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Identification of Two Minor Subunits in the Pilus of *Haemophilus influenzae*

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Haemophilus influenzae **type b (Hib) organisms produce pili, which mediate attachment to human cells and are multimeric structures composed of a 24-kDa subunit called pilin or HifA. Although pili from other organisms contain additional proteins accessory to pilin, no structural components other than pilin have been identified in Hib pili. Previous analysis of a Hib pilus gene cluster, however, suggested that two genes, *hifD* and *hifE*, may encode additional pilus subunits. To determine if *hifD* and *hifE* encode pilus components, the genes were overexpressed in *Escherichia coli* and the resulting proteins were purified and used to raise polyclonal antisera. Antisera raised against C-terminal HifD and HifE fragments reacted with *H. influenzae* HifD and HifE proteins, respectively, on Western immunoblots. Western immunoblot analysis of immunoprecipitated Hib pili demonstrated that HifD and HifE copurified with pili. In enzyme-linked immunosorbent assays, antisera raised against a recombinant HifE protein that contained most of the mature protein reacted more to piliated Hib than to nonpiliated Hib or to a mutant containing a *hifE* gene insertion. Immunoelectron microscopy confirmed that the HifE antiserum bound to pili and demonstrated that the antiserum bound predominantly to the pilus tips. These data indicate that HifD and HifE are pilus subunits. Adherence inhibition studies demonstrated that the HifE antiserum completely blocked pilus-mediated hemagglutination, suggesting that HifE mediates pilus adherence.**

The pilus of *Haemophilus influenzae*, composed of multimers of a 24-kDa protein called pilin, mediates adherence of the organism to human cells in vitro (8, 20). We have previously defined a gene cluster responsible for pilus expression in *Haemophilus influenzae* type b (Hib) Eagan (E1a) (5, 16, 25). The cluster contains five genes required for pilus expression (Fig. 1A). *hifA* encodes pilin, *hifB* and *hifC* encode putative periplasmic chaperone and outer membrane usher-like proteins, respectively, and *hifD* and *hifE* encode putative pilus structural proteins.

The proposed functions of the *hif* genes are largely based on amino acid sequence homologies to a number of *Escherichia coli* pilus components, and *E. coli* pili have therefore been useful models for the study of Hib pili. *E. coli* pili require a number of structural proteins for pilus biogenesis and adherence (18). For example, four pilus subunits form an adhesive fibrillar tip structure at the distal ends of Pap pili (11). Two minor subunits of *E. coli* S pili combine to initiate pilus formation, and one of these molecules is the adhesin (19). In addition, minor pilus components also function as adhesins in the type 1 (1) and F17 (14) pili of *E. coli*. No pilus structural components other than pilin, however, have been identified in *H. influenzae* pili.

hifD and *hifE* have been proposed to encode structural components of Hib pili (16). The deduced amino acid sequences of the *hifD* and *hifE* gene products have homology to each other and to pilin (HifA), the major pilus structural component. In addition, Hib mutants containing insertionally inactivated *hifD* or *hifE* genes have reduced pilus expression, and these strains are no longer able to mediate adherence (16). These results indicate that the *hifD* and *hifE* gene products are required for the expression of functional pili and suggest that they are pilus structural components. Identification and characterization of the *hifD* and *hifE* gene products should therefore provide additional understanding of *H. influenzae* pilus structure and adherence.

The objective of this study was to determine if *H. influenzae* pili contain HifD and HifE. Polyclonal antisera raised against the cloned *hifD* and *hifE* gene products overexpressed in, and purified from, *E. coli* were used to demonstrate that HifD and HifE are structural components of *H. influenzae* pili. In addition, we found that antibodies reactive to the native HifE subunit inhibited pilus adherence, suggesting that HifE is a pilus adhesin.

MATERIALS AND METHODS

Bacterial strains and plasmids. All Hib strains and mutants were grown on Levinthal agar (17) containing brain heart infusion broth, Bacto Agar (Difco Laboratories, Detroit, Mich.), a horse erythrocyte lysate, and NAD. The creation of the E1a *hifA* kanamycin resistance (Kan^r) cassette insertion mutant (designated E101 in this study) and the *hifD* and *hifE* mγδ transposon mutants (E114 and E113, respectively) has been described previously (7, 16). *E. coli* DH5α was used as a host strain for genetic manipulations and was grown in Luria broth (Difco) containing 100 μg of carbenicillin per ml. For fusion protein overexpression and purification, DH5α was grown in enrichment medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl, 0.2% [wt/vol] glucose) containing 100 μg of carbenicillin per ml. All bacteria were grown at 37°C.

Recombinant proteins were purified from *E. coli* containing the *hifD* or *hifE* gene cloned into the pMAL-cRI expression vector (New England Biolabs, Inc., Beverly, Mass.). Various lengths of the *hifD* or *hifE* gene (Fig. 1B) were placed downstream of, and in frame with, the vector *malE* gene (*malE* encodes maltose-binding protein [MBP] and is under the control of an inducible *tac* promoter). Three such subclones, pMED2, pMEE1, and pMEE2, were made (described below), and DNA sequence analysis was used to confirm that each construct contained an in-frame *malE-hif* gene fusion.

$pMED_2$ (Fig. 1B) contains a *malE-hifD* gene fusion that encodes 42.9% of the predicted mature HifD protein, and $pMEE_1$ and $pMEE_2$ (Fig. 1B) contain *malE-hifE* gene fusions that encode 99.5 and 38.6%, respectively, of the pre-

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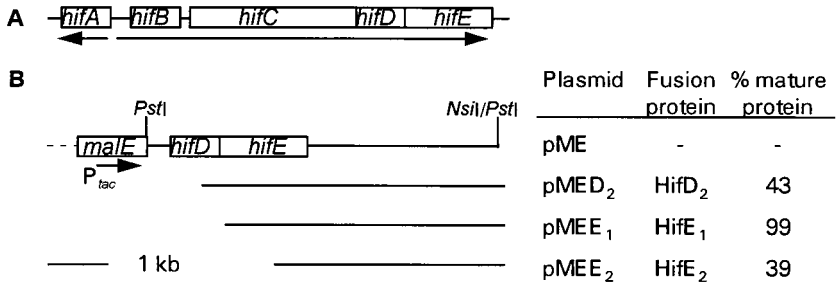

FIG. 1. Physical map of the *hif* gene cluster and genetic maps of the pMAL-*hif* gene plasmid constructs used in this study. (A) *hif* gene cluster, with transcriptional activity noted by arrows. (B) Genetic maps of each pMAL-*hif* construct. The name and percentage of mature Hif protein expressed by each construct are also indicated. pME contains the pMAL-cRI expression vector (partially shown by a dotted line) with an inducible *malE* gene (encoding MBP) and a *Pst*I/*Nsi*I *hif* DNA insert originating from pWW4 (16) (not shown). $pMED_2$, $pMEE_1$, and $pMEE_2$ are all unidirectional exonuclease deletions derived from pME. The length of insert DNA within each construct is represented by a single line beneath the appropriate gene(s).

dicted mature HifE protein. These three constructs were obtained by generating unidirectional exonuclease deletion derivatives (Erase-a-Base System; Promega, Madison, Wis.) from a common pMAL-cRI subclone containing the 3′ end of the *hifC* gene, the *hifD* gene, the *hifE* gene, and DNA downstream of the *hifE* gene (pME) (Fig. 1B). The insert DNA of pME was obtained from a previously described *hif* gene clone, pWW4 (16, 25), as follows: pWW4, which contains the entire *hif* gene cluster, was digested with *Pst*I to remove most of the DNA 5′ of *hifD*. The vector and remaining DNA insert were then self-ligated. The insert of this construct (not shown) was excised with *Pst*I and *Nsi*I and ligated into pMAL-cRI cut with *Pst*I. A clone (pME) that contained the *hifD* and *hifE* genes in the correct orientation was selected. The DNA downstream of *hifE* encodes the 5′ end of a gene with homology to an *E. coli* housekeeping gene called *pepN*, and mutations in this gene do not effect pilus expression in *H. influenzae* (16).

Production of $HifD_2$, $HifE_1$, and $HifE_2$ antisera. Recombinant *E. coli* containing $pMED_2$, $pMEE_1$, or $pMEE_2$ was used to generate the $HifD_2$, $HifE_1$, or $HifE_2$ protein, respectively (Fig. 1B). Overexpression and purification of each protein were performed according to the New England Biolabs protocol. Briefly, recombinant *E. coli* was grown and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and the cells were harvested and frozen overnight at −20°C. After thawing, the cells were sonicated and centrifuged. The supernatant was diluted and applied to an amylose resin column. The proteins were eluted with maltose-containing buffer, and the eluate was concentrated in a Centriprep-30 or -50 concentrator (Amicon, Inc., Beverly, Mass.).

Each of the purified fusion proteins was subjected to 1% factor Xa protease overnight in order to cleave the maltose-binding domain from the recombinant protein. Each protein ($HifD_2$, $HifE_1$, and $HifE_2$) (0.5 mg) was emulsified in 1 ml of Freund's complete adjuvant (Pierce, Rockford, Ill.) and administered subcutaneously to New Zealand White rabbits (Hazelton Research Products, Denver, Pa.) at four to five sites. Booster immunizations were given three times at 3-week intervals with Freund's incomplete adjuvant.

Antiserum absorptions. The $HifD_2$, $HifE_1$, and $HifE_2$ antisera used in the immunologic assays described below were extensively preabsorbed with an insoluble fraction of IPTG-induced *E. coli* containing the pMAL-cRI vector to remove nonspecific reactivity. In addition, control experiments used $HifE_1$ antiserum that was absorbed with a fraction of *E. coli* containing $pMEE_1$ (which expresses the $HifE_1$ fusion protein) similar to that used with the pMAL-cRI vector. Briefly, insoluble fractions were obtained from 1-liter cultures of recombinant *E. coli* following IPTG induction, sonication, and centrifugation. The pellets were resuspended in 5 ml of phosphate-buffered saline (PBS), and 300-μl aliquots were pelleted in microcentrifuge tubes. Each pellet was resuspended in 1 ml of antiserum and mixed for 1 h at room temperature. Following centrifugation, the antiserum was removed and absorbed three to five more times.

Western immunoblot analysis. All Western immunoblots used in this study contained proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes as previously described (6). Resolved proteins from whole-cell lysates of E1a test strains were reacted with antisera diluted 1:200.

Immunoprecipitation of pili. Immunoprecipitation of piliated E1a ($E1ap^+$) or nonpiliated E1a ($E1ap^-$) was performed by incubating 0.5 ml of a dense suspension of bacteria (A_{560} = 0.523) in PBS with antiserum directed against purified E1a pili (purified pilus antiserum) (6) (Table 1) at a dilution of 1:10 for 1 h. The bacteria were washed once, resuspended in 1 ml of PBS, and sonicated for 30 s on ice. The sonicated bacteria were centrifuged, and the supernatant (crude soluble fraction) was incubated with protein A-conjugated agarose beads (Sigma Chemical Co.) (40 μl of a 25% [wt/vol] PBS suspension) for 1 h. The beads were washed twice with 1 ml of PBS, and the immunopurified proteins were eluted by SDS-PAGE. The eluted proteins were analyzed on a Western immunoblot with combined $HifD_2$-, $HifE_2$-, and pilin (HifA)-specific antisera (5) (Table 1), each diluted 1:200. Purity of the immunoprecipitated pili was demonstrated by Western immunoblot analysis with an antiserum from a conventionally housed rabbit which had previously been shown to react nonspecifically with *H. influenzae* (3) (Table 1).

ELISA. Enzyme-linked immunosorbent assays (ELISA) were done as previously described (4). Various HifE antisera were diluted 1:500 in PBS containing 0.2% gelatin as a blocking agent. Each assay was done in duplicate or triplicate.

Immunoelectron microscopy. Immunoelectron microscopy was performed by incubating 50 μl of $E1ap^+$ suspended in PBS (A_{560} = 0.300) with $HifE_1$ or purified-pilus antiserum diluted 1:50 for 15 min. The bacteria were centrifuged, resuspended in 50 μl of PBS containing rabbit immunoglobulin G-conjugated 10-nm colloidal gold (final dilution, 1:25; Sigma Chemical Co.), and incubated for 15 min. Following centrifugation, the bacteria were resuspended in 50 μl of PBS and 10 μl of the suspension was applied to Formvar, carbon-coated specimen grids (Electron Microscopy Sciences, Fort Washington, Pa.) for 2 min. The grids were stained with two drops of 0.5% phosphotungstic acid (pH 4.0), air dried, and examined with a Zeiss model EM 10CR electron microscope.

Hemagglutination inhibition assays. The hemagglutination inhibition assay was performed similarly to a previously described hemagglutination assay (15) except that suspensions of $E1ap^+$ were preincubated with 1:10 dilutions of various $HifE_1$ antisera or purified-pilus antiserum for 1 h at room temperature. Following this incubation, the bacteria were centrifuged, resuspended in the original volume of PBS, and used in the hemagglutination assays in twofold serial dilutions.

RESULTS

Detection of HifD and HifE by Western immunoblot. Previous work from our laboratory demonstrated that the *hifD* and *hifE* genes are part of the *H. influenzae* pilus gene cluster and

TABLE 1. Descriptions of the antisera used in this study

Antiserum	Immunogen	Specificity	Reference
$HifD_2$	$HifD_2$ (43% of mature HifD)[a]	Denatured HifD	This study
$HifE_2$	$HifE_2$ (39% of mature HifE)[a]	Denatured HifE	This study
$HifE_1$	$HifE_1$ (99% of mature HifE)[a]	Native HifE	This study
Purified pilus	E1a purified pili	E1a pili	6
Pilin (HifA)	Pilin band cut from an SDS-polyacrylamide gel	Denatured pili (pilin)	5
Nonspecific	None (serum from a conventionally housed rabbit)	Numerous *H. influenzae* antigens	3

[a] Protein deletion is at the N terminus.

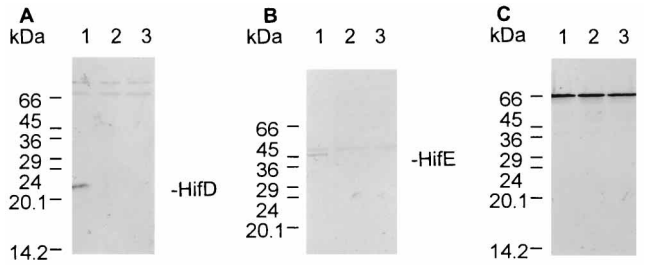

FIG. 2. Reactivities of HifD and HifE antisera to E1a strains on Western immunoblots. (A) $HifD_2$ antiserum reacted to $E1ap^+$ (lane 1), $E1ap^-$ (lane 2), and a *hifD* insertion mutant, E114 (lane 3). HifD marks the 22-kDa band present in lane 1. (B) $HifE_2$ antiserum reacted to $E1ap^+$ (lane 1), $E1ap^-$ (lane 2), and a *hifE* insertion mutant, E113 (lane 3). HifE marks the 45-kDa band present in lane 1. (C) $HifE_1$ antiserum reacted to the same strains as in panel B. Molecular mass markers are shown on the left of each panel.

are required for pilus expression (16). No *hifD* or *hifE* gene product, however, has been identified in *H. influenzae*.

To determine if the *hifD* and *hifE* genes are expressed in *H. influenzae*, we tested antisera raised against a recombinant MBP-HifD fusion protein ($HifD_2$) and two different MBP-HifE fusion proteins ($HifE_1$ and $HifE_2$) (Materials and Methods; Table 1) on Western immunoblots containing *H. influenzae* $E1ap^+$, $E1ap^-$, and E1a insertion mutants. Antiserum directed against $HifD_2$ (Table 1) reacted with a 22-kDa band in whole-cell lysates of $E1ap^+$ (Fig. 2A, lane 1). The molecular mass of 22 kDa approximates the 20.6-kDa mass predicted for the mature *hifD* gene product (16). The 22-kDa band was not present in $E1ap^-$ (Fig. 2A, lane 2) or E114 (a *hifD* insertion mutant) (lane 3). The $HifD_2$ antiserum did, however, react with a 22-kDa band in E101 (a *hifA* insertion mutant), demonstrating that the antiserum was not cross-reacting with the 23.5-kDa pilin (HifA) protein (data not shown). Antiserum directed against $HifE_2$ (Table 1) reacted with a 45-kDa band in $E1ap^+$ (Fig. 2B, lane 1) that was not present in $E1ap^-$ (lane 2) or E113 (a *hifE* insertion mutant) (lane 3). The 45-kDa band has a molecular mass similar to the mass predicted for the mature *hifE* gene product (45.5 kDa) (16). These data suggest that the *hifD* and *hifE* genes are expressed in *H. influenzae* $E1ap^+$. $HifE_1$ antiserum did not react differently among $E1ap^+$, $E1ap^-$, and a *hifE* insertion mutant (E113) (Fig. 2C, lanes 1 to 3), indicating that this antiserum does not recognize the denatured HifE subunit on Western immunoblots.

In spite of extensive preabsorption of the antisera with lysates of *E. coli* containing the pMAL-cRI vector to remove nonspecific antibodies, each antiserum reacted with one or more bands (having molecular masses greater than 45 kDa) common to $E1ap^+$, $E1ap^-$, and the HifD (E114) or HifE (E113) insertion mutants. These bands were also present with each preimmune antiserum (data not shown) and are therefore considered to be nonspecific.

Detection of HifD and HifE in immunoprecipitated pili. Previous studies suggested that HifD and HifE may be integral components of the pilus. These proteins would therefore be expected to copurify with pili. Analysis of purified pilus preparations, however, have never detected HifD or HifE (2, 9, 22, 23). This may have been due to relatively few HifD and HifE subunits within the pilus structure, making them difficult to detect, or to loss of the proteins during harsh purification procedures. We therefore used the sensitive $HifD_2$ and $HifE_2$ antisera to determine if the subunits could be detected in pili that had been purified by a nondenaturing immunoprecipitation method.

The pili of $E1ap^+$ were immunoprecipitated with antiserum directed against purified E1a pili and analyzed on Western immunoblots with antisera against pilin (HifA), $HifD_2$, and $HifE_2$. The immunoblot in Fig. 3A demonstrates that HifA, HifD, and HifE were present both in the crude $E1ap^+$ soluble fraction obtained from lysates of $E1ap^+$ incubated with purified pilus antiserum (lane 1) and in the $E1ap^+$ immunoprecipitate obtained from incubating the crude soluble fraction with protein A-agarose beads (lane 3). The absence of these bands in an $E1ap^-$ soluble fraction (lane 2) and immunoprecipitate (lane 4) demonstrates that the reactive bands did not originate from the purified-pilus antiserum or protein A beads. Pili were extensively purified by immunoprecipitation as demonstrated by the ability of a nonspecific *H. influenzae* antiserum (Table 1) to react with numerous bands in the crude soluble fraction of $E1ap^+$ (Fig. 3B, lane 1) that were absent or greatly diminished in the immunoprecipitate (Fig. 3B, lane 2). The immunoprecipitation experiments reveal that HifD and HifE copurify with the major structural subunit in pili, HifA, and suggest that these proteins are pilus structural components.

Detection of HifE on native pili. To further demonstrate that HifE is a pilus subunit, we investigated the ability of an antiserum raised against the recombinant $HifE_1$ protein to bind to piliated *H. influenzae*.

In whole-cell ELISA, the $HifE_1$ antiserum reacted more to $E1ap^+$ than to $E1ap^-$ or a *hifE* insertion mutant (E113) (Fig. 4A). $HifE_1$ antiserum absorbed with the $HifE_1$ fusion protein (Fig. 4B), and $HifE_1$ preimmune antiserum (not shown) reacted similarly to $E1ap^+$ and $E1ap^-$. These results suggest that the $HifE_1$ immune antiserum binds to pili.

Immunoelectron microscopy was used to confirm that the $HifE_1$ antiserum binds specifically to pili. The electron micrograph in Fig. 5A shows that antibodies in the $HifE_1$ antiserum bound to pili on $E1ap^+$ and that antibodies in the antiserum bound predominantly to the pilus tips. In contrast, antibodies in the purified-pilus antiserum agglutinated pili and reacted along the entire length of the pilus structure (Fig. 5B). These results indicate that the $HifE_1$ antiserum binds to pili, providing additional evidence that HifE is a pilus structural component that is localized predominantly at the pilus tip.

Inhibition of hemagglutination by the $HifE_1$ antiserum. Since *H. influenzae* pili are genetically similar to various *E. coli* pili that employ minor pilus components to adhere to host

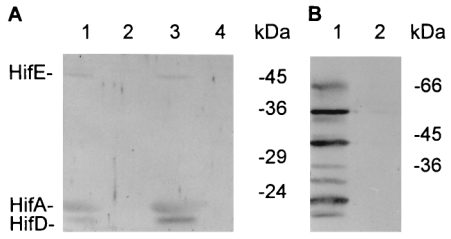

FIG. 3. Western immunoblot analysis of pili immunoprecipitated from $E1ap^+$. (A) An immunoblot was reacted with pooled pilin (HifA), $HifD_2$, and $HifE_2$ antisera to demonstrate the presence of all three subunits in the immunoprecipitate. Lanes 1 and 2 contain soluble fractions of $E1ap^+$ and $E1ap^-$, respectively, that were obtained by incubating bacteria with purified-pilus antiserum and then sonicating and centrifuging them. Lanes 3 and 4 contain the immunoprecipitates obtained from $E1ap^+$ and $E1ap^-$ soluble fractions, respectively. The name of each reactive band is indicated at the left of the figure. (B) A nonspecific *H. influenzae* antiserum was reacted to the $E1ap^+$ soluble fraction (lane 1) and immunoprecipitate (lane 2) to demonstrate the extent of purification of the immunoprecipitate. Molecular mass markers for both Western blots are shown to the right of each blot.

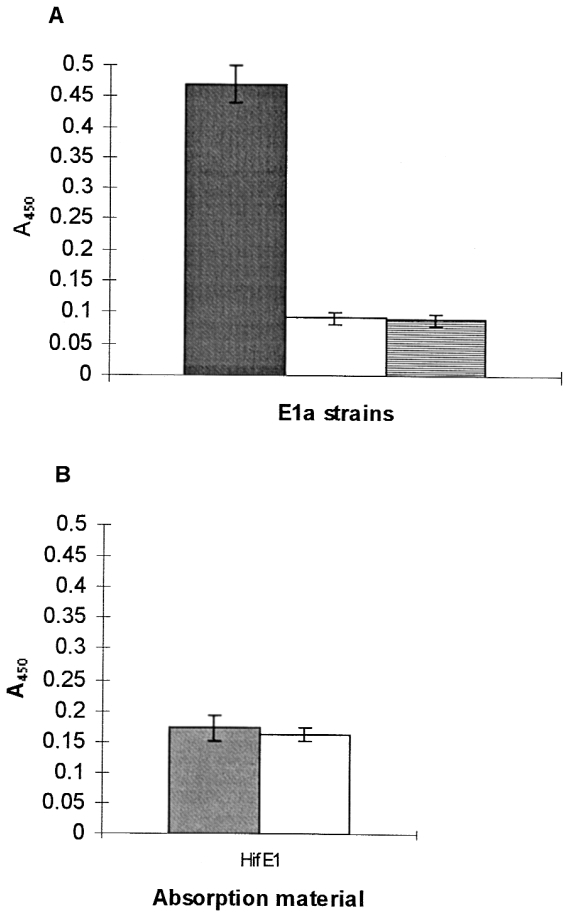

FIG. 4. Reactivities of $HifE_1$ antiserum to various E1a strains. (A) $E1ap^+$ (solid bar), $E1ap^-$ (open bar), and a *hifE* insertion mutant (E113) (hatched bar) reacted with $HifE_1$ antiserum. (B) Reactivities of the $HifE_1$ antiserum to $E1ap^+$ and $E1ap^-$ when the serum was absorbed with the $HifE_1$ fusion protein. Each strain of bacteria was used to coat eight microtiter wells. The error bars represent the standard deviations for the data sets.

cells, we hypothesized that the *H. influenzae* pilus subunit HifE mediates adherence (16).

To determine if HifE can bind to human cells, we attempted to inhibit pilus-mediated hemagglutination by preincubating $E1ap^+$ with various $HifE_1$ antisera. Preincubation of $E1ap^+$ with the $HifE_1$ preimmune antiserum did not inhibit hemagglutination; the titer (1:16) was the same as that of nontreated $E1ap^+$. The $HifE_1$ immune antiserum, however, inhibited hemagglutination below the detection limit (<1:1). In addition, absorption of the antiserum with the $HifE_1$ fusion protein resulted in an increased hemagglutination titer (1:8), suggesting that the antibodies blocking adherence are specific for HifE. In contrast to the ability of the $HifE_1$ antiserum to completely inhibit hemagglutination in this assay, the purified-pilus antiserum reduced the hemagglutination titer of $E1ap^+$ only to 1:2, indicating that pilus adherence was not completely blocked by this antiserum. Taken together, these results suggest that HifE mediates pilus adherence.

DISCUSSION

The pilus structures of various gram-negative bacteria have a number of common features. They are composed of multimers of the major subunit protein that forms the bulk of the pilus shaft. In addition, minor protein subunits contribute to pilus assembly, structure, and adherence and these subunits are localized at the pilus tip and/or along the pilus shaft. Minor subunits can also compose short, thin, fibrillar structures at the distal ends of *E. coli* P, type 1, and S pili (10, 12, 13). The location of HifE at the distal ends of pili may suggest that this subunit resides in a fibrillar tip.

Our data support the hypothesis that HifD and HifE are components of the pilus structure. HifD and HifE copurified with HifA when Hib pili were immunoprecipitated with an antiserum raised against purified pili, suggesting that HifD and HifE are integrally associated with pili. An alternative explanation for this observation is that the purified pilus antiserum contained antibodies directed against HifD or HifE and that these proteins were directly immunoprecipitated instead of copurifying with pili. In this event, however, the precipitating antibodies originated from a humoral immune response to the HifD or HifE proteins contained in the purified pili originally used to make the antiserum, again implicating them as structural components.

The observation that $HifE_1$ antiserum reacts to intact pili but not to denatured pilus subunits on Western immunoblots suggests that the antiserum is specific for conformational pilus epitopes. Conversely, $HifD_2$ and $HifE_2$ antisera recognize their respective subunits on Western blots but do not react differ-

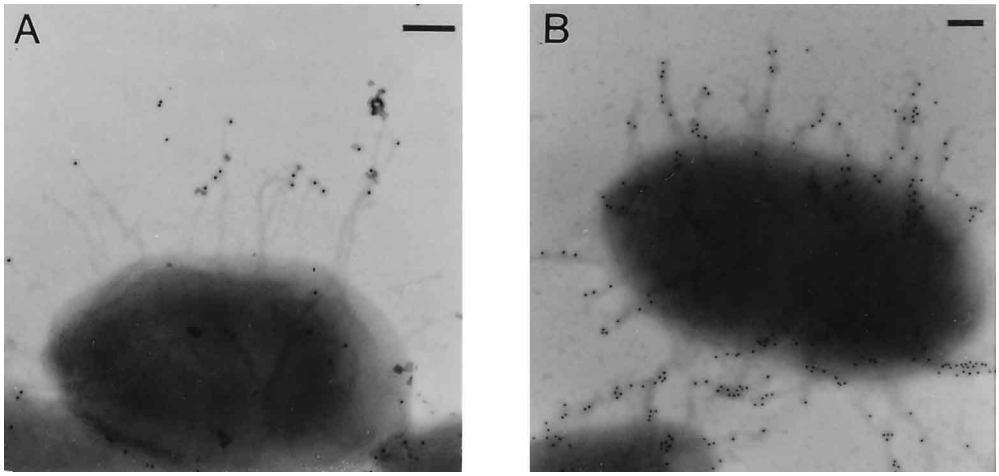

FIG. 5. Immunoelectron microscopy of $E1ap^+$ with $HifE_1$ (A) or purified-pilus (B) antiserum. Bars, 0.2 μm.

ently to E1ap$^+$ and E1ap$^-$ cells in ELISA (unpublished observations), suggesting that these antisera react with linear or denatured epitopes. These results are reminiscent of the results of previous immunologic studies of *H. influenzae* pili (6, 15) in which antibodies specific for native (assembled) pilus epitopes did not react with denatured pilin (HifA) and antiserum specific for denatured pilin did not react with assembled pili.

This study demonstrates that $HifE_1$ antibodies completely block adherence in a hemagglutination assay. One interpretation of this observation is that antibodies bound to HifE sterically interfered with an adhesive domain located on HifA. This is unlikely, however, since antibodies in the purified-pilus antiserum, which bound along the entire pilus structure (composed largely of HifA), did not inhibit hemagglutination to the same extent as antibodies in the HifE antiserum. We believe that the partial inhibition seen with the purified-pilus antiserum resulted from steric interference of HifE binding to its erythrocyte receptor or from decreased pilus valency due to agglutination of pili by antibodies.

van Ham et al. (24) recently proposed that the Hib pilus adhesive site is present on the HifA protein. Our studies, however, demonstrate that antibodies directed against HifE completely inhibited hemagglutination, suggesting that HifE rather than HifA contains an adhesive site. We present one possible explanation for this discrepancy. The conclusions of van Ham et al. were based on the ability of recombinant *E. coli* DH5α, expressing Hib pili that contained only HifA, to mediate moderate adherence to human cells. In this system, HifA was expressed from an entire *hif* gene cluster that contained a Kan^r cassette within the *hifD* gene and this DNA was present in a high-copy-number plasmid. HifA was believed to be the only expressed pilus structural protein, since the *hifD* insertion presumably terminated *hifE* transcription by polar effects. The authors, however, did not rule out the possibility that HifE was expressed at low levels by normal *hif* gene transcription proceeding through the Kan^r cassette or by transcription originating within the Kan^r cassette or from a cryptic promoter located within the insert DNA. If indeed HifE was expressed even at low levels, it may have mediated the adherence that was reported in their study. In contrast, our results are based on the inhibition of adherence of native pili expressed by *H. influenzae*. Additional studies that determine the direct binding potentials of purified HifA, HifD, and HifE are necessary to completely resolve this discrepancy.

In conclusion, we have identified two structural components of Hib pili and provide evidence that one of these components, HifE, mediates pilus adherence.

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ADDENDUM

After submission of the manuscript, an article describing the presence of the HifD subunit in a pilus fibrillar tip structure was published (21).

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