INFECTION AND IMMUNITY, May 1993, p. 2233–2237
0019-9567/93/052233-05$02.00/0

Vol. 61, No. 5

Isolation, Expression, and Nucleotide Sequencing of the Pilin Structural Gene of the Brazilian Purpuric Fever Clone of *Haemophilus influenzae* Biogroup aegyptius

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Received 18 December 1992/Accepted 20 February 1993

In this study we isolated the pilin gene from the Brazilian purpuric fever (BPF) clone of *Haemophilus influenzae* biogroup aegyptius, expressed the gene in *Escherichia coli*, and determined its nucleotide sequence. Comparison of the nucleotide sequence of the BPF pilin gene with the sequences of pilin genes from strains of *H. influenzae* sensu stricto demonstrated a high degree of identity. Consistent with this observation, hemagglutination inhibition studies performed with a series of glycoconjugates indicated that BPF pili and *H. influenzae* type b pili possess the same erythrocyte receptor specificity.

Brazilian purpuric fever (BPF) is a fulminant septicemic illness of young children that is usually preceded by purulent conjunctivitis. This disease was first recognized in 1984 when an outbreak occurred in Promissao, a rural town in Sao Paulo State, Brazil (3). In this outbreak, 10 children developed high fever associated with abdominal pain and vomiting. Within 12 h of the onset of fever, these patients developed purpura and vascular collapse, and all 10 died. In 1986 the etiology of BPF was established when another outbreak occurred, this time in the town of Serrana in Sao Paulo State, Brazil (4). Nine blood cultures and one hemorrhagic cerebrospinal fluid culture from 10 clinically ill children grew *Haemophilus influenzae* biogroup aegyptius. Extensive analysis of these strains, including examination of whole bacterial protein profiles, plasmid restriction patterns, multilocus enzyme electrophoresis types, rRNA hybridization patterns, and seroagglutination reactions suggested that they are members of the same clone (the BPF clone) and are distinct from other strains of *H. influenzae* biogroup aegyptius in Brazil and other countries (5). Further examination of the BPF clone by multilocus enzyme electrophoresis demonstrated that this clone is genetically quite divergent from other strains of *H. influenzae* biogroup aegyptius and instead more closely related to serotype c strains of *H. influenzae* (18).

In the process of comparing BPF and non-BPF isolates of *H. influenzae* biogroup aegyptius, Brenner et al. identified a 25-kDa protein with distinctive silver staining properties that was present in the majority of BPF clone strains but was generally absent from non-BPF strains (5). The association of this protein with BPF but not non-BPF strains of *H. influenzae* biogroup aegyptius suggested that it may play an important role in the pathogenesis of BPF. Subsequently, Weyant et al. reported that this protein is a pilin with an amino acid composition very similar to that of the *H. influenzae* type b pilin (27). In addition, they found that expression of this protein is associated with piliation and agglutination of human erythrocytes, indicating further resemblance to the *H. influenzae* type b pilin. Consistent with these observations, upon probing chromosomal DNA from BPF strains we found evidence of homology with the pilin structural gene of *H. influenzae* type b (22). In the present study we exploited this homology and isolated the pilin structural gene from a BPF clone strain by using the polymerase chain reaction (PCR). We report the results of our characterization of the gene and its product.

H. influenzae biogroup aegyptius strains F3037, F2052, and F3050 were all isolated from Brazilian children and share the BPF clone phenotype (5). Strain F3037 was recovered from the bloodstream of a child with the clinical syndrome of BPF and was obtained from L. Rubin (Albert Einstein Medical School, New Hyde Park, N.Y.). Strains F2052 and F3050 were isolated from the conjunctivas of patients who had conjunctivitis but were not systemically ill and were obtained from D. Brenner (Centers for Disease Control, Atlanta, Ga.).

H. influenzae M43p$^+$ is a serotype b isolate that was originally recovered from the nasopharynx of a child with meningitis and was obtained from J. Gilsdorf (University of Michigan, Ann Arbor, Mich.). This strain expresses long pili that mediate agglutination of AnWj-positive human erythrocytes (10, 24, 25). The M43p$^+$ structural pilin gene has been cloned, and its nucleotide sequence has been determined (9). H. influenzae Rd is a capsule-deficient serotype d strain that has been in the laboratory for over 40 years (1) and was provided by J. Setlow (Brookhaven National Laboratory, Upton, N.Y.) from her strain collection (21). This strain is unable to express long pili and fails to hybridize in Southern blots with the pilin gene from M43p$^+$ (21a).

Escherichia coli DH5α was obtained from Bethesda Research Laboratories. E. coli XL-1 Blue and the plasmid pKS$^-$ were obtained from Stratagene. Plasmid pT7-7 and phage mGP1-2 were provided by S. Tabor (Harvard Medical School, Boston, Mass.).

H. influenzae strains were grown on chocolate agar supplemented with supplement VX (Difco Laboratories, Detroit, Mich.) or in brain heart infusion broth supplemented

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with hemin and NAD (2). *E. coli* strains were grown on L agar or in L broth, supplemented with ampicillin at a concentration of 100 μg/ml when appropriate.

On the basis of our previous study demonstrating homology between the *H. influenzae* type b pilin gene and chromosomal DNA from BPF clone strains (22), we reasoned that we might be able to isolate the BPF clone pilin gene by the PCR. In the present experiments, *H. influenzae* type b strain M43p$^+$ served as a positive control, and the laboratory *H. influenzae* strain Rd served as a negative control. Amplification was carried out with oligonucleotide primers that correspond to regions just flanking the pilin structural genes of *H. influenzae* type b strains 770235f$^+$b^o, M43p$^+$, and MinnA (the sequences of the pilin genes from these three strains are identical) (6, 9, 26). The sequence of the 5′ oligonucleotide is 5′AACGAATTCTGCTGTTTATTAAG GCTTTAG and contains an *Eco*RI site to facilitate subcloning. The sequence of the complementary 3′ oligonucleotide is 5′AGCTGGATCCTTGTAGGGTGGGCGTAAGCC and contains a *Bam*HI site for subcloning purposes. By using these primers, a 0.8-kb fragment was amplified from chromosomal DNA from strain M43p$^+$ and the BPF clone strain F3037. In contrast, no product was amplified from strain Rd.

To examine expression of the DNA fragment amplified from strain F3037, the 0.8-kb PCR product from this strain was digested with *Eco*RI and *Bam*HI and ligated into the bacteriophage T7 expression vector pT7-7. The ligation mixture was then electroporated into *E. coli* XL-1 Blue (7). The resulting plasmid, designated pT7-BPFpil, contains the cloned fragment downstream of the T7 phage ϕ10 promoter and ribosome binding site (23). As a negative control, pT7-7 containing no insert was also introduced into XL-1 Blue, producing XL-1 Blue/pT7-7. The T7 promoter was induced in these two strains by infection with the recombinant M13 phage mGP1-2 and addition of isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM). Phage mGP1-2 contains the gene encoding T7 RNA polymerase, which activates the ϕ10 promoter in pT7-7 (23). After 30 min at 37°C, rifampin was added to a final concentration of 200 μg/ml. Thirty minutes later, 1 ml of the culture was pulsed with 10 μCi of [^{35}S]methionine for 5 min. Bacteria were harvested and resuspended in Laemmli buffer for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (12). As shown in Fig. 1, induction of XL-1 Blue/pT7-BPFpil resulted in expression of a novel protein estimated to be approximately 22 kDa in size. In contrast, when XL-1 Blue/pT7-7 was induced, there was no expression of this protein. This result confirmed that the PCR product amplified from strain F3037 contains an open reading frame encoding a protein with a size appropriate for the BPF pilin.

To determine the nucleotide sequence of the strain F3037 PCR product, the 0.8-kb fragment from this strain was cloned into pKS$^-$ and sequencing was performed along both strands, using a double-stranded plasmid template. The sequence of the amplified segment of DNA is shown in Fig. 2. Sequencing of the product from a second PCR revealed the identical sequence, indicating that no PCR errors had occurred. The sequence contains a 633-bp open reading frame that encodes a 22-amino-acid leader peptide followed by a 189-amino-acid mature protein. The calculated molecular mass of the mature gene product is 20.4 kDa. To confirm that we had indeed isolated the BPF pilin gene, we compared the derived amino acid sequence with the chemically determined N-terminal sequence reported by Weyant et al. (27). As shown in Fig. 2, the two sequences are in complete

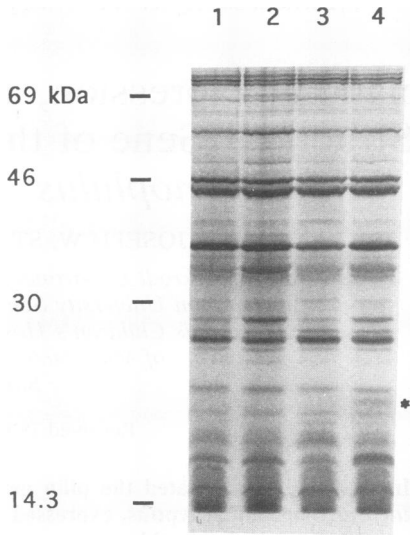

FIG. 1. Autoradiogram of a 10% polyacrylamide gel demonstrating expression of the recombinant BPF strain F3037 pilin in the bacteriophage T7 expression system. Lanes 1 and 2, whole bacterial lysates of the control strain, *E. coli* XL-1 Blue/pT7-7, uninduced and induced, respectively; lanes 3 and 4, whole bacterial lysates of *E. coli* XL-1 Blue/pT7-BPFpil, uninduced and induced, respectively. The asterisk indicates the novel 22-kDa protein induced in XL-1 Blue/pT7-BPFpil.

agreement, except that chemical analysis failed to reveal the identity of the residues in positions 1 and 2.

The nucleotide sequences have been determined for the structural pilin genes from four different *H. influenzae* type b strains (M43p$^+$ [= AO2], 770235f$^+$b^o, MinnA, and Eagan) (6, 8, 9, 13, 26) and one nontypeable strain (M37) (6). The M43p$^+$, 770235f$^+$b^o, and MinnA genes have the identical sequence, while the Eagan and M37 gene sequences differ somewhat. Comparison of the sequence of the BPF pilin gene with the sequences of the genes from these five strains demonstrated 79 to 80% identity. Interestingly, the leader peptide of the BPF pilin is two amino acids longer than the leader peptide in the type b strains and four residues longer than the leader sequence in nontypeable strain M37. Despite the difference in leader peptide length, the first 15 amino acids of the leader are completely conserved among all these strains and the first 20 amino acids are the same for the BPF pilin and the four type b pilins. As in the case of the other *H. influenzae* pilins, the mature BPF clone protein contains two cysteine residues and a penultimate tyrosine (positions 21, 41, and 188, respectively).

In an effort to identify common regions of type b pili that are surface exposed and represent antigenic epitopes, Forney et al. analyzed the hydrophilicity of the pilin proteins expressed by the type b strains M43p$^+$ and Eagan (8). These investigators found hydrophilicity profiles for the pilins from these two strains to be very similar. They identified three hydrophilic domains that were located between residues 15 and 33, residues 79 and 96, and residues 120 and 131 and proposed that these domains might constitute conserved antigenic epitopes. The corresponding amino acid stretches in the BPF pilin are 15 to 33, 77 to 94, and 116 to 127, respectively. As shown in Fig. 3, the BPF pilin and the type b pilins show a high degree of sequence similarity in these regions, suggesting conservation of hydrophilic domains. It will be interesting to determine whether peptides corre-

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       10         20         30         40         50         60
ATGAAAAAAACACTTCTTGGTAGCTTAATTTTATTGGCATTTGCGGGAAATGTGCAGGCG
MetLysLysThrLeuLeuGlySerLeuIleLeuLeuAlaPheAlaGlyAsnValGlnAla

       70         80         90        100        110        120
GCAACTACAACTAATGCTGAAACCTCTGGTAAAGTTACTTTCTTTGGTAAGGTTGTTGAG
AlaThrThrThrAsnAlaGluThrSerGlyLysValThrPhePheGlyLysValValGlu

      130        140        150        160        170        180
AATACTTGTAAAGTGAAAACGGATAATCAAAATATGAGCGTTGTGTTAAATGATGTAGGT
AsnThrCysLysValLysThrAspAsnGlnAsnMetSerValValLeuAsnAspValGly

      190        200        210        220        230        240
AAAAATAGCTTATCTACTAAAGGAAATACAGCAATGCCAACGCCATTTACTATTTCATTG
LysAsnSerLeuSerThrLysGlyAsnThrAlaMetProThrProPheThrIleSerLeu

      250        260        270        280        290        300
CAAGACTGTAATAATGTTACTACAACTGCTAATAAAGCATCTAAAGTTGGCGTTTATTTC
GlnAspCysAsnAsnValThrThrThrAlaAsnLysAlaSerLysValGlyValTyrPhe

      310        320        330        340        350        360
TATTCTTGGGAAAATGCGGATAAAGACAATGACTATACATTGAAAAATAAAATGAGTAAT
TyrSerTrpGluAsnAlaAspLysAspAsnAspTyrThrLeuLysAsnLysMetSerAsn

      370        380        390        400        410        420
GATTTTGCGACTATGGTTAATATCCAACTTATGGAAAGTGATGGCACAACTCCAATCAAA
AspPheAlaThrMetValAsnIleGlnLeuMetGluSerAspGlyThrThrProIleLys

                                                    PvuII
      430        440        450        460        470        480
GTTGTAGGCAAAACAACAGAAGATTTTGTTCATAAAGGTGCTAATGGATCAGCTGTAAAT
ValValGlyLysThrThrGluAspPheValHisLysGlyAlaAsnGlySerAlaValAsn

      490        500        510        520        530        540
TCAACTGCTGTAACTAGTAAACATATTTCGGGCAACACAGCATTAAATGGTAATACCTCA
SerThrAlaValThrSerLysHisIleSerGlyAsnThrAlaLeuAsnGlyAsnThrSer
          SpeI

      550        560        570        580        590        600
ATTGACCTCCATTTTATTTCTCAATATTACGCAACAGGTATAGCAACAGCGGGTAAAGTA
IleAspLeuHisPheIleSerGlnTyrTyrAlaThrGlyIleAlaThrAlaGlyLysVal

      610        620        630        640        650        660
GAATCCTCAGTAAATTTCCAAATTGCTTACGAATAA
GluSerSerValAsnPheGlnIleAlaTyrGluEND
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FIG. 2. Nucleotide and derived amino acid sequences of the BPF strain F3037 pilin. The first 22 residues represent the leader peptide. The underscored residues correspond to those previously determined by chemical analysis and represent positions 3 through 20 of the mature protein. The locations of the unique *Pvu*II and *Spe*I sites are as indicated.

Hydrophilic Domain 1

F3037	KVVENTCKVKTDHQNMSVV
M43p+	KVVENTCKVKTEHKNLSVV
Eagan	KVVENTCQVKTDHKNLSVV

Hydrophilic Domain 2

F3037	YFYSWENADKDNDYTLKN
M43p+	YFYSWKNVDKENNFTLKN
Eagan	YFYSWENADKENNFTLKN

Hydrophilic Domain 3

F3037	PIKVVGKTTEDF
M43p+	AISVVGKETEDF
Eagan	EIKVVGKETEDF

FIG. 3. Hydrophilic domain amino acid sequence homology between the pilins produced by BPF strain F3037, type b strain M43p+, and type b strain Eagan. Identical residues are highlighted in open type.

sponding to these regions elicit antibodies that recognize native pili from the divergent BPF clone and type b strains.

Southern blotting results reported in previous studies indicate that type b strains contain a single copy of the pilin structural gene (8, 9, 13). The observations for nontypeable strain M37 are ambiguous, with one study providing evidence for a single copy (8) and a second study suggesting the possibility of multiple copies (6). In an earlier study, we found that two distinct *Eco*RI fragments of chromosomal DNA from BPF clone strains hybridized with the M43p+ pilin gene (22). Interestingly, based on the nucleotide sequence we have reported here, there is no *Eco*RI restriction site within the BPF pilin gene, indicating that there are two copies of this gene in the BPF clone genome. To determine whether these two copies are identical, we digested chromosomal DNA from BPF strain F3037 with *Pvu*II or *Spe*I, enzymes predicted from the nucleotide sequence to cut within the pilin gene. After Southern transfer, we probed this DNA with the 0.8-kb *Eco*RI-*Bam*HI fragment containing the BPF pilin gene (20). To confirm our earlier observation, we also probed chromosomal DNA that had been digested with *Eco*RI. As shown in Fig. 4, there were three *Pvu*II fragments and three *Spe*I fragments that hybridized with the probe, suggesting polymorphism between the two copies of the BPF pilin gene, with only one copy containing *Pvu*II and *Spe*I restriction sites. Alternatively, there may be comigrating bands that cannot be resolved on the Southern blot. Whether the two copies of the BPF pilin gene are both functional will require additional study. As predicted from the restriction map of strain M43p+, probing of M43p+ chromosomal DNA digested with *Eco*RI, *Pvu*II, or *Spe*I revealed a single hybridizing band in all cases (Fig. 4).

Recently, van Alphen et al. reported that pili expressed by *H. influenzae* type b strains mediate attachment to erythrocytes and oropharyngeal epithelial cells by binding to sialic acid-containing lactosylceramide structures (24). These workers observed that attachment was inhibited by glycoconjugates such as the gangliosides GM1 and GM2, which contain the sequence (NeuAcα2-3)Galβ1-4Glcβ1-1Cer. In contrast, molecules lacking sialic acid (e.g., the asialo derivative of GM1), the ceramide backbone (e.g., sialyl-lactose), or glucose (e.g., glycophorin) failed to inhibit attachment. On the basis of the sequence similarity between the BPF pilin gene and the pilin genes of type b strains, we hypothesized that BPF pili might recognize the same receptor. To test this possibility, we examined the effect of GM1, GM2, asialo-GM1, sialyl-lactose, and glycophorin on hemagglutination by the BPF clone strain F3037. As shown in Table 1, when used at a concentration of 100 μg/ml, both GM1 and GM2 completely inhibited hemagglutination, while asialo-GM1, sialyl-lactose, and glycophorin had no effect. Two additional BPF clone strains, F2052 and F3050, showed the same pattern of inhibition, as did the control type b strain M43p+. Consistent with previous observations, a nonpiliated variant of strain F3037 failed to cause hemagglutination (data not shown). Whether the similar receptor specificity of BPF and type b pili is determined by the structural pilin protein or instead a distinct adhesive protein like those described for other species of bacteria (14, 17) is currently under investigation.

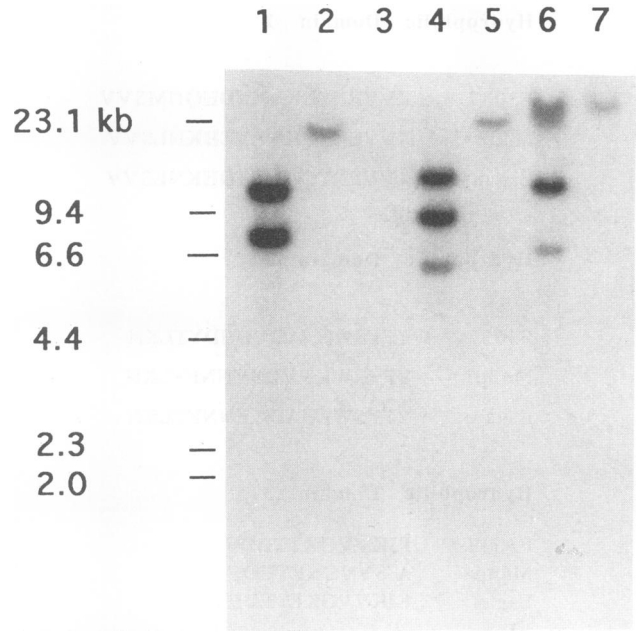

FIG. 4. Southern analysis of BPF strain F3037, *H. influenzae* type b strain M43p$^+$, and *H. influenzae* Rd, using the PCR-amplified *Eco*RI-*Bam*HI fragment containing the BPF pilin gene as a probe. Lanes 1, 4, and 6, chromosomal DNA from strain F3037 digested with *Eco*RI, *Pvu*II, and *Spe*I, respectively; lanes 2, 5, and 7, chromosomal DNA from strain M43p$^+$ digested with *Eco*RI, *Pvu*II, and *Spe*I, respectively; lane 3, chromosomal DNA from strain Rd digested with *Eco*RI.

In the present study we isolated, expressed, and sequenced the gene encoding the pilin subunit protein from the BPF clone strain F3037. Our results indicate marked similarity with the pilin genes characterized from strains of *H. influenzae* sensu stricto. In the past, there has been considerable controversy regarding the taxonomic classification of *H. influenzae* biogroup aegyptius isolates (11, 19). Some have argued that these strains should be considered a separate species (19). The observations we report here are consistent with the multilocus enzyme electrophoresis findings of Musser and Selander (18) and support the position that at least the BPF clone of *H. influenzae* biogroup aegyptius belongs to the species *H. influenzae*.

TABLE 1. Effects of glycoconjugates on agglutination of AnWj-positive human erythrocytes

Glycoconjugate[a]	Hemagglutination titer[b] for strain[c]:			
	F3037	F2052	3050	M43p$^+$
None	1:32	1:32	1:16	1:16
Ganglioside GM1	<1:1	<1:1	<1:1	<1:1
Ganglioside GM2	<1:1	<1:1	<1:1	<1:1
Asialo GM1	1:32	1:32	1:16	1:16
Sialyl-lactose	1:32	1:32	1:16	1:16
Glycophorin MN	1:32	1:32	1:16	1:16

[a] Glycoconjugates were used at a concentration of 100 μg/ml, and bacterial suspensions were preincubated with these compounds at 37°C for 45 min before quantitation of hemagglutination.

[b] Hemagglutination titers were determined by using V-bottom 96-well microtiter plates and AnWj-positive human erythrocytes as previously described (22).

[c] Strains F3037, F2052, and F3050 all share the BPF clone phenotype and express long pili. Strain M43p$^+$ is a type b strain that also expresses long pili; these pili are known to recognize sialic acid-containing lactosylceramide structures on human erythrocytes.

In addition to examining the structure of the BPF clone pilin gene, we have examined the receptor specificity of BPF pili. Our results suggest that these bacterial appendages bind to the same host cell structure as do pili from *H. influenzae* type b strains. Given the similarities between the pilin subunit proteins in BPF and type b strains, this observation is not surprising. However, it is notable that BPF clone strains isolated from the blood of patients with BPF are generally piliated (16), while serotype b strains recovered from the bloodstream are consistently nonpiliated (15). The suggestion is that pili confer a survival advantage to intravascular BPF clone organisms but a disadvantage to type b bacteria. The explanation for this apparent paradox may provide important insights into the pathogenesis of BPF.

We thank D. Cutter for technical assistance and R. Munson for critical reading of the manuscript.

This work was supported by the following Public Health Service grants: HD29678 to J. W. St. Geme III and AI26195 to S. Falkow.

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