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Neonatal, Urogenital Isolates of Biotype 4 Nontypeable *Haemophilus influenzae* Express a Variant P6 Outer Membrane Protein Molecule

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The P6 outer membrane protein is a highly conserved molecule which is present on the surface of all strains of *Haemophilus influenzae*. Sixty strains of nontypeable *H. influenzae* which caused invasive disease or colonized the female urogenital tract were studied with monoclonal antibodies 7F3 and 4G4, which recognize different surface-exposed epitopes on the P6 molecule. All 60 strains expressed the epitope recognized by 4G4, whereas 47 of 60 strains expressed the epitope recognized by antibody 7F3. The 7F3-nonreactive strains were all biotype 4 and were recovered from the blood of neonates or postpartum women or from the female urogenital tract. The P6 genes from two 7F3-nonreactive strains were cloned, and the nucleotide sequences were determined. Analysis of amino acid sequences, immunoassays with synthetic peptides, and site-directed mutation of the P6 gene indicate that the epitope recognized by antibody 7F3 is conformational and that the sequence Asp-Ile-Thr is critical in maintaining the conformation of the epitope. We conclude that the unusually virulent clone family of biotype 4 strains of nontypeable *H. influenzae* express a variant P6 molecule which has an alteration in a highly conserved surface-exposed epitope.

Neonatal sepsis caused by *Haemophilus influenzae* has been recognized with increasing frequency during the past decade (4, 12, 17, 28, 30). The clinical manifestations of this disease are similar to those of group B streptococcal sepsis in the newborn except that meningitis is seen less frequently in *H. influenzae* neonatal sepsis (30). The infection is associated with a 50% mortality overall and a 90% mortality among premature infants (12). A characteristic feature of the illness is its early onset, originating before or at the time of delivery. *H. influenzae* colonizes the female urogenital tract and also causes urogenital infections and postpartum sepsis (3, 7–9, 11, 29, 31). Infection in the neonate appears to result from transmission of the organism from the mother.

Most invasive *H. influenzae* infections in older infants are caused by encapsulated type b strains. By contrast, most strains of *H. influenzae* that cause neonatal and postpartum infections are serologically nontypeable (30). Some of the strains of nontypeable *H. influenzae* (NTHI) that cause neonatal sepsis share several phenotypic characteristics. (i) They are biotype 4. (ii) They express peritrichous fimbriae. (iii) They have a homogeneous outer membrane protein pattern in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (28). These same characteristics are shared by strains that are recovered from the female urogenital tract. Indeed, recent studies involving electrophoretic typing and DNA hybridization indicate that these strains are genetically distinct from other *H. influenzae* strains and actually represent a new species of *Haemophilus* (22, 27).

The P6 protein of *H. influenzae* is a 16,000-Da outer membrane protein which expresses epitopes on the surface of the intact bacterium. P6 demonstrates a high degree of antigenic conservation among strains of *H. influenzae* (18, 24). Monoclonal antibody 7F3 recognizes a P6 epitope that is highly specific for *H. influenzae* strains and that is present in strains from diverse geographic and clinical origins (21, 24). Assays of several hundred strains of *H. influenzae* showed that the epitope recognized by antibody 7F3 was present in all strains. However, to our surprise, we recently identified a strain of *H. influenzae* that was nonreactive with antibody 7F3. Since this strain was isolated from the bloodstream of a neonate, we studied other such strains and found an association between the absence of the 7F3 epitope and strains causing neonatal sepsis.

The goal of the present study is to characterize the P6 molecule in strains of NTHI that are associated with neonatal and urogenital infections. Two different epitopes on the P6 protein will be studied in isolates recovered from neonates and adults with invasive disease and isolates from the adult female urogenital tract. The epitope recognized by antibody 7F3 will be identified by determination and analysis of DNA sequences and by site-directed mutagenesis of the P6 gene.

MATERIALS AND METHODS

Bacteria. Table 1 shows the clinical sources of the 60 strains of NTHI used in this study. They included strains from the following cities: Buffalo, N.Y. (16 strains); Houston, Tex. (29 strains); St. Louis, Mo. (5 strains); Chapel Hill, N.C. (9 strains); and Johnson City, Tenn. (1 strain). Strain 1479 was used as a prototype strain of NTHI since the sequence of the P6 gene from this strain has been determined and its P6 protein has been studied extensively (18, 23, 24).

Bacteria were grown on chocolate agar at 35°C in an atmosphere of 5% carbon dioxide. Isolates were stored in Mueller-Hinton broth plus 10% glycerol at −70°C.

Antibodies. Monoclonal antibodies 7F3 and 4G4 recognize different surface-exposed epitopes on the P6 protein of *H. influenzae* and have been described previously (18, 21, 24). Both monoclonal antibodies recognize epitopes that are

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TABLE 1. Clinical source, biotype, and P6 monoclonal antibody reactivity of strains of NTHI

Clinical source	No. of strains	No. (%) of biotype 4 strains	No. (%) reactive with:	
			7F3	4G4
Blood, neonate[a]	19	9 (47)	11 (58)	19 (100)
Blood, postpartum adult	3	2 (67)	1 (33)	3 (100)
Blood, adult[b]	26	0 (0)	26 (100)	26 (100)
Cervix, adult	12	3 (25)	9 (75)	12 (100)

[a] Includes one isolate from the cerebrospinal fluid of a neonate.
[b] Includes one isolate from a 3-month-old infant and one isolate from a 4-month-old infant (10).

present in all strains of *H. influenzae* tested prior to this study. The epitopes recognized by antibodies 7F3 and 4G4 are highly specific for *H. influenzae*. Both are of the immunoglobulin G isotype.

Immunodot and immunoblot assays. The strain specificity of the monoclonal antibodies was tested by immunodot assay using whole-organism lysates. Bacteria were harvested from a plate of chocolate agar after overnight incubation and were suspended in ~15 ml of 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4. Cells were centrifuged at 5,000 × *g* for 15 min, and the supernatant was discarded. The resulting pellet of cells was suspended in 1 ml of HEPES buffer and sonicated at 100 W for 30 s. The lysate was stored at −20°C.

To perform an immunodot assay a 2-μl volume of lysate was dotted onto nitrocellulose and allowed to air dry. The nitrocellulose sheet was incubated in 3% BLOTTO (nonfat dry milk) for 30 min at room temperature to block reactive sites. After washing with 0.01 M Tris–0.15 M NaCl, pH 7.4 (buffer A), the dots were incubated overnight at room temperature in antibody diluted in buffer A. The nitrocellulose was washed with buffer A and then incubated for 1 h with protein A-peroxidase. The immunodots were developed with horseradish peroxidase color developer.

SDS-PAGE and immunoblot assays were performed with 15% gels as previously described (18). After electrophoretic transfer to nitrocellulose, immunoblot assays were treated exactly as described for the immunodots. Outer membrane complex was prepared by previously described methods (16).

ELISAs. Enzyme-linked immunosorbent assays (ELISAs) were performed to further assess the specificity of the antibodies for the P6 protein of two strains. Microtiter plates were coated with outer membrane complex or purified P6. P6 was purified by a modification of our previously described method (18, 19). Wells were coated with antigens diluted in 0.05 M carbonate buffer (pH 9.6) by overnight incubation at room temperature. Purified P6 was used in different assays at a concentration that varied from 1 to 10 μg/ml, and outer membrane complex was used at a concentration of 100 to 800 μg/ml. Wells were washed three times with phosphate-buffered saline (PBS) plus 0.05% Tween 20 (PBS-Tween) between all steps. Unbound sites were blocked with 1% bovine serum albumin in PBS for 2 h at room temperature. After washing, antibodies were added to wells and incubated at room temperature for 3 h. After washing, protein A-peroxidase was added and incubated for 1 h at room temperature. Color was developed by the addition of *o*-phenyldiamine substrate (16). Optical density was read on an MR 6000 Microplate reader (Dynatech Laboratories, Inc., Alexandria, Va.). Assays were performed in duplicate. Results are expressed as the mean, plus or minus the range of the values. Controls for each assay included wells which were coated with carbonate buffer only (no antigen) and wells to which PBS was added in place of antibody after coating with antigen.

Determination of biotypes. Biotypes were determined by using the Minitek system (BBL Microbiology Systems, Cockeysville, Md.) described by Oberhofer and Back (1, 26). Discs impregnated with the substrates ornithine, urea, and *o*-nitrophenyl-β-D-galactopyranoside were placed in plastic wells of Minitek trays. A suspension of the organism to be tested was prepared by suspending two loopfuls of colonies from chocolate agar in 0.5 ml of brain heart infusion broth supplemented with hemin and NAD, both at 10 μg/ml. Fifty microliters of this suspension was placed into wells with the discs and overlaid with 2 drops of mineral oil. The results with ornithine decarboxylase, urease, and *o*-nitrophenyl-β-D-galactopyranoside were read by observing the color after overnight incubation at 37°C. The test for indole was performed by adding 2 drops of Kovács reagent to the wells containing *o*-nitrophenyl-β-D-galactopyranoside discs and observing the color after 30 s.

PCR. The Gen Amp PCR reagent kit (Perkin-Elmer Cetus, Norwalk, Conn.) was used to amplify the P6 genes of strains 1595 and 1610 by the polymerase chain reaction (PCR). Thirty cycles were performed with denaturing at 95°C, annealing at 55°C, and elongation at 72°C, each for 1 min, except for the first two cycles. The first cycle was allowed to denature for 2.5 min, and the second cycle was allowed to denature for 1.5 min. The reaction was performed in a volume of 100 μl, and the result was monitored by agarose gel electrophoresis.

Molecular cloning and DNA sequencing. The PCR yielded a 560-bp DNA fragment which contained the P6 gene. The fragment was excised from an agarose gel and ligated into the TA cloning vector (Invitrogen, San Diego, Calif.). Dideoxy sequencing was performed with Sequenase 2.0 (U.S. Biochemicals, Cleveland, Ohio). The sequence of the P6 gene which was subjected to site-directed mutagenesis was determined first in the bacteriophage vector M13 and subsequently in pGEM 7Zf$^-$ (Promega, Madison, Wis.).

Computer-assisted analysis of the protein and nucleotide sequences was performed with the Genetics Computer Group Sequence Analysis Software Package.

Oligonucleotide-directed mutagenesis of the P6 gene. To determine whether the epitope recognized by antibody 7F3 was localized to amino acids 59 through 61 of the P6 protein, the P6 gene of strain 1479 (7F3 reactive) was subjected to oligonucleotide-directed mutagenesis. The region encoding amino acids 59 to 61 was altered to correspond to the sequence of this region of the P6 gene in strain 1595 (7F3 nonreactive). Figure 1 shows the 36-base oligonucleotide which was used to substitute 4 bases in the P6 gene of strain 1479. This oligonucleotide is complementary to the antisense strand of the 1479 P6 gene in the bacteriophage vector M13mp18. Mutations in the single-stranded template DNA were introduced by the double-primer method (25, 33). Briefly, the phosphorylated mutagenic oligonucleotide and the M13 universal sequencing primer were annealed to the M13 single-stranded template containing the wild-type P6 gene of strain 1479. Subsequent to the annealing reaction, primer extension with Sequenase, deoxynucleoside triphosphates, T4 DNA ligase, and ATP was performed at 0°C for 5 min, room temperature for 5 min, and then 37°C for 2 h. The resulting double-stranded heteroduplex DNA was then di-

	227*					(Asp)		(Thr)				262*
1479 P6 Sequence	GGT	TTT	GAT	AAA	TAC	GAC	ATC	ACC	GGT	GAA	TAC	GGT
mutagenic oligonucleotide	...	...	...	...	...	A.. (Asn)	...	GAA (Glu)	...	...	...	...
oligonucleotide used to screen mutant clones				AAA	TAC	AAC	ATC	GAA	GGT			

***nucleotide position in pBUD 5**

FIG. 1. Nucleotide sequences of the P6 gene from NTHI strain 1479 (the numbers denote nucleotide position in pBUD5 [23]), the oligonucleotide used to generate the mutant (it is identical to the 1479 P6 sequence except for the 4 nucleotides noted), and the oligonucleotide used to screen mutant clones.

rectly transfected into competent *Escherichia coli* DH5αF′ (15).

Plaque lifts of the resultant clones were screened by hybridization with the 18-base oligonucleotide shown in Fig. 1. The oligonucleotide was labeled with [^{32}P]ATP by using T4 kinase. By gradually increasing the wash temperature in the presence of 3 M tetramethylammonium chloride, the background from clones expressing the wild-type gene was eliminated and several plaques hybridized with the oligonucleotide (32). These plaques were picked, and the nucleotide sequence of one was determined to confirm that the desired mutation was obtained.

Expression of recombinant P6. Recombinant plasmids expressing the two forms of the P6 gene were constructed using pGEM 7Zf$^-$. The insert containing the mutant P6 gene was recovered from M13 replicative-form DNA by digestion with *Bam*HI. The resultant 580-bp fragment containing the P6 gene was isolated by electrophoresis on a 1.5% agarose gel. The band was excised from the gel and ligated into pGEM 7Zf$^-$ that was digested with *Bam*HI. Recombinant clones were recovered by transformation of *E. coli* DH5αF′. One of the recombinant clones was picked, and the nucleotide sequence of the entire P6 gene insert was determined. This clone containing the oligonucleotide-directed mutation of the P6 gene was named pDAN1.

A similar strategy was used to subclone the corresponding 580-bp fragment containing the P6 gene of strain 1479 from pBUD5 (23) into pGEM 7Zf$^-$. This clone containing the wild-type P6 gene of strain 1479 was named pDJS1.

RESULTS

Reactivity of strains with P6 monoclonal antibodies. Whole-organism lysates of 60 strains of NTHI were tested by immunodot assay for the presence of the epitopes recognized by antibodies 7F3 and 4G4. As noted in Table 1, the sources of the isolates included invasive isolates in neonates, invasive isolates in adults, and cervical isolates in women. All strains were reactive with antibody 4G4, and 47 strains were reactive with antibody 7F3 in immunodot assays. The results of these immunodot assays were further tested by subjecting whole-organism lysates of 3 of the 13 nonreactive strains to immunoblot assays. Figure 2 shows that these strains were nonreactive in immunoblot assay and that the molecular mass of the P6 protein is identical in these strains regardless of antibody 7F3 reactivity.

Phenotypic characteristics of strains. Biotyping of strains revealed that all 13 strains that were nonreactive with antibody 7F3 were biotype 4. Two additional strains were biotype 4 but expressed the epitope recognized by antibody 7F3 (Table 1). Excluding the 7F3-nonreactive strains, the biotypes of the remaining isolates were distributed in a pattern that is typical of nontypeable strains of *H. influenzae* in that biotypes 2 and 3 predominated (2, 5, 20).

The outer membranes of selected strains were purified by previously described methods and subjected to SDS-PAGE. Figure 3 shows that the 7F3-nonreactive, biotype 4 strains have outer membrane protein patterns that are similar to one another (lanes f through i) but distinctly different from the patterns of typical strains of NTHI (lanes a through e). The two strains which are biotype 4 and 7F3 reactive have outer membrane protein patterns which resemble those of typical strains of NTHI (lanes d and e).

The colony morphology and the gross appearance of the 7F3-negative, biotype 4 bacterial cells differed from those of typical strains of NTHI. The colonies appear somewhat drier than typical strains. When cells are harvested from agar plates, the 7F3-negative, biotype 4 strains are more easily lifted onto glass slides and seem stickier, whereas typical strains tend to slide off the glass slide. The character of the pellet after centrifugation of bacterial cells is distinctly different. The 7F3-negative, biotype 4 strains form a pellet

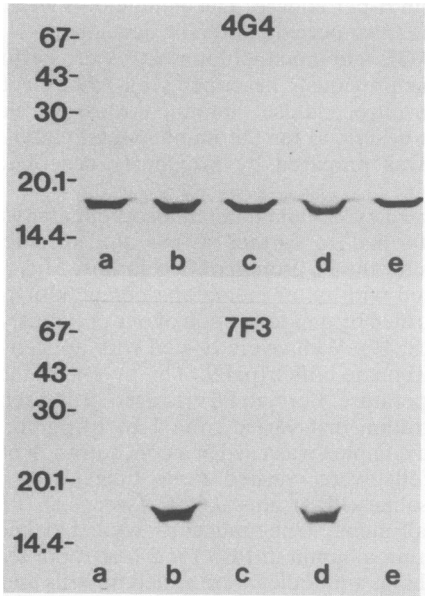

FIG. 2. Immunoblot assay of whole-organism lysates of five strains of NTHI. Lanes: a, 1610; b, 3198; c, 1595; d, 3524; e, 799. The top panel was developed with antibody 4G4, and the bottom panel was developed with antibody 7F3. Molecular mass markers are noted on the left in kilodaltons.

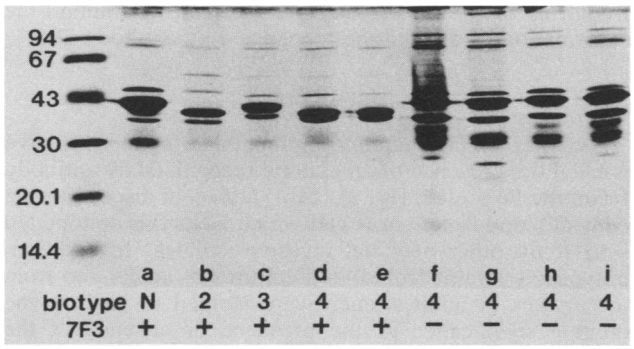

FIG. 3. SDS-polyacrylamide gel stained with Coomassie blue. Lanes contain the outer membrane complexes of the following strains: 1479 (a), 615 (b), 1161 (c), 1217 (d), 59 (e), 597 (f), 756 (g), 1595 (h), and 6351 (i). The biotype of each strain is noted (N, nonbiotypeable). The reactivity with antibody 7F3 is noted at the bottom (+, reactive; −, nonreactive). Molecular mass markers are on the left in kilodaltons.

which is less firm and more diffuse and actually require a longer time to centrifuge. The pellets have a subtle orange hue, in contrast to typical strains, which form a white pellet.

P6 gene sequence determination and analysis. PCR was used to amplify the genes from two 7F3-negative strains. Genomic DNA from strains 1595 and 1610 was isolated, digested with *Bgl*II and *Bam*HI, and subjected to agarose gel electrophoresis. The P6 gene is located on a 738-bp *Bgl*II-*Bam*HI fragment (24). The DNA from this region of the gel was excised and used as the template in the PCR to amplify the P6 genes from the two strains. Oligonucleotide primers corresponding to sequences immediately upstream and downstream of the P6 gene were used in the reaction, and this resulted in a 560-bp DNA fragment which contained the P6 gene. After this fragment was cloned, the DNA sequences were determined. The sequences of both strands of the two P6 genes were determined initially from two independent clones from each ligation. Both clones from strain 1595 yielded the identical sequences. The first two clones from strain 1610 had a single discrepancy within the sequence of the P6 gene. Therefore, the sequence of both strands of a third independent clone was determined. Since it corresponded with the sequence of one of the first two clones, it was assumed that this was the correct sequence.

The DNA sequences of the P6 genes of strains 1595 and 1610 were identical to one another. The predicted amino acid sequence from these two strains was compared with the sequences of 7F3-reactive P6 proteins that have been previously published (6, 23, 24). The sequences differed by 5 of 153 amino acids, and these are shown in Table 2. The amino acid differences at positions 33, 42, and 152 are isolated from one another, and they involve small, uncharged amino acids. However, the amino acid differences at positions 59 and 61 are clustered at what appears to be an antigenic site and involve charged amino acids. On the basis of the comparison of these sequences, we hypothesized that the epitope recognized by antibody 7F3 is in or around positions 59 through 61.

ELISAs. To further characterize the P6 molecule in biotype 4, 7F3-nonreactive strains, the P6 proteins from strain 1479 (7F3 reactive) and strain 1595 (7F3 nonreactive) were purified by a modification of our previously described method (18, 19). Figure 4 shows that this method is effective in purifying P6 from both strains. Since the method of purifying P6 is based on the association of P6 with peptidoglycan, this observation indicates that P6 is associated with peptidoglycan in 7F3-nonreactive strains. However, the yield of P6 from strain 1595 was significantly lower than that from strain 1479, suggesting that the association of P6 and peptidoglycan is different in these strains.

TABLE 2. Amino acid sequence of P6 proteins from antibody 7F3-reactive and -nonreactive strains

Amino acid position[a]	Amino acid in:	
	7F3-reactive strains[b]	7F3-nonreactive strains[c]
33	Ala	Gly
42	Ala	Ser
59	Asp	Asn
61	Thr	Glu
152	Ala	Ser

[a] Position 1 is defined as the start codon (methionine) (23).
[b] Previously published sequences of nontypeable strain 1479 and type b strains Minn A and Eagan (6, 23, 24).
[c] Sequence of nontypeable, biotype 4 strains 1595 and 1610.

Purified outer membrane (Fig. 3) and purified P6 (Fig. 4) from both strains were used in an ELISA. Table 3 shows that strain 1595 lacks the 7F3 epitope but expresses the 4G4 epitope, confirming the results of the immunodot and immunoblot assays. Antibody 4G4 was less reactive with antigens from strain 1595 than with those from strain 1479. Outer membrane and purified P6 from strain 1479 were reactive with both antibodies in ELISA.

Synthetic peptides were studied to determine whether antibodies 7F3 and 4G4 recognize linear epitopes in the region of the protein corresponding to the amino acid differences between 7F3-reactive and -nonreactive strains. Two 20-mers corresponding to the regions of the protein at amino acid positions 59 through 61 and flanking residues in 7F3-reactive and -nonreactive strains were obtained. These peptides did not bind antibodies 7F3 and 4G4 directly in immunodot assays or ELISAs. Furthermore, neither peptide

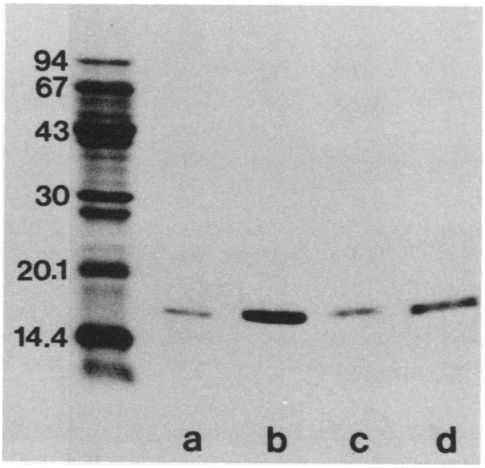

FIG. 4. SDS-polyacrylamide gel stained with Coomassie blue. Lanes contain purified P6 from strains 1479 (1 μg) (a), 1479 (5 μg) (b), 1595 (1.2 μg) (c), and 1595 (12 μg) (d). Molecular mass markers are noted in kilodaltons on the left.

TABLE 3. Results of ELISA with monoclonal antibodies to P6

Coating antigen	Optical density with:	
	7F3 antibody	4G4 antibody
1479 P6	0.713 ± 0.070	0.834 ± 0.037
1595 P6	0.000 ± 0.000	0.126 ± 0.014
1479 OMC[a]	0.387 ± 0.005	0.492 ± 0.004
1595 OMC	0.019 ± 0.001	0.254 ± 0.022
Buffer[b]	0.037 ± 0.005	0.001 ± 0.000

[a] OMC, outer membrane complex.
[b] Wells were coated with carbonate buffer in place of antigen.

caused inhibition in the ELISAs employing purified outer membrane or purified P6 as the antigen.

Analysis of recombinant wild-type and mutant P6. Sequence analysis and comparison of the P6 genes of 7F3-reactive and -nonreactive strains suggested that the 7F3 epitope was located in or around amino acids 59 through 61. However, the P6 protein of 7F3-nonreactive strains contained three additional amino acid differences compared with P6 in the 7F3-reactive strains (Table 2). To directly assess whether the epitope recognized by antibody 7F3 was localized to amino acids 59 to 61, oligonucleotide-directed mutagenesis was performed.

The P6 gene of strain 1479 was mutagenized such that the sequence was altered exclusively in the region encoding amino acids 59 to 61. Dideoxy sequencing confirmed that the P6 gene insert in pDAN1 contained the desired mutation and that the remainder of the P6 gene is identical to the wild-type P6 gene of strain 1479. The P6 gene insert in pDJS1 contains the wild-type P6 gene in strain 1479.

Expression of the recombinant P6 proteins in pDAN1 and pDJS1 was analyzed by immunoblot assay with antibodies 7F3 and 4G4. Figure 5 is an immunoblot assay which shows that, as expected, both recombinant P6 proteins express the epitope recognized by antibody 4G4. Immunoblot analysis with antibody 7F3 shows that the recombinant P6 protein which contains the mutation in amino acids 59 to 61 is nonreactive with antibody 7F3 (Fig. 5). Immunodot assays of a whole-organism lysate of *E. coli* DH5αF′ containing pGEM 7Zf$^-$ were nonreactive with both antibodies. We conclude that the sequence Asp-Ile-Thr of amino acids 59 through 61 of the P6 protein is critical in maintaining the conformation of the epitope recognized by antibody 7F3.

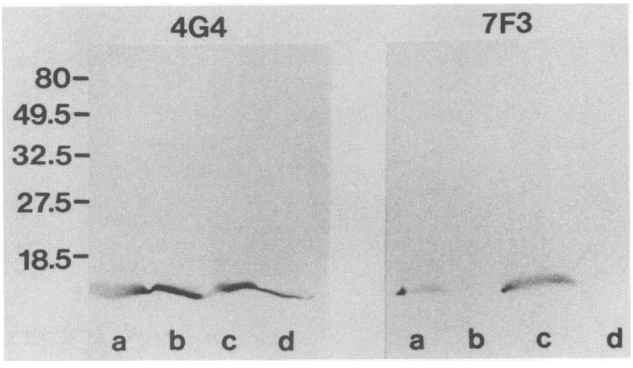

FIG. 5. Immunoblot assay with antibodies 4G4 (left) and 7F3 (right). Lanes contain whole-organism lysates of NTHI strain 1479 (a), NTHI strain 1595 (b), *E. coli* containing plasmid pDJS (wild-type P6 gene) (c), and *E. coli* containing pDAN1 (mutant P6 gene) (d). Molecular mass markers are noted in kilodaltons on the left.

DISCUSSION

Assays of several hundred strains of *H. influenzae* have revealed the presence of an epitope recognized by antibody 7F3 on the P6 protein (18, 21, 24). The recent discovery of a neonatal blood isolate of NTHI which lacks this epitope led us to study other neonatal invasive isolates. In addition, nontypeable strains from blood cultures of adults and from the cervices of adult women were studied to analyze the potential significance of the presence or absence of the epitope in invasive and urogenital disease. The data reported here establish that the absence of the 7F3 epitope on P6 is characteristic of the unusually virulent clone family of biotype 4 strains that are associated specifically with invasive disease in the neonate and postpartum women.

Wallace et al. (30) first reported the association of biotype 4 strains with invasive infection in the neonate. Subsequent studies from several centers have confirmed this association and have further characterized these strains (12, 27, 28). The biotype 4 strains which cause neonatal sepsis have a homogeneous outer membrane protein pattern by SDS-PAGE, in contrast to strains of NTHI which are recovered from the respiratory tract (2, 5, 20, 28). Electrophoretic typing has shown that these strains are indeed different from other nontypeable strains and actually represent a separate *Haemophilus* species (22, 27). In view of the extensive molecular conservation of the P6 gene among strains of *H. influenzae*, the presence of a variant P6 molecule in these strains is consistent with the observation that the strains are genetically distinct from other strains of *H. influenzae*.

The P6 outer membrane protein of NTHI has been the subject of intensive investigation over the past 5 to 7 years. The protein has several features which suggest that it plays an important role in pathogenesis and in the human immune response to infection. P6 is a target of human bactericidal antibody, and antibody to P6 is bactericidal for most strains of NTHI (13, 19). Despite the observation that strains of NTHI show extensive outer membrane protein and genetic diversity, the P6 protein shows a remarkable degree of molecular conservation among strains (14, 18, 24). The sequences of the P6 proteins of three geographically and epidemiologically unrelated strains show 100% homology at the amino acid level (24). Therefore, there is enormous selective pressure on the organism to express a P6 protein that does not vary in its amino acid sequence.

In this study, the 7F3-negative, biotype 4 phenotype was observed only in isolates recovered from invasive neonatal or postpartum infections and from the female urogenital tract. Invasive isolates in adults and respiratory tract isolates from patients of all ages express the 7F3 epitope (18, 21, 24). Since antibody 7F3 recognizes an epitope that is exposed on the surface of the intact bacterium (18), the alteration in the P6 molecule in neonatal, urogenital strains is on the surface-exposed region of the molecule. This is the region that is most available for interaction with host molecules. This observation suggests that the variant molecule is important in the organism's ability to cause disease in this specific clinical setting.

Comparison of the P6 gene sequences and immunoblot analysis of the recombinant P6 genes (Fig. 5) indicate that the 7F3 epitope is located at or around amino acid residues 59 to 61. The epitope is expressed in whole-cell lysates (Fig. 2), purified outer membrane preparations (Table 3), and

purified P6 (Table 3). Immunoreactivity persists in these preparations despite boiling in the presence of SDS. The inability of synthetic peptides to bind the antibody and to inhibit the binding of antibody 7F3 to P6 suggests that the antibody recognizes a conformational epitope that cannot be formed by a peptide consisting of 20 amino acids. The epitope apparently depends on the Asp-Ile-Thr sequence for its conformation.

Finally, the identification of a variant P6 molecule in the unusually virulent biotype 4 clones of NTHI may have practical implications. After further basic and clinical studies it may be possible to screen pregnant women specifically for these strains. Those women in whom such strains are identified can receive prophylactic antibiotics with the prospect of preventing neonatal and postpartum infections. Future studies will reveal whether this strategy will be feasible and effective.

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