 2 to 5) and post-$rP1_{BCH-3}$-immune chinchilla serum (lanes 6 to 9). The wt BCH-3 outer membrane was also probed with the P1-specific AD4 MAb as a control (lane 10). Lane 1, molecular mass standards; P1 and ΔP1, non-heat-modified and heat-modified forms of OMP P1, respectively (25).

end of $rP1_{BCH-3}$ expressed from pBJG2 (lane 2). The AD4 MAb, specific to P1, recognized the major rP1 band with the appropriate molecular mass (lane 3). This MAb also bound to lower-molecular-mass bands thought to be breakdown products of rP1. The immune chinchilla serum, included in the analysis as a source of polyclonal antibodies, also recognized the major band and lower-molecular-mass bands (lane 1), similar to those recognized by the two MAbs.

Serum antibody response to $rP1_{BCH-3}$. Twenty-eight chinchillas received seven doses of 25 μg of BCH-3 rP1 (i.m.) in combination with RIBI adjuvant on days 0, 35, 57, 79, 99, 120, and 141. Simultaneously, eight chinchillas were immunized with RIBI adjuvant only, serving as controls. Preimmune (collected on day 0) and postimmune (collected prior to BCH-3 challenge on day 148) sera were used to measure individual antibody responses to $rP1_{BCH-3}$ by ELISA. Mean values for the change in optical density per minute ± standard errors are shown in Table 2 for sera diluted 1:1,000.

The same pre- and postimmune sera were used for Western blots to analyze outer membrane preparations from wt BCH-3 and the *ompP1*$_{BCH-3}$ mutant (Fig. 6). Antibodies present in the postimmune serum reacted only with the normal and heat-modified (Δ) forms of wt $P1_{BCH-3}$ (lanes 7 and 9, respectively). All animals lacked preimmune antibodies to P1 (lanes 2 to 5), indicating that the immune response to rP1 was highly specific.

TABLE 2. Serum antibody levels against $rP1_{BCH-3}$

Immunogen	n^a	Mean antibody level (ΔOD/min) ± SE in[b]:	
		Preimmune sera	Postimmune sera
rP1/RIBI	28	0.002 ± 0.001	0.281 ± 0.053
RIBI	8	0.001 ± 0.001	0.002 ± 0.001

[a] *n*, number of animals immunized.
[b] Antibody levels against $rP1_{BCH-3}$ at a 1:1,000 serum dilution. OD, optical density.

Specificity was confirmed in lanes 6 and 8, where postimmune antibodies did not bind to the outer membrane preparations from the *ompP1*$_{BCH-3}$ mutant.

Transbullar challenge with NTHI strain wt BCH-3 and the BCH-3 *ompP1* isogenic mutant. Seven days after the final immunization (day 148), 36 chinchillas immunized with either rP1-RIBI or with RIBI alone and 4 nonimmunized animals were challenged with an inoculum containing 50 to 60 CFU of wt BCH-3 or the BCH-3 *ompP1* isogenic mutant by direct inoculation into the right middle-ear cavity. Each animal was evaluated for the development of otitis media by otoscopy, tympanometry, and middle-ear cultures on days 2, 4, 6, 8, 10, 14, and 18 postchallenge. The numbers and percentages of animals with culture-positive middle ears versus the total number of animals challenged are shown in Table 3. The peak number of culture-positive animals previously immunized with $rP1_{BCH-3}$ and homologously challenged with wt BCH-3 occurred on day 4 (8 out of 18 animals infected). By day 6, the number of animals infected had been reduced to 7 of 18 (39%), and by day 18 only 2 of 16 (12%) animals had culture-positive middle-ear disease. The difference between the proportion of $rP1_{BCH-3}$-immunized animals with culture-positive disease and that of animals immunized solely with RIBI adjuvant was significant ($P < 0.05$) at all time points except on day 4 when 8 of 18 animals were infected ($P = 0.067$). All animals challenged with the BCH-3 *ompP1* isogenic mutant as well as all unimmunized animals (with the exception of one animal immunized with adjuvant alone that never developed infection) were culture positive by day 2. By day 18, four of six rP1-immunized animals and two of three animals given RIBI adjuvant alone remained culture positive for the BCH-3 *ompP1* mutant challenge strain.

In addition to the finding that a reduced number of animals developed culture-positive acute otitis media, it was found that $rP1_{BCH-3}$-immunized animals experienced delayed onset of infection and milder disease due to wt BCH-3, as evidenced by tympanometry, otoscopy, and direct examination of the middle-ear cavity. All immunized animals had normal (type A) tympanograms (22) on day 2 after challenge with wt BCH-3, and 16 (80%) had normal otoscopic examinations. As the course of disease progressed, their tympanograms resembled that of either normal pressure with reduced compliance or negative pressure (type C) but were never flat (type B), indicative of unmodified otitis media in this model. Three of eight

TABLE 3. Transbullar homologous challenge with wt NTHI strain BCH-3 and an otherwise isogenic BCH-3 *ompP1* mutant

Challenge strain	Immunogen	No. of animals with culture-positive middle-ear fluid/no. challenged (%), P^a, on day:						
		2	4	6	8	10	14	18
wt BCH-3	$rP1_{BCH-3}$ + adjuvant	1/20 (5)	8/18 (44)	7/18 (39)	5/16 (31)	5/16 (31)	2/16 (12)	2/16 (12)
	Adjuvant alone	4/4 (100), 0.001	4/4 (100), 0.067	4/4 (100), 0.045	4/4 (100), 0.026	4/4 (100), 0.026	3/4 (75), 0.032	2/2 (100), 0.039
	Nonimmunized	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	1/1 (100)	1/1 (100)	1/1 (100)
BCH-3 *ompP1* mutant	$rP1_{BCH-3}$ + adjuvant	8/8 (100)	8/8 (100)	8/8 (100)	8/8 (100)	8/8 (100)	6/7 (86)	4/6 (67)
	Adjuvant alone	3/4 (75), 0.333	3/4 (75), 0.333	3/4 (75), 0.333	3/4 (75), 0.333	2/3 (67), 0.273	2/3 (67), 0.533	2/3 (67), 0.774
	Nonimmunized	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	1/2 (50)

[a] *P* values are based on a comparison of data for chinchillas immunized with rP1 plus adjuvant with those for chinchillas immunized with adjuvant alone, as determined by the Fisher exact test (80). Note that the incidence of culture-positive effusions from chinchillas immunized with rP1 plus RIBI adjuvant and challenged with wt BCH-3 was statistically significant. The incidence of culture-positive effusions from the contralateral unchallenged ears (bilateral effusion) was 5% in the group challenged with wt BCH-3 and 62% in the group challenged with the isogenic BCH-3 *ompP1* mutant on days 6 and 8 ($P = 0.0045$).

(37.5%) animals immunized with $rP1_{BCH-3}$ and challenged with the BCH-3 *ompP1* mutant had normal tympanograms on day 2, but all eight had abnormal otoscopic examinations. The tympanograms observed in these animals became flat (type B) as disease progressed. Experimental otitis media in the chinchilla due to NTHI initially develops with a translucent serous middle-ear effusion, which is followed by a turbid, low-viscosity, white fluid (16). In the partially protected rP1-immunized animals, middle-ear fluid remained serous throughout the course. Both the control unimmunized animals and those challenged with the BCH-3 *ompP1* mutant strain developed turbid, purulent middle-ear fluid typical of first infection in pristine animals. The incidence of culture-positive effusions from the contralateral unchallenged ears (bilateral effusion) was 5% (1 of 18 animals) in the group challenged with wt BCH-3 and 62% (5 of 8 animals) in the group challenged with the otherwise isogenic BCH-3 *ompP1* mutant on days 6 and 8 ($P = 0.0045$). These observations indicate that immunization with $rP1_{BCH-3}$ modifies both the proportion and duration of culture-positive disease as well as the otomicroscopic and tympanometric indicators of inflammation and the likelihood of developing infection in the contralateral (unchallenged) middle ears.

Transbullar heterologous challenge with NTHI strains BCH-1 and BCH-2. Ten of 20 chinchillas immunized with $rP1_{BCH-3}$ that had not developed culture-positive otitis media by day 14 were rechallenged (in the previously unchallenged left middle ear) with either wt BCH-1 or wt BCH-2 expressing variant *ompP1* alleles, as described above. All animals were evaluated for the development of otitis media using otoscopy, tympanometry, and middle-ear cultures on days 2, 4, and 6.

Four of the five animals rechallenged with BCH-1 had negative middle-ear cultures on day 2 (Table 4), while all five animals rechallenged with BCH-2 became culture positive. Although all the animals had been completely protected against initial BCH-3 challenge, only one animal rechallenged with BCH-1 remained culture negative through day 6. However, all of the animals challenged with BCH-1 developed clear, serous middle-ear fluid and had tympanograms of normal pressure and reduced compliance (type A_s) (22), suggesting modified disease. In contrast, animals challenged with BCH-2 demonstrated middle-ear effusions typical of unmodified, natural infection in pristine animals. Although these results are limited by the small number of animals and are not statistically significant, the observations nevertheless suggest that immunization with $rP1_{BCH-3}$ elicits a partially protective immune response against NTHI isolates with nearly identical (99.6%) amino acid sequences, such as BCH-1, but not against isolates with variability as great as that seen with BCH-2 (91%).

DISCUSSION

Our study provides conclusive evidence that immunization with $rP1_{BCH-3}$ elicits protection against mucosal surface infection with NTHI in a homologous-challenge experimental model. Specificity of the protection was demonstrated by (i) the use of an otherwise isogenic *ompP1* mutant capable of producing unmodified disease in animals immunized with rP1 from the wild-type strain and (ii) heterologous challenge with two NTHI strains, one with a single amino acid change in variable region I of OMP P1 and a second that has significant amino acid changes in four of the surface-exposed regions of OMP P1 (Fig. 1). These strains produced modified and full disease, respectively, in immunized chinchillas. When considered together, the findings involving the specificity of the rP1 immune response, the variability of P1 epitopes, and the virulence of the *ompP1* mutant that we constructed have fundamental implications in the development of OMP P1 as a vaccine candidate against NTHI.

Diseases associated with NTHI are primarily mucosal infections of the respiratory tract. They occur most commonly as otitis media in young children and infectious complications in adults with chronic pulmonary disease; both are characterized by frequent recurrences. Disease in infants typically begins after maternal antibodies have dissipated and before their own immune systems are fully matured (42). While the conjugate *H. influenzae* serotype b vaccines that have evolved from the prototype developed in the 1980s by Anderson (1; P. Anderson, Letter, J. Infect. Dis. **149:**1034–1035, 1984) and Claesson et al. (15) are efficacious, unencapsulated NTHI remains responsible for ~30% of the >45 million episodes of acute bacterial otitis media yearly in North America and Europe (43, 45, 58, 68) and is implicated in as many as ~20% of the 4 million fatal acute respiratory infections per annum worldwide occurring mostly in children (9). Studies of these infections demonstrate that NTHI usually elicits bactericidal antibodies (71, 72). However, protection appears to be strain specific, with

TABLE 4. Transbullar heterologous OMP P1 challenge with NTHI wt strains BCH-1 and BCH-2

Challenge strain	No. of culture-positive animals/ no. challenged (*P*) on day:		
	2	4	6
BCH-1	1/5	3/5	4/5
BCH-2	5/5 (0.024)	5/5 (0.222)	5/5

subsequent infections due to antigenically variant, unrelated strains (11).

A number of studies have suggested that OMPs of *H. influenzae* might be efficacious vaccine candidates. Increases in specific antibodies to NTHI in sera and middle-ear fluids from children recovering from otitis media have been reported (71, 72). These results are paralleled by our study of an adult patient indicating that the majority of serum bactericidal antibody activity after recovery from NTHI disease was directed to OMPs (24). Furthermore, passive immunoprophylaxis in the chinchilla model has provided evidence that humoral antibodies are sufficient to prevent experimental otitis media due to NTHI. Protection in these studies was a result of antibodies directed mainly to OMPs and less so to lipooligosaccharide antigens (6, 32).

Several reports have characterized aspects of OMP P1 as an experimental vaccine candidate. Polyclonal antibodies and a MAb specific for P1, when used for passive immunization, protected against *H. influenzae* serotype b bacteremia in the infant rat model (25, 29, 48). Active immunization with P1 was also found to induce partial protection against bacteremia in this model (41). We took a different approach to the question of P1 as an immunogen by analyzing the variability of P1-encoding genes (Table 1 and Fig. 1) found among a phylogenically diverse collection of typeable *H. influenzae* and NTHI isolates (13; unpublished data). We also examined the capacity of OMP P1 to prevent experimental otitis media due to NTHI in chinchillas actively immunized with a recombinant form of P1 (rP1).

Our preliminary studies with OMP P1 had shown that a $P1_{BCH-1}$-enriched protein preparation from NTHI protected against experimental disease following homologous challenge but that the antibody response lacked specificity (data not shown). To overcome this problem and clarify the results, we subsequently cloned *ompP1* from the clinical NTHI strain BCH-3 and expressed P1 in the cytoplasm of *E. coli*, thus avoiding contamination with other *H. influenzae* OMPs. As summarized in Tables 2 and 3, this $rP1_{BCH-3}$ preparation induced antibodies in all actively immunized chinchillas and protected them against otitis media due to the wt NTHI isolate BCH-3. Fifty-six percent (10 of 18) of the chinchillas immunized with $rP1_{BCH-3}$ demonstrated complete protection against disease. The remaining 44% (8 of 18) demonstrated modified disease, expressed as delayed onset and milder infection defined by standardized tympanometric criteria (22) to supplement microbiological evidence of infection. The absence of protection in $rP1_{BCH-3}$-immunized animals challenged with an otherwise isogenic BCH-3 *ompP1* mutant confirmed the specificity of the protective response (Table 3).

The isogenic BCH-3 *ompP1* mutant produced disease in the $P1_{BCH-3}$ immune chinchilla model similar to that produced by wt BCH-3 in nonimmunized animals (Table 3). Although the pathogenicity of an *H. influenzae* serotype b *ompP1* mutant in the infant rat model for invasive disease had been described (29), the virulence of an *ompP1* mutant in a chinchilla model for otitis media mucosal infection has not been reported previously. The viability and unimpeded virulence of these *ompP1* mutants should elicit concern regarding the use of P1 as an immunogen because of the possibility for inadvertent selection of virulent *ompP1* escape mutants. Evaluation of BCH-3 isolates recovered from the animals immunized with rP1 that demonstrated modified disease (delayed onset and less purulent effusion) will provide initial insight as to whether selection for P1 variants accounts for the dichotomous results (complete protection versus modified disease). An example of such modified disease was found in the case of immunization with $rP1_{BCH-3}$ followed by experimental challenge and infection with NTHI BCH-1, a strain differing from BCH-3 by only 1 aa in immunogenic loop 2 (aa 97) and 1 aa in an otherwise conserved region (aa 395) of OMP P1 (Fig. 1). In contrast, while 8 of 18 animals immunized with $rP1_{BCH-3}$ and infected with BCH-3 eventually displayed such modified disease, none showed full disease similar to that found following challenge with the isogenic BCH-3 *ompP1* mutant with an outer membrane devoid of OMP P1.

The 10 animals that were fully protected from disease due to wt NTHI strain BCH-3 were rechallenged in the opposite middle ear cavity with either wt NTHI strain BCH-1 or BCH-2 to determine the effect of variable *ompP1* alleles and consequently P1 antigen expression (Table 4). $P1_{BCH-1}$ and $P1_{BCH-2}$ display 99.6 and 91% amino acid identity to $P1_{BCH-3}$, respectively (Table 1 and Fig. 1). P1s of BCH-1 and BCH-3 differ by only 1 of the 20 putative surface-exposed amino acid residues located within variable region I, suggesting that cross-protection against BCH-1 might occur in chinchillas immunized with $rP1_{BCH-3}$. In contrast, the four identified variable regions of $P1_{BCH-2}$ differ considerably from those of $P1_{BCH-3}$, with the percent identities between their individual variable regions being 45 (region I), 38 (region II), 31 (region III), and 50% (region IV). BCH-2 was included in the rechallenge to determine if this considerable degree of variation circumvented protection induced by $rP1_{BCH-3}$ and also to ensure that a vaccine-independent positive control was present in the rechallenge experiments. While infection with BCH-1 resulted in a mild disease with delayed onset, BCH-2 caused infection and disease typical of first infection in pristine animals. It is possible that BCH-3 challenge may have stimulated immunity in these animals even though no infection was produced. However, we have previously shown that heterologous antibodies against BCH-2 OMPs were not raised in two-thirds (four of six) of the animals challenged and infected with BCH-1 (32). Therefore we have reason to believe that cross-reactive antibodies were not likely to have been raised against BCH-1 and BCH-2 when BCH-3 was used as a challenge, particularly because an infection with BCH-3 did not ensue. Also, in unpublished studies we have found that no cross-protection between BCH-1 and BCH-2 exists when animals initially infected with one strain are challenged with the other. These results suggest that immunity generated by $rP1_{BCH-3}$ is not broadly protective. This indicates the necessity for studies that further define protective epitopes within the four variable regions and that identify functional aspects of OMP P1 and the anti-P1 immune response. Likewise, the development of an extensive NTHI *ompP1* allele-specific typing system applied to a phylogenically diverse population of strains would be necessary for a more complete understanding of the potential of OMP P1 as a vaccine candidate.

A high degree of conservation throughout the natural population structure of the pathogenic species is a critical property of any vaccine candidate. Previous phenotypic studies have shown that *H. influenzae* serotype b P1 varies both antigenically and in molecular weight, with size differences having been exploited as a rudimentary subtyping scheme for *H. influenzae* serotype b isolates (8). Our DNA sequence-based analysis directly examined the precise degree of conservation of *ompP1* among 42 phylogenically variant *H. influenzae* isolates (13). The calculated percent identities for the DNA sequences and their corresponding amino acid sequences range from 87.8 to 100% and from 82.5 to 100% (Fig. 1), respectively. We identified 23 P1 variants by grouping the amino acid sequences based on their percentages of similarity, using 95% similarity as the cutoff point (Table 1). Additional *ompP1* sequences

from our phylogenically organized *H. influenzae* collection will be needed to test whether a limited number of such variants might provide a reasonable degree of protection when considered as possible polyvalent immunogens.

The study described in this report involved a multifaceted approach to vaccine development. This included a broad phylogenic perspective integrated with DNA sequencing, cloning, mutational inactivation, and animal modeling. Cloning and expression of the immunogen allowed for its isolation in the absence of all other antigens of the pathogenic species and its subsequent use in studies that tested an OMP as a protective immunogen. The otherwise isogenic mutant strain allowed for several insights regarding the immunogen of interest, providing the means to directly test its specificity while also revealing whether its encoding gene is necessary for cell viability and virulence. The phylogeny-based DNA-sequencing survey proved equally revealing, making possible unique heterologous challenge studies involving isolates with known minor and major differences in surface-exposed amino acid residues of the native antigen. This provided the means to test the range of specificity of the isolated immunogen. Furthermore, identifying a range of immunogen-encoding alleles with significant variability unambiguously demonstrates that phylogenic screening of the immunogen-encoding gene for any vaccine candidate is a rational prerequisite, particularly for OMPs that are subject to diversifying antigenic selection. This novel strategy based on microbial population structure represents the means to determine a priori a critical aspect of the likely efficacy of any immunogen candidate, thus saving time and expense involved with animal testing until the decisive degree of immunogen conservation has been unambiguously established. As such, we refer to this previously untested approach as the phylogenic strategy for vaccine development.

Based on this rational new approach we have constructed a relatively large-scale model of the natural population structure of typeable *H. influenzae* and NTHI involving more than 500 phylogenically characterized isolates from different patient populations and geographical locations, isolated over a 30-year span (13; unpublished data). This is currently being used to carry out an initial screen of potential immunogen vaccine candidates for the essential nature of their encoding genes and for their natural conservation. Those immunogens encoded by genes demonstrated to be essential for both viability and virulence, while also exhibiting a relatively high degree of conservation across the natural population structure, are then to be used for animal testing thus eliminating a major factor, i.e., antigenic variability, that has typically confounded vaccine development.

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