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# Identification of a Gene Essential for Piliation in *Haemophilus influenzae* Type b with Homology to the Pilus Assembly Platform Genes of Gram-Negative Bacteria

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***Haemophilus influenzae* type b (Hib) pili are complex filamentous surface structures consisting predominantly of pilin protein subunits. The gene encoding the major pilin protein subunit of Hib adherence pili has been cloned and its nucleotide sequence has been determined. In order to identify specific accessory genes involved in pilus expression and assembly, we constructed isogenic Hib mutants containing insertional chromosomal mutations in the DNA flanking the pilin structural gene. These mutants were screened for pilin production, pilus expression, and hemagglutination. Pili and pilin production were assessed by immunoassays with polyclonal antisera specific for pilin and pili of Hib strain Eagan. Hemagglutination was semiquantitatively evaluated in a microtiter plate assay. Six Hib mutants produced proteins immunoreactive with antipilin antiserum but no longer produced structures reactive with antipilus antiserum. In addition, the mutants were unable to agglutinate human erythrocytes. Nucleotide sequence analysis localized the insertion sites in the six mutants to a 2.5-kb open reading frame upstream of the pilin structural gene and immediately downstream of an Hib pilin chaperone gene. The amino acid sequence encoded by this open reading frame has significant homology to members of the pilus assembly platform protein family, including FhaA of *Bordetella pertussis*, MrkC of *Klebsiella pneumoniae*, and the *Escherichia coli* assembly platform proteins FimD and PapC. This open reading frame, designated *hifC*, appears to represent a gene essential to Hib pilus biogenesis that has genetic and functional similarity to the pilus platform assembly genes of other gram-negative rods.**

*Haemophilus influenzae* is a gram-negative, nonenteric rod that contributes to significant morbidity in children and adults. The encapsulated *H. influenzae* type b (Hib) has been a leading cause of bacterial meningitis in children and an important cause of other invasive diseases such as epiglottitis, cellulitis, and septic arthritis. The specific bacterial factors that enhance the pathogenesis of Hib are currently undergoing intense study. One of these factors is Hib adherence pili, which are complex surface structures composed predominantly of pilin protein subunits. The pili are associated with the adherence of Hib to human erythrocytes and to human buccal epithelial cells (12, 34), which may contribute to Hib colonization of the human respiratory tract, ultimately promoting its ability to establish infection (2, 38). Although Hib pili have been well described and characterized (32), little is known about the genetic mechanisms involved in Hib pilus biogenesis.

The pilin genes of several Hib strains, including Eagan, have been cloned (10, 13, 22, 35), and the nucleotide sequences show a high degree of homology with each other as well as with the pilin genes of nontypeable *H. influenzae* (3, 7). In addition, the Hib pilin proteins show some similarity to the F17, type 1C, and PapA pilins of *Escherichia coli*. In an attempt to localize areas of DNA that code for Hib pilus expression, van Alphen et al. transformed *E. coli* DH5α with plasmids containing Hib strain 77032b$^0$f$^+$ DNA. The resultant transformants agglutinated human erythrocytes and possessed assembled Hib pili on their surface, as detected by electron microscopy with immunogold staining techniques (35). Through successive deletions, they determined that an 8-kb fragment carrying the Hib pilin gene, *hifA*, and extending ~7.0 kb 5′ of the gene contained the minimum amount of DNA that coded for detectable Hib pilus expression. This suggests that the genes related to assembly and function are organized in a cluster or operon in proximity to the Hib pilin gene.

The gene clusters involved in pilus biogenesis have been characterized for several pilus-producing organisms, including *E. coli* and *Klebsiella* (1), *Salmonella* (6, 28), and *Bordetella* (25, 39) spp. Most extensively studied have been the *fim* and *pap* gene clusters associated with *E. coli* type 1 and P pili, respectively (18, 20, 23). Both of these clusters contain genetic information for the pilin protein subunits, chaperone proteins, and assembly platform proteins, as well as minor pilins and adhesins. Recently a gene, *hifB*, encoding a protein with functional and sequence similarity to the pilin chaperone protein family has been identified in Hib strain Eagan (17, 31). This suggests that Hib pilus biogenesis may involve multiple genes, as in the *E. coli* systems, located on the chromosome in the area flanking the Hib pilin gene.

From the results of insertion mutagenesis and DNA sequence analysis, we report here an additional Hib gene, *hifC*, that is essential for pilus production and has homology to the assembly platform genes of *Bordetella*, *Klebsiella*, and *Salmonella* spp. and *E. coli*.

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FIG. 1. Map of plasmids containing Hib insert DNA. pBB16A contains a 16-kb Hib DNA insert obtained by *Bam*HI digestion of cosmid pCosXV-12. Portions of the insert were subcloned to constructs pBaX5.5 and pPP6.2. The Hib DNA insert from the cosmid was subsequently found to contain two unlinked fragments. pWW1 was obtained by ligating a 9.5-kb *Bgl*II chromosomal fragment that hybridized to a probe made from the *Eco*RI-*Xho*I fragment of pBaX5.5 into pGEM 7. A 2.7-kb *Eco*RI fragment was deleted from pWW1 to create pWW2. pWW4 contains the entire Hib fragment from pWW1 ligated to the 1.6-kb *Bgl*II-*Pst*I fragment from pPP6.2. pGEM7 is represented by the dark hatched boxes. pGEM5 is represented by the light hatched boxes. The DNA unlinked to the pilin gene is represented by the unboxed hatched line. Restriction enzyme sites are abbreviated as follows: Ap, *Apa*I; Av, *Avr*II; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; Nd, *Nde*I; Ns, *Nsi*I; P, *Pst*I; S, *Sac*I; and X, *Xho*I.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture methods.** Hib strain Eagan was provided by Terry Stull (Medical College of Pennsylvania), and a stably piliated variant (E1a$^+$) was obtained by an erythrocyte enrichment procedure (32). *E. coli* DH2 and LW68 were provided by Claire Berg, University of Connecticut (5), and served as the donor and recipient, respectively, for γδ-1 transposon mutagenesis. *E. coli* HB101 and DH5α were used as hosts for construction of specific insertional mutations and for amplification of plasmid DNA for nucleotide sequence analysis. The Hib and nontypeable *H. influenzae* strains used in the Southern analysis have been described previously (8, 11, 14, 32).

The plasmids containing Hib DNA were derived from two sources. pBB16A (Fig. 1) was constructed by digesting the cosmid pCosXV-12 (10) carrying a 16-kb insert of Hib strain Eagan chromosomal DNA with *Bam*HI. The resulting 16-kb fragment, which carries the pilin gene, was then ligated into the *Bam*HI site of pGEM7. Two subclones were constructed from pBB16A: pBaX5.5 (Fig. 1) contains the 5.5-kb *Bam*HI-*Xho*I fragment 5′ of the pilin gene inserted into pGEM7, and pPP6.2 contains the 6.2-kb *Pst*I fragment carrying the pilin gene inserted into the *Pst*I site of pGEM5 (Fig. 1). The other source of Hib DNA, pWW1 (Fig. 1), was constructed by ligating a 9.5-kb *Bgl*II chromosomal fragment of Hib strain Eagan DNA directly into the *Bam*HI site of pGEM7. This plasmid contains a portion of the pilin gene as well as DNA upstream of the gene. pWW2 and pWW4 are subclones of this plasmid (Fig. 1). pWW2 was constructed by deletion of the 2.7-kb *Eco*RI fragment of pWW1. pWW4 was constructed by ligating the *Apa*I-*Xho*I fragment of pPP6.2 containing the pilin gene into pWW1 cut with *Apa*I and *Xho*I to produce a large plasmid containing the intact pilin gene.

Site-specific insertional mutations were constructed by introduction of a kanamycin resistance (Km$^r$) gene cassette, from plasmid pUC-4-K (26), or the mγδ-1 element into specific restriction sites on the subclones. Plasmids used for mγδ-1 conjugational mutagenesis were provided by C. Berg and included pIF200, a conjugation-proficient plasmid containing the Tn*1000* derivative mγδ-1; pXRD4043, carrying the γδ transposase; and pNG54, carrying the γδ resolvase (5).

Hib strains were grown on brain heart infusion (BHI) broth or agar (Difco, Detroit, Mich.) supplemented with hemin (10 μg/ml) and NAD (4 μg/ml) (BHIs) at 37°C in 5% $CO_2$. Kanamycin-resistant Hib mutants were selected on BHIs agar with kanamycin (25 μg/ml). The Hib strain were made competent by growth in MIV medium as described by Herriot et al. (16). *E. coli* strains containing subclone plasmids or mutagenized subclones were grown in LB medium (GIBCO, Madison, Wis.) supplemented with 50 μg of carbenicillin per ml or with 250 μg of carbenicillin and 25 μg of kanamycin per ml, respectively. LB medium supplemented with chloramphenicol (2.5 μg/ml), kanamycin (25 μg/ml), carbenicillin (250 μg/ml), nalidixic acid (20 μg/ml), and 1 mM isopropylthio-β-D-galactoside (IPTG; Sigma Chemical Co., St. Louis, Mo.) in various combinations was used for the mutagenesis procedure. Hemin, NAD, and the antibiotic reagents were obtained from Sigma Chemical Company.

**Conjugational mutagenesis of subclones.** Mutagenesis was carried out by the liquid mating procedure of Berg et al. (5)

with specific modifications. In brief, plasmid pBaX5.5 or pWW2 was transformed into *E. coli* DH2 containing pIF200, the conjugative plasmid carrying mγδ-1, and pXRD4043, encoding the γδ transposase, made competent by the $CaCl_2$ shock method (29). The transformants were grown overnight in LB broth containing kanamycin, chloramphenicol, and carbenicillin diluted 1:6 with LB containing chloramphenicol and 1 mM IPTG, and allowed to incubate in a petri dish at 37°C for 3 h. Then, 0.5 ml of this culture was combined with 0.2 ml of a stationary-phase culture of the recipient strain LW68 grown in LB plus chloramphenicol. This mating mixture was diluted with 5 ml of LB plus chloramphenicol and 1 mM IPTG and incubated for 3 h to allow expression of the γδ resolvase by pNG54. After incubation, the cells were diluted and plated on LB agar containing kanamycin, chloramphenicol, carbenicillin, and nalidixic acid to select the LW68 hosts carrying the mutagenized plasmids. The transconjugants were purified on LB agar plus kanamycin and carbenicillin. The isolates were preserved in LB with 20% glycerol at −70°C.

In addition to the random mutations, three site-specific mutations were constructed from the subclones. pBB16/K, which has been described previously (15), was created by ligating a $Km^r$ cassette with *Bam*HI ends into the *Bgl*II site within the pilin gene on pBB16. pWW1/k:P was created by digesting pWW1 with *Pst*I, which produces a 12.2-kb fragment plus a 312-bp fragment. A $Km^r$ gene cassette with *Pst*I ends was ligated to the large pWW1 *Pst*I fragment, creating a deletion-insertion mutation. pWW4/k:E was created by introducing the $Km^r$ gene cassette into the *Eco*RI site of pWW4.

**Transformation of Hib with mutagenized DNA.** Plasmid DNA was purified from the individual transconjugants by the alkaline lysis miniprep technique (29). The entire 20-μl sample was then digested with specific restriction endonucleases to separate the pGEM vectors from the mutagenized inserts of Hib DNA. After overnight incubation, the linear DNA was added to a 200-μl aliquot of competent $E1a^+$ Hib. The transformation mixture was allowed to incubate for 90 min at 37°C with 5% $CO_2$. After the incubation, 800 μl of BHIs was added, and the tubes were incubated with shaking for an additional hour. The cells were then diluted and plated on BHIs agar with kanamycin. The plates were examined for kanamycin-resistant transformants after 24 to 48 h of incubation.

To verify that the alteration from the parental phenotype was due to the mγδ-1 insertion and not to phase variation, we performed a back-transformation. A pure culture of each nonpiliated, $Km^r$ Hib mutant was streaked to obtain isolated colonies. A crude chromosomal DNA lysate was obtained by adding six or seven colonies to 0.015% sodium dodecyl sulfate and 0.01× SSC (1.5 mM sodium chloride, 0.15 mM sodium citrate) and incubating at 65°C for 2 h. Then, 15 μl of each lysate was mixed with a loopful of $E1a^+$ cells and allowed to grow overnight on BHIs agar. A lawn of the overnight growth was spread onto BHIs agar containing kanamycin and incubated overnight. From 10 to 30 randomly selected kanamycin-resistant transformants from each plate were examined for phenotype.

**Immunoassays, hemagglutination, and buccal epithelial cell adherence assays.** Immunoassays were performed by the immunodot technique as described previously (24). To assay mutants for pilin production, a bacterial suspension was boiled for approximately 12 min to disrupt the pili and denature the pilin protein and was then applied to nitrocellulose membranes. The membranes were incubated with Hib pilin-specific antiserum R20 (24). Pilus production was assayed in the same manner except that the bacterial suspension was not boiled and the membranes were reacted with polyclonal antibody R19, specific for native pili of strain Eagan (24).

The hemagglutination assays were performed as described previously (24). Briefly, twofold serial dilutions of a standardized bacterial suspension were placed into Cooke U-shaped microtiter plate wells (Dynatech Laboratories, Inc., Chantilly, Va.), and washed human erythrocytes were added to each well. Hemagglutination was detected after 1 h of incubation at room temperature.

**DNA sequence determination.** The nucleotide sequence was determined by the dideoxy nucleotide chain termination method of Sanger et al. (30). The reagents for the reactions included the Sequenase 2.0 sequencing kit (United States Biochemical Corp., Cleveland, Ohio) with T7 and SP6 primers and [α-$^{35}$S]dATP (New England Nuclear, Boston, Mass.). Additional oligonucleotide primers were synthesized by the University of Michigan Core Facility to facilitate analysis in some regions. The DNA sequence was determined by using plasmid DNA from specifically constructed subclones and nested serial deletions of the 16-kb Hib chromosomal DNA fragment constructed by exonuclease III digestion (Erase-A-Base System; Promega, Madison, Wis.). The reactions were performed on circular plasmid DNA isolated by the alkaline lysis miniprep technique and large plasmid prep column technique (Qiagen Inc., Chatsworth, Calif.). The reaction products were analyzed on 5% polyacrylamide gels. In order to localize the sites of the mγδ-1 insertions, sequence reactions were performed on the mutagenized plasmid DNA with specific oligonucleotide primers, 5′GTAGGGAGCCTGATATG (res primer) and 5′GCTATCCGCGCATCCAT (kan primer), that were synthesized from the known nucleotide sequence proximal to each end of mγδ-1 (5). The DNA and protein sequences were analyzed with software from DNASTAR (Madison, Wis.).

**Southern analysis.** Genomic DNA from Hib strain Eagan, Hib mutants, previously described laboratory strains, and clinical isolates of *H. influenzae* was purified (29). The DNA was digested with specific restriction endonucleases, electrophoresed on an agarose gel, and transferred to a nylon membrane (Hybond N; Amersham, Arlington Heights, Ill.). pWW4 was digested with *Eco*RI and *Nde*I, and the 945-kb fragment was purified and used as the DNA probe. Hybridization was performed overnight at 65°C.

**Nucleotide sequence accession number.** The nucleotide sequence reported in this article has been submitted to the GenBank data bank with accession number U02932.

## RESULTS

**Subcloning of Hib DNA fragments flanking the pilin gene.** A 16-kb fragment of Hib DNA known to contain the pilin structural gene at its midpoint was cloned from the cosmid pCosXV-12 to generate pBB16A. Subclones pBaX5.5 and pPP6.2 were constructed from pBB16A. Later analysis showed that the Hib DNA fragment in the cosmid actually represented two noncontiguous DNA fragments ligated at the *Eco*RI site (Fig. 1). Subsequently, pWW1 was constructed by directly ligating a 9.5-kb fragment of a *Bgl*II digest of Hib strain Eagan chromosomal DNA containing the pilin gene into pGEM7. Subclones pWW2 and pWW4 were constructed from this clone.

**Construction of Hib mutants.** A set of mγδ-1 insertion mutations were created in the target plasmids pBaX5.5 and pWW2 by conjugational mutagenesis. In addition, specific mutations were created by insertion of a $Km^r$ cassette into known restriction enzyme sites on pBB16A, pWW1, and

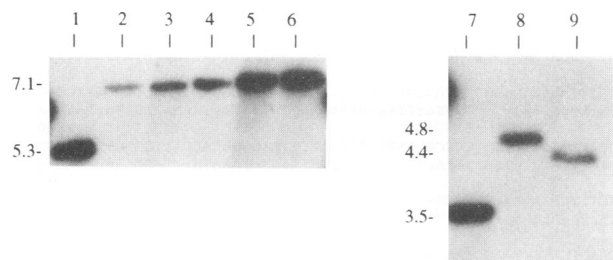


FIG. 2. Southern analysis of mutants, demonstrating allelic exchange. This Southern blot was prepared with chromosomal DNA from Hib strain Eagan and isogenic mutants carrying mγδ-1 or the Km$^r$ cassette. Variations in band intensity reflect variations in genomic DNA quantity. Lanes 1 to 6 contain chromosomal DNA cut with *Avr*II and *Xho*I. Lane 1, Hib strain Eagan DNA; lanes 2 to 5, DNA from individual Hib mutants containing mγδ-1 insertions within the *hifC* gene; lane 6, DNA from the mutant carrying an mγδ-1 insertion between *hifB* and *hifC*. Lanes 7 to 9 contain chromosomal DNA digested with *Nde*I and *Nco*I. Lane 7, Hib strain Eagan DNA; lane 8, DNA from the Hib mutant containing the Km$^r$ cassette placed within the *Eco*RI site; 9, DNA from the Hib mutant with the insertion-deletion mutation created by insertion of a Km$^r$ cassette into the *Pst*I sites in *hifC* with loss of a 312-bp *Pst*I fragment. Sizes are shown in kilobases.

pWW4. Aliquots of competent, stably piliated Hib strain Eagan (E1a$^+$) were transformed with linear mutagenized plasmid DNA. Hib mutants resulting from allelic exchange were identified by selection with kanamycin. Southern analysis was performed on selected Hib mutants to confirm that allelic exchange had occurred, with the introduction of a single insertion into the chromosome and without evidence of gene duplication (Fig. 2). As demonstrated by the blot, Hib strain Eagan DNA digested with *Avr*II and *Xho*I contains a single hybridizing band of 5.3 kb, while the mutants each contain a single 7.1-kb band that represents a unique mγδ-1 (1.8-kb) insertion. With the *Nco*I and *Nde*I double digest, the predicted 3.5-kb fragment was identified in Hib strain Eagan DNA, while a single 4.7-kb fragment corresponding to a unique Km$^r$ cassette (1.2 kb) insertion is noted in the mutant in lane 8. The 4.4-kb fragment seen in lane 9 corresponds to a single Km$^r$ cassette insertion with simultaneous loss of a 312-bp *Pst*I fragment.

**Characterization and localization of mγδ-1 insertions.** The resulting Km$^r$ Hib mutants were characterized for their ability to react with antiserum to the pilin structural protein, to react with antiserum directed against assembled pili, and to agglutinate human erythrocytes. One mutant had strong reactivity to the pilus-specific antiserum but had a hemagglutination titer of only 1:4. This was much weaker than that of the piliated parental strain, which hemagglutinated at a dilution of 1:16. Electron microscopic examination of this mutant demonstrated the presence of assembled pili (data not shown). Three mutants did not react with antipilin antibody, and six Hib mutants produced pilin, but no pili or hemagglutination was detected.

To verify that the alteration from the parental phenotype was due to the mγδ-1 insertion and not to phase variation, we performed an additional back-transformation with chromosomal DNA from the nonpiliated, kanamycin-resistant Hib mutants. The back-transformants uniformly exhibited the pilus-negative phenotype, demonstrating linkage of kanamycin resistance and loss of pilus expression.

The nucleotide sequence for the area flanking the mγδ-1 insertions was determined by using primers complementary to and directed away from the mγδ-1 element. The insertion sites were then localized by comparing this sequence with the known nucleotide sequence of the flanking area.

Figure 3 shows the sites of the mγδ-1 insertions and the site-specific insertional mutations as well as the resulting phenotypes of the corresponding Hib mutants. Two of the mutants that exhibited loss of pilin production had insertional mutations within the Hib chaperone gene *hifB*; the third mutant contains an insertional mutation within the pilin structural gene. The six mutants that produced pilin but no pili or hemagglutination had insertional mutations in the region of the chromosome downstream of *hifB*, localizing another gene involved in the expression of pili.

**DNA sequence analysis of the targeted area.** The nucleotide sequence of the DNA surrounding these mutations was determined on both strands and revealed an open reading frame containing 2,514 bp, encoding an 837-amino-acid protein of approximately 92.7 kDa (Fig. 4). The open reading frame starts with a GTG codon 107 bp 3′ of the *hifB* terminus and has a

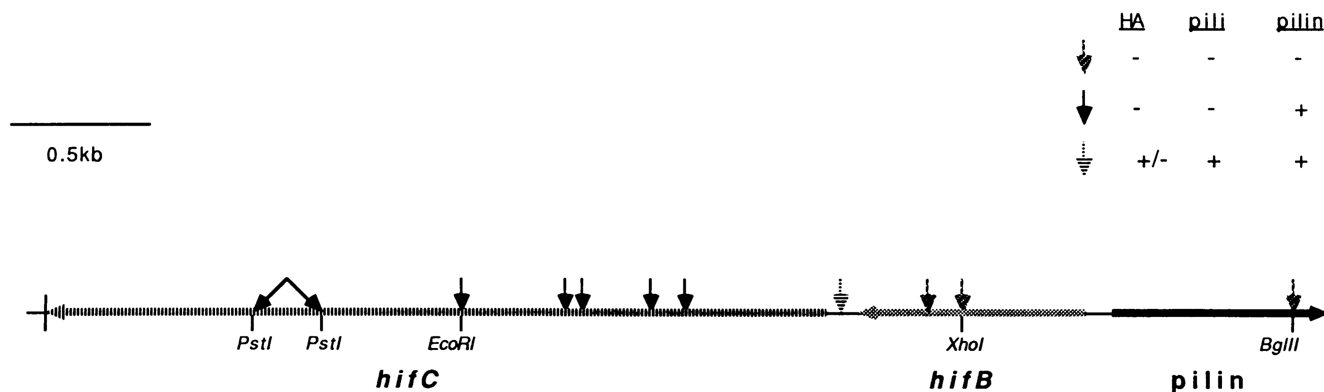


FIG. 3. Map of insertional mutations. This map demonstrates the location and orientation of the two open reading frames located in the region upstream of the pilin gene. *hifB* has been described previously. The sites of mγδ-1 and Km$^r$ mutations and the resulting phenotypes of the Hib mutants are marked with arrows. Hatched arrows mark mutations that produce Hib mutants that no longer hemagglutinate (HA) or react with antiserum directed against pili or pilin antigens; solid arrows mark mutants that no longer hemagglutinate or produce pili but do react with antipilin antiserum; and the striped arrow marks a mutant that reacts with pili and pilin-specific antiserum but hemagglutinates at a significantly lower titer than piliated Hib controls.

FIG. 4. Nucleotide sequence of Hib gene *hifC*, with the predicted amino acid sequence. The start codon (GTG), the potential Shine-Dalgarno (S.D.) site of *hifC*, and the termination (Ter) codons of *hifB* and *hifC* are underlined. The *Eco*RI and *Pst*I cleavage sites are also indicated.

TAA stop codon. A sequence (GAGAA) with homology to an *E. coli* ribosome-binding site was found 9 bp upstream of the initiation codon; this sequence has been found previously in *Haemophilus* transformation genes (ORF F) by Tomb et al. (33). The open reading frame is read on the same strand as *hifB*, and therefore it is oriented in the opposite direction from the pilin structural gene. This open reading frame has been designated *hifC*.

The predicted amino acid sequence of the *hifC* gene product (Fig. 5) has 30% homology to MrkC (1) and FhaA (25), the platform assembly proteins of *Klebsiella pneumoniae* and *Bordetella pertussis*, respectively. Additionally, there is homology with *E. coli* PapC (28%) (27) and FimD (29%) (20) and the *Salmonella enteritidis* protein SefC (24%) (6). The *hifC* open reading frame has GTG as its initiating codon. Interestingly, a putative platform assembly gene in *Salmonella typhimurium* (28) with modest homology to *hifC* is also initiated by GTG. The sequence contains a likely peptide signal sequence, with the predicted cut site at the tryptophan-alanine-aspartate residues, based on data from von Heijne (37). The predicted protein contains two cysteine residues, located 27 amino acids apart, near the N terminus and two cysteine residues at the C terminus; this arrangement has been identified in all pilus assembly platform protein sequences to date (25).

**Southern analysis of Hib strains.** In order to define the prevalence of *hifC* in strains of *H. influenzae*, Southern analysis

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Predicted Amino Acid sequence of HifC-                    MKTKNFP
Amino Acid sequence of MrkC-                         MKQRSICPGRLSTAI

                  ↓
LNKIAFACTLLLANPVAWAEDQFDASLWGGGSVLGIDFARFNVKNAVLPGRYEAQIYVNNEEK
AVALCCFPPFSSGQESPGTIYQFNDGFIVGSRE-KVDPSRFST-SAISEGVYSLDVYTNGEWK

GESDIIFADNPATGRAELCFTPKLQEMLDLMDEAIVKSPNSEDDTCVFASDAIPKGTF--DYQ
GRYDLKITAGKD-GKMGVCYTKAMLMQYGISPEKLNPQLSEKEGFCGRLQEWRHEDNVKDTLI

GGDMKLKLEIPQALTIRRPRGYIAPSRWQTGTNA---AFANYDINYYRSGNPEVKSESLYVGL
QSSLRLDIAVPQIYEDQRLKNFVSPQFWDKGVAALNLGWMANAWNSHISSANGSDNSSAYLGV

RGGVNFGNWALRHNGSFSRFENQSSSGFTDKGKNHYERGDTYLQRDFALLRGNVTVGDFFSTA
NAGLSWDGWLLKHIGNLNW--------QQQQGKAHWNSNQTYLQRPIPQINSIVSGGQIFTNG

RIGENFGLRGLRIASDDRMLAPSQRGFAPVVRGVANTNAKVSIKQNGYTIYQITVPAGPFVIN
EFFDTIGLRGVNLATDDNMFPDGMRSYAPEIRDVAQSNALATVRQGSNIIYQTTVPPGPFTLQ

DLYASGYSGDLTVEIQESDGKVRSFIVPFSNLAPLMRVGHLRYQLAGGRYRIDSRTFDERVLQ
DVYPSGYGSDLEVEVKEGDGSVEVFSVPYASVAQLLRPGMTRYALSAGKVDDSSLRNKPMLYQ

GVLQYGLTNHLTLNSSLLYTRHYRAGLFGFGLNTPIGAFSADATWSHAEFPLKKVSKNGYSLH
GTWQHGLNNLFTGYTGVTGFDDYQAFLLGTGMNTGIGALSFDVT--HTRLKSDTLDEHGQSYR

GSYSINFNEIGTNLTLAAYRYSSRDFYTLSDTIGLNRTFRQFSGAYLPEIYRPKNQFQVSLSQ
ATFNRMFTETQTSIVLAAYRYSTKGYYNLNDALYAVDQEKNYNSNY--TVWRQKNGMTFTVNQ

SL-GNWGNLYLSGQTYNYWEKRGTNTQYQVAYSNSFHILNYSVNLSQ--SIDKETGKRDNSIY
NLPDGWGGFVYCGRVADYWNRSGTEKQYQFSYNNMYGRLSWSVDAQRVYTPDSSGHRRDDRVS

LSLSLPL--GDNHSA----DSSYSRSGNDINQRLGVNGSFGERHQWSYGINASRNNQGYRSYD
LNFSYPLWFGENRTANLTSNTAFNNSRFASSQ-IGVNGSLDSENNLNYGVSTTTATGRQHDVA

GNLSHNNSIGSYRASYSRDSLKNRSTSLGVSGAVVAHKYGITLSQPVGESFALIHAKDAAGAK
LNGSYRTPWTTLNGSYSGQE-GYRQSGVGASGTLIAHQHGVVFSPETGPTMALIEAKDAAGVM

VESGANVSLDYFGNAVVPYTSPYEINYIGINPSDAEANVEFEATERQIIPRANSISLVDFRTG
LPGSPGTRIDDSNGYAILPYLRPYRINSVEIDPKGSNDDVAFGSTVAQVVPWEGSVVKVSFDFT

KNTMVLFNLTLPNGEPVPMASTAQDSEGAFVGDVVQGGVLFANKLTQPKGELIVKWGERESEQC
LQNNITLRARQANGLPLPFAATIFGPSGKEIGVVGQGSMMFISDASAPKAT--VKWSGGQCSVE

RFHYQVDLDNAQIQNHDIQCKTAE
LSQEKTKETLCR
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FIG. 5. Predicted amino acid sequence of HifC and comparison with the MrkC amino acid sequence of the type 3 pilus of *K. pneumoniae*. The potential signal peptide cleavage site is marked with an arrow. Identical amino acids are shaded.

was performed with an intragenic *hifC* probe to hybridize with chromosomal DNA from a variety of isolates, both type b and nontypeable. The results (Fig. 6) demonstrate that a region of DNA homologous to *hifC* is present in all the Hib strains tested; however, the region is present only in some of the nontypeable strains.

## DISCUSSION

In this article, we describe *hifC*, a gene essential to Hib pilus expression with functional and sequence similarity to the pilus assembly platform protein genes of other gram-negative bacilli. The most extensively studied pilus assembly platform protein is PapC. PapC is a large outer membrane-associated protein that plays a role in extracellular transport of the growing pilus structure of P pili. *papC* and *hifC* mutants produce the pilin structural subunit but no longer assemble pili (27; this study). A recent review (19) suggests that PapC and analogous proteins be referred to as ushers, as they appear to direct the ordered interaction between the various pilin subunits that is essential for native pilus formation in addition to their role in facilitating extracellular translocation.

Genes encoding pilus assembly platform proteins have been identified in a variety of gram-negative bacteria. We have compared the amino acid sequence of *hifC* with the products of the pilus assembly platform genes from *E. coli*, *K. pneumoniae*, *S. typhimurium*, *S. enteritidis*, and *B. pertussis*. HifC seems to be most similar to MrkC of *K. pneumoniae.*

Among Hib strains, the prevalence of a region homologous to *hifC* appears to be quite high, as all the strains tested had areas that hybridized to the intragenic *hifC* probe. The variable results obtained with the nontypeable strains may reflect the heterogeneity of the group as a whole. Interestingly, the state of piliation did not correlate with the hybridizing patterns of the type b or nontypeable strains. The identification of a pilus assembly platform gene in Hib further expands the list of organisms that may assemble native pili by a similar mechanism.

Recently, Smith et al. (17, 31) identified a pilin chaperone protein gene, *hifB*, in Hib strain Eagan. This gene is located in

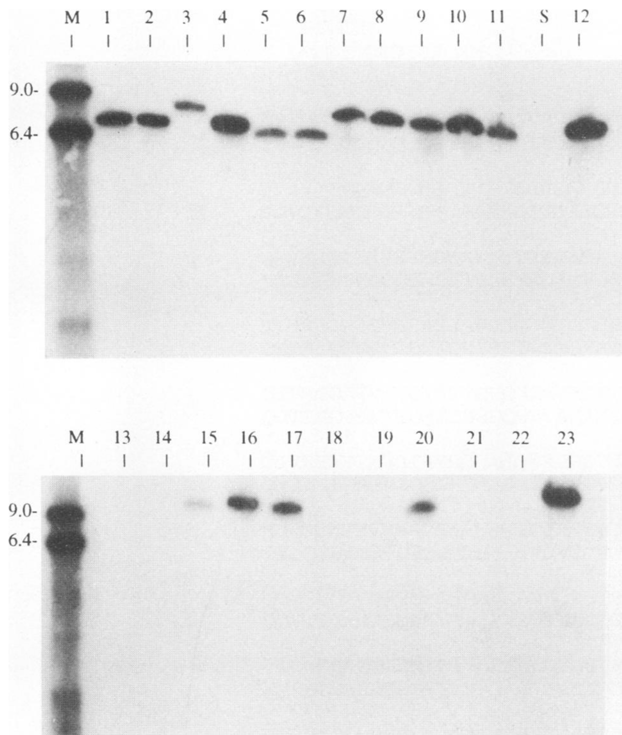

FIG. 6. Southern analysis of Hib and nontypeable *H. influenzae* strains. This blot contains *Sna*BI-digested genomic DNA from a variety of *H. influenzae* strains described previously (8, 11, 14, 32). The DNA probe was derived from the intragenic region of *hifC*. (Top) Laboratory strains and invasive clinical isolates of Hib. (Bottom) Clinical isolates of nontypeable strains. Lanes M, marker DNA. (Top) Lanes: 1, E1a$^+$; 2, nonpiliated variant of Hib strain Eagan; 3, M43; 4, AAr9; 5, AAr13; 6, AAr61; 7, AAr106; 8, AAr108; 9, AAr119; 10, AAr147; 11, DL42; S, space; 12, C54. (Bottom) Lanes: 13, Mr24; 14, Mr31; 15, AAr39; 16, AAr45; 17, AAr 73; 18, AAr91; 19, AAr169; 20, AAr176; 21, AAr 180; 22, LB1; 23, LB2. Sizes are shown in kilobases.

the region immediately upstream of the pilin structural gene. We found that Hib strains with mutations in this gene do not react with antipilin antiserum, presumably because of rapid degradation of the unprotected pilin protein. This is consistent with the results of pulse-chase experiments done with *papD* mutants of *E. coli*, which lack the pilin chaperone protein associated with P pili. These mutants exhibit delayed processing and rapid degradation of the mature pilin subunit (27). The identification of two Hib pilus assembly genes, *hifC* and *hifB*, in the region adjacent to the pilin structural gene suggests that the genes of Hib pilus biogenesis are organized in a cluster, similar to the type 1 and P pili clusters of *E. coli*. However, certain differences are seen in the organization of these clusters and of the Hib pilus biogenesis gene cluster. One difference is that the Hib strain Eagan structural pilin gene is transcribed in the opposite direction from that proposed for the chaperone (31) and assembly platform (this study) genes. Recently, van Ham et al. described the divergent transcription of *hifB* and *hifA* in Hib strains A860268 and 770235 (36).

Our findings provide evidence that a third gene in the Hib strain Eagan pilin gene cluster, *hifC*, conforms to the orientation of the other known Hib pilus accessory gene, *hifB*. A similar genetic configuration of the orientation of the pilin and *hifC* genes has also been found in Hib strain M43 (data not shown). The genes present in the pilus biogenesis clusters of other gram-negative bacilli are transcribed as single or multiple transcripts (9, 21). The transcriptional start site for *hifC* has not been identified at this time. We did identify one mutant with an insertional mutation in the intergenic area between *hifB* and *hifC* that did produce pili; however, its ability to hemagglutinate was significantly lower than that of the piliated controls. This suggests that the platform assembly protein was being transcribed in this mutant, but an alteration of the quality or quantity of the transcript may have resulted in a decrease in the hemagglutination properties. This could be the result of incomplete disruption of the native promoter or the introduction of an artificial promoter site created by the insertion mutation itself (4).

Another point of interest is that the type 1 and P pilus gene clusters of *E. coli* have areas immediately preceding the major pilin subunit gene that contain genetic elements known to play a role in pilus regulation. No comparable region exists immediately upstream of the Hib pilin gene, as this region contains *hifB* and *hifC*. Although this area is involved in phase variation (36), no genes actively involved in specific regulation of expression have been identified. Preliminary analysis of the DNA region downstream of the Hib pilin gene reveals the presence of two open reading frames with sequence homology to the *purE* and *purK* housekeeping genes of *E. coli* (37a). The significance of the pattern of genes in the Hib cluster and the characterization of the specific regulatory elements involved in Hib pilus expression remain open for study.

In the study presented here, we have identified a gene linked to the pilin structural gene in Hib strains that has sequence homology and functional similarity to the pilus assembly platform genes of other gram-negative bacteria. Taken together with the finding of a chaperone gene immediately upstream of this gene, the genes for Hib pilus synthesis appear to be organized in a gene cluster. Work is currently in progress to identify additional genes involved in Hib pilus biogenesis.

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