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Comparison of Hemagglutinating Pili of *Haemophilus influenzae* Type b with Similar Structures of Nontypeable *H. influenzae*

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Thirty-eight clinical isolates of nontypeable *Haemophilus influenzae* were tested for the presence of hemagglutinating pili similar to those of *H. influenzae* type b (Hib) that mediate buccal epithelial cell adherence. Four endogenously hemagglutinating (HA^+) strains were identified, and eight additional HA^+ variants were obtained from HA^- strains by erythrocyte enrichment. All 12 HA^+ nontypeable *H. influenzae* isolates bound antisera directed against denatured pilins of Hib, but none bound antisera against assembled native pili of Hib. In erythrocyte- and buccal-cell-binding assays, HA^+ nontypeable *H. influenzae* binding was reduced compared with HA^+ Hib binding and was not significantly different from HA^- nontypeable *H. influenzae* binding. Both HA^- and HA^+ nontypeable *H. influenzae* binding was increased over binding of HA^- Hib. HA^+ nontypeable *H. influenzae* strains agglutinated adult erythrocytes that possess the Anton antigen, which is thought to be the receptor for Hib pili, and did not agglutinate cord or Lu(a^-b^-) dominant erythrocytes, which lack the Anton antigen. Electron microscopy of HA^- and HA^+ variants of three nontypeable *H. influenzae* strains showed few or no surface appendages on the HA^- organisms, but piluslike structures were seen on many organisms from two HA^+ nontypeable *H. influenzae* strains and on a few organisms from one strain. Thus, nontypeable *H. influenzae* appears to possess structures that are immunologically similar to the pilins that make up the hemagglutinating pili of Hib. However, nontypeable *H. influenzae* appears to also possess mechanisms for erythrocyte and buccal cell adherence that are not directly correlated with the presence of a hemagglutinating pilus.

Haemophilus influenzae type b (Hib) possesses filamentous surface proteins called pili that mediate adherence of Hib to respiratory epithelial cells (16, 19) and have been implicated in promoting Hib colonization of the respiratory tract (1). In addition to binding to epithelial cells, these pili also bind to the Anton antigen on human erythrocytes (25). Although many clinical isolates of Hib don't express pili (19), piliated variants can often be selected for by hemagglutination among populations of nonpiliated organisms (6). Expression of pili by Hib appears to undergo phase variation, with the p^- phenotype occurring in about 3×10^{-4} p^+ cells per generation and the p^+ phenotype occurring in about 7×10^{-4} p^- cells per generation (8).

Similar to pili of other bacteria, Hib pili are bacterial surface structures assembled from subunit polypeptides called pilins (18). Previous studies from our laboratory have demonstrated that the immunodominant epitopes on assembled native Hib pili differ in various strains (15). In contrast, the immunodominant epitopes of denatured pilins are conserved on all type b strains and appear to be masked or internalized upon assembly of pilins into pili (15).

Nontypeable (NT) *H. influenzae* differs from type b organisms in several ways. NT *H. influenzae* lacks a polysaccharide capsule, and the outer membrane protein patterns vary more widely than those of Hib (3). Hib causes invasive bacteremic infections and meningitis in normal hosts, whereas bacteremic infections with NT *H. influenzae* organisms usually occur only in immunocompromised patients (11). NT organisms, however, are a prominent cause of localized upper respiratory tract infections such as sinusitis and otitis media. Despite a difference in the diseases caused by these strains, NT *H. influenzae* and Hib occupy a similar ecologic niche, as both types of organisms colonize the respiratory tracts of humans. The molecular mechanisms that promote successful colonization have not been well defined, and the adhesive molecules on Hib and NT *H. influenzae* that bind to respiratory epithelial cell receptors have not been identified. The goal of this study was to determine whether or not NT *H. influenzae* possesses the HA^+ pili of Hib that mediate buccal epithelial cell adherence. To accomplish this, we identified hemagglutinating (HA^+) NT isolates and compared them with HA^+ Hib isolates in terms of erythrocyte and buccal epithelial cell binding characteristics and agglutination, reactivity with antisera specific for pili and pilins of Hib, and presence of pilus structures by electron microscopy.

MATERIALS AND METHODS

Bacteria and identification of hemagglutinating NT *H. influenzae*. Thirty-eight NT *H. influenzae* clinical isolates were obtained from a variety of clinical sources, including specimens from the middle ear, eye, throat, nasopharynx, trachea, and sinuses. The organisms had been retrieved from the clinical diagnostic laboratory after two or three subcultures on chocolate agar and were stored in skim milk at −70°C. Prior to use in the hemagglutination assays, the organisms were thawed and cultured on Levinthal agar (15). The erythrocyte selection technique to identify hemagglutinating (HA^+) variants was performed as previously described (22). Hemagglutination was tested in a semiquantitative microtiter well assay as previously described (22), and positive hemagglutination was defined as a titer of ≥1:8.

Immunologic analyses. The immunologic assays in this study utilized rabbit antisera that have been previously

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described (13, 15). Two of the antisera recognize epitopes on assembled pili; antiserum R1 was made against native pili on the surface of Hib strain M43p$^+$ and adsorbed with its p$^-$ variant (18), and antiserum R19 was made against purified pili of Hib strain Eagan. R1 recognizes pili of many other type b strains but not those of strain Eagan. Similarly, R19 recognizes pili of some other type b strains but not those of strain M43. R1 or R19, or both, recognized all type b strains tested in our previous study (15). Two other antisera recognized epitopes on pilin subunit proteins; antiserum R20 was made against the 24-kDa pilin band of Hib strain M43p$^+$ cut from a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel, and antiserum R13 was made against a 13-amino-acid peptide of Hib strain M43p$^+$ pilin called peptide I. The immunoassays used included an immunodot assay, as previously described (15), in which a suspension of whole bacteria was placed on nitrocellulose membranes and reacted with the various antisera. In addition, Western blot (immunoblot) assays, in which the bacterial proteins of whole bacteria were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and reacted with the various antisera, were performed as previously described (15).

Hib and NT *H. influenzae* binding to erythrocytes and epithelial cells. Enzyme-linked immunoassays were used to quantitate Hib and NT *H. influenzae* binding to human buccal epithelial cells and human erythrocyte ghosts. Buccal epithelial cells from five donors were collected, prepared as previously described (12), and pooled. Erythrocyte ghosts were prepared (7) from erythrocytes of an Anton-positive adult donor, from cord erythrocytes, and from erythrocytes of two individuals with Lu(a$^-$b$^-$) dominant phenotype. Cord and Lu(a$^-$b$^-$) dominant erythrocytes do not express the Anton antigen (25). The erythrocyte ghosts and the epithelial cells were bound to 96-well, U-bottom, polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) by the method of Ofek et al. (20). The unoccupied binding sites on the microtiter wells were blocked with 100 μl of 0.2% gelatin in 0.01 M phosphate-buffered saline (PBS), pH 7.5. The PBS–0.2% gelatin (100 μl) containing 10^8 CFU of HA$^+$ or HA$^-$ Hib or NT *H. influenzae* was added to the microtiter wells. The plates were incubated at room temperature for 1 h, and the wells were washed with PBS containing 0.3% Tween-20 (PBS-T). In order to detect the adherent bacteria, 100 μl of a 1:100 dilution of serum from a rabbit colonized with *Pasteurella multocida*, which contains many antibodies that cross-react with Hib and NT *H. influenzae*, was added to the wells. Preliminary data showed that this serum bound similarly to all Hib and NT strains tested, with a mean optical density of 1.050, a standard deviation of 79, and a coefficient of variation of 7.6%. After 1 h of incubation, the wells were again washed with PBS-T. Goat anti-rabbit immunoglobulin (100 μl) labeled with horseradish peroxidase (Cappel Laboratories, Malvern, Pa.) diluted 1:4,000 in PBS-T was added, and the plates were incubated for 1 h. Following washing with PBS-T, 100 μl of the enzyme substrate (34 μg of *o*-phenylenediamine [Sigma Chemical Co., St. Louis, Mo.] in 100 μl of 0.1 M citrate phosphate buffer [pH 5.01] with 10 μl of 30% H_2O_2) was added to each well. The reaction was allowed to proceed for 15 min in the dark and was then terminated by adding 50 μl of 4 N H_2SO_4. The optical densities of the fluids in the wells were determined at a wavelength of 490 nm on a Dynatech automated enzyme-linked immunosorbent assay (ELISA) spectrophotometer (Dynatech). Control wells were coated with buccal cells or erythrocyte membranes, but no *H. influenzae* was added. Each experimental variable was tested in six duplicate wells, and the mean optical densities were calculated.

TABLE 1. NT *H. influenzae* strains used in this study, their sources, and their hemagglutination capability before and after being subjected to erythrocyte enrichment

Strain	Source	Hemagglutination titer of ≥1:8[a]	
		Before enrichment	After enrichment
Mr13	Middle ear	−	+
Mr31	Middle ear	−	−
AAr14	Eye	−	−
AAr39	Throat	−	+
AAr45	Nasopharynx	−	+
AAr49	Throat	+	ND
AAr73	Nasopharynx	+	ND
AAr91	Throat	−	+
AAr100	Nasopharynx	+	ND
AAr154	Nasopharynx	−	−
AAr157	Nasopharynx	−	−
AAr160	Trachea	−	+
AAr169	Sinus	−	+
AAr176	Nasopharynx	−	+
AAr180	Sputum	−	−
Mr24	Middle ear	−	−
LB1(86-042)	Middle ear	−	−
LB2(1128)	Middle ear	−	−
LB3(287)	Middle ear	−	−
LB4(1590)	Middle ear	−	+
LB5(1712)	Middle ear	+	ND

[a] −, titer of <1:8; +, titer of ≥1:8; ND, enrichment not done.

Buccal epithelial cell slide agglutination was performed by mixing a suspension of piliated or nonpiliated Hib or NT *H. influenzae* at a concentration of 10^9/ml with an equal volume of buccal epithelial cell suspension containing 1,000 buccal epithelial cells per ml on a glass slide. Positive buccal epithelial cell agglutination was defined as the presence of visible clumps.

Electron microscopy. Negative-staining electron microscopy of selected NT organisms (HA$^+$ and HA$^-$ strains Mr13, AAr91, and AAr169) was performed as previously described (2).

Statistics. The optical density values of the enzyme-labeled binding assays were compared by using Student's *t* test and correlation analyses by StatView, Brain Power, Calabasas, Calif.

RESULTS

Identification of hemagglutinating NT *H. influenzae* variants. Of the 38 NT *H. influenzae* clinical isolates, 4 were hemagglutination positive (HA$^+$) and 34 were hemagglutination negative (HA$^-$). Following selection for HA$^+$ variants by erythrocyte agglutination, eight additional hemagglutination-positive isolates were identified. The 12 HA$^+$ organisms as well as 9 HA$^-$ isolates were selected for further study (Table 1). Results of buccal epithelial cell agglutination (data not shown) were 100% concordant with those of hemagglutination for all NT strains tested.

Immunologic analyses. Table 2 shows the reaction of hemagglutinating NT organisms with antisera that are specific for Hib pili (and react only with native pili as tested by dot blot assay) or pilin (and react only with denatured pilins as tested by Western blot assay). The two antisera that

TABLE 2. Reactivity of polyclonal antipilus, antipilin, and antipeptide I antisera in immunodot and Western blot immunoassays[a]

Antiserum	Antiserum specificity	No. (%) positive	
		HA$^+$ NT strains (n = 12)	HA$^+$ Hib (n = 22)
R1	Native pili (Hib M43p$^+$)	0	18 (82)
R19	Purified pili (Hib E1ap$^+$)	0	11 (50)
R20	Pilin (Hib M43p$^+$)	12 (100)	22 (100)
R13	Peptide I (Hib M43p$^+$)	12 (100)	22 (100)

[a] HA$^+$ NT isolates in this study are compared with 22 HA$^+$ Hib isolates from a previously published study (9).

recognize assembled pili of strain M43 or Eagan (R1 and R19, respectively) bound to all HA$^+$ Hib strains (15) but did not bind to any of the HA$^+$ NT strains on dot blot assay. In contrast, the antisera R20 and R13, which recognize pilin or peptide I, respectively, bound to an ~24-kDa band from all HA$^+$ Hib and HA$^+$ NT strains on Western blot assay.

Binding of NT *H. influenzae* to human buccal epithelial cells and erythrocytes. Table 3 compares the binding of HA$^+$ NT *H. influenzae* and HA$^-$ Hib organisms to human buccal epithelial cells by the adherence ELISA method. HA$^+$ Hib adhered significantly better than HA$^+$ NT strains and HA$^-$ Hib. Although HA$^+$ NT strains adhered only somewhat better than HA$^-$ NT strains, the degree of adherence was greater than that of HA$^-$ Hib, suggesting that the NT organisms possess an adherence mechanism significantly reduced or not present on nonhemagglutinating type b organisms.

Table 3 also compares the binding of HA$^+$ and HA$^-$ NT *H. influenzae* and Hib to adult human erythrocyte ghosts. These results are concordant with those seen in the buccal epithelial cell binding assays, corroborating previously published observations that HA$^+$ Hib binds to human buccal epithelial cells and to human erythrocytes, although the receptors on these two cell types may be different (24).

Figure 1 shows that erythrocyte and buccal epithelial cell binding of Hib strains correlated well (correlation coefficient, 0.909; $P < 0.01$), but this correlation was weaker when NT strains were used (correlation coefficient, 0.364; $P = 0.06$).

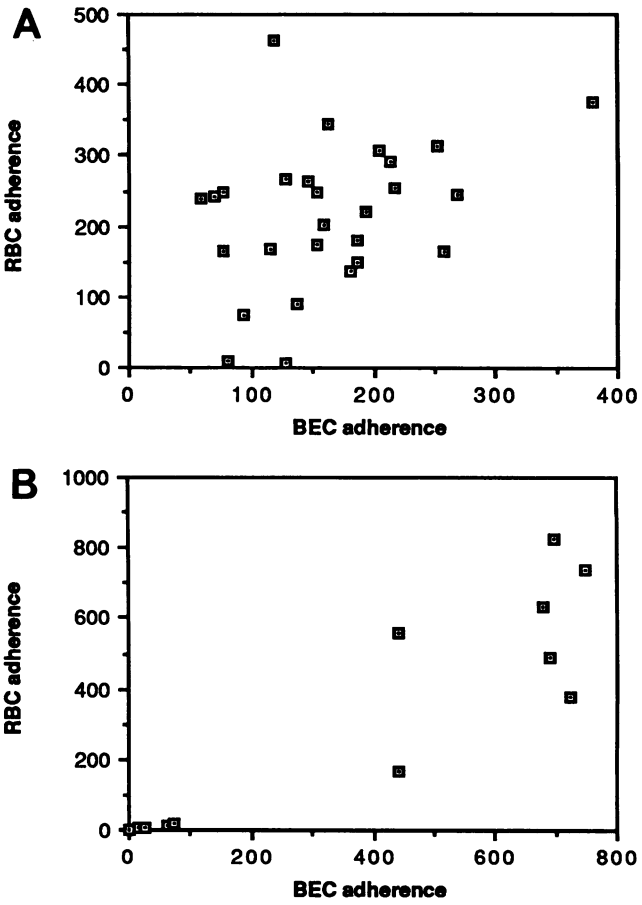

FIG. 1. Correlation of erythrocyte (RBC) adherence with buccal epithelial cell (BEC) adherence. Values are optical density at 490 nm × 1,000. (A) Adherence of NT strains; (B) adherence of Hib strains.

TABLE 3. Binding of HA$^+$ and HA$^-$ NT *H. influenzae* and Hib to human buccal epithelial cells and human erythrocyte ghosts

Organism	Binding of[a]:		
	Buccal epithelial cells	RBC ghosts	
		Adult	Cord
NT *H. influenzae*			
HA$^+$	194 ± 84[b,c]	236 ± 77[e,f]	137 ± 122[h,i]
HA$^-$	137 ± 53[b,d]	183 ± 126[e,g]	215 ± 137[h,j]
Hib			
HA$^+$	612 ± 120[c]	508 ± 200[f]	131 ± 142[i]
HA$^-$	44 ± 29[d]	24 ± 38[g]	65 ± 57[j]

[a] Binding = mean optical density at 490 nm × 1,000 (±standard deviation). RBC, erythrocyte.
[b] $0.10 > P > 0.05$.
[c] $P < 0.001$.
[d] $P < 0.001$.
[e] $P > 0.10$.
[f] $P < 0.001$.
[g] $P < 0.01$.
[h] $0.10 > P > 0.05$.
[i] $P > 0.10$.
[j] $P < 0.02$.

We also measured (Table 3) the binding of HA$^+$ and HA$^-$ NT strains and Hib to cord erythrocyte ghosts, which lack the Anton antigen to which Hib pili adhere (25). HA$^+$ Hib adheres better to adult erythrocytes (mean optical density ± standard deviation = 0.508 ± 0.200) than to cord cells (0.131 ± 0.142; $P < 0.01$). HA$^+$ NT strains also adhere better to adult erythrocytes (0.236 ± 0.77) than to cord cells (0.137 ± 0.122; $P < 0.05$), suggesting that, as with HA$^+$ Hib, hemagglutinating NT strains also bind to an antigen present on adult erythrocytes that is not present on cord cells (such as the Anton antigen). Although HA$^+$ Hib adheres somewhat better to cord cells than HA$^-$ Hib, the difference is not nearly as great as the difference in their binding to adult erythrocytes. The binding of HA$^+$ and HA$^-$ NT strains to cord cells did not differ.

Table 4 shows the hemagglutination of adult erythrocytes, cord erythrocytes, and Lu(a$^-$b$^-$) dominant blood cells by HA$^+$ and HA$^-$ NT strains and Hib organisms. These results show that the HA$^+$ NT strains, like the HA$^+$ Hib, agglutinated erythrocytes possessing the Anton antigen but did not agglutinate erythrocytes [cord and Lu(a$^-$b$^-$) dominant erythrocytes] that lack the Anton antigen.

Electron microscopy. Three HA$^+$ NT *H. influenzae* iso-

TABLE 4. Hemagglutination of erythrocytes by HA^+ and HA^- NT *H. influenzae* and Hib

Organism	No. of bacterial strains that agglutinated RBC no. of bacterial specimens[a]		
	Adult RBC	Cord RBC	Lu(a^-b^-) dominant RBC
NT *H. influenzae*			
HA^+	7/7	0/8	0/8
HA^-	0/20	0/20	0/6
Hib			
HA^+	10/10	0/10	0/2
HA^-	0/7	0/7	ND

[a] RBC, erythrocyte; ND, not determined.

lates and their HA^- variants were examined by electron microscopy. The enriched HA^+ variants of strains Mr13 and AAr91 showed pili of ~2.4 to 2.9 nm in diameter evenly distributed over the bacterial surface (Fig. 2). The HA^- variants of these two strains were predominantly nonpiliated but contained rare cells that were piliated. Strain AAr169 cells showed no pili on HA^- variants and showed some pili on HA^+ variants.

DISCUSSION

The data presented in this paper suggest that NT *H. influenzae* expresses pili similar in some ways to the hemagglutinating-adherence pili of Hib. As in Hib, the expression of these hemagglutinating pili by NT strains was not constant, but hemagglutinating variants, as defined by a hemagglutinating titer of ≥1:8, could be selected for among many NT clinical isolates. All HA^+ NT organisms expressed a 24-kDa protein that bound antibodies specific for the pilin of Hib strains; this protein was not present on HA^- NT strains. In addition, the three HA^- NT strains examined by electron microscopy showed few or no pilus structures, whereas two of the three HA^+ NT variants had many surface pili, and the third had some surface pili.

In addition to these similarities, some significant differences were also noted between Hib and NT hemagglutinating organisms. The binding of HA^+ NT strains to both buccal epithelial cells and adult erythrocyte ghosts was considerably weaker than that of HA^+ Hib, suggesting that the adhesive molecules of NT *H. influenzae* may not bind as efficiently as and may, in fact, be distinct from those of Hib. Although the antisera against Hib pilin bound to the putative pilin band of all HA^+ NT strains, the antisera against two immunologically distinct Hib pili did not bind to any of the HA^+ NT strains, suggesting that when immunologically similar pilins of NT strains are assembled into pili, immunologically different epitopes are expressed, which is similar to the situation seen when Hib pilins are assembled into pili (13). Additional explanations for the absence of binding of antipilus antisera to NT *H. influenzae* pili include low-level affinity of the antibodies or low levels of pili produced by NT strains. Furthermore, the pili (or fimbriae) seen on electron microscopy of the HA^+ NT strains are typically thinner and lack the hollow core and helical appearance of the classic HA^+ Hib pilus (2). Type b strains do, however, express an appendage morphologically similar to that noted on NT strains (10), which further suggests the possibility of distinct and multiple pilus expression or altered assembly of pilins into pili by these two groups of organisms.

One of us (2) has described 60 NT *H. influenzae* strains isolated from the middle ears or nasopharynges of children with otitis media. All of these strains possessed a variable number of surface structures that were termed fimbriae, and all adhered to human and chinchilla respiratory epithelial cells, but many did not hemagglutinate. Five of the strains (LB1 through LB5) in the present series were among those described previously (2). Of the strain LB1(86-042) cells, 100% possessed fimbriae, as described previously, but did not hemagglutinate in the microtiter plate assays, did not possess pilin on immunoassay, and could not be enriched to hemagglutinate to a titer of ≥1:8 in the semiquantitative assay. In contrast, 75% of cells of strain LB5(1712) possessed fimbriae, as previously described (2); this strain had a hemagglutination titer of 1:16 without enrichment in the microtiter plate assay and reacted with the antipilin antisera. From these observations, we believe that the fimbriae described by Bakaletz et al. (2) represent different structures from those of the NT strains characterized in the present study, which appear to be responsible for hemagglutination and reactivity with antipilin antisera. Brinton et al. (4) described *H. influenzae* pili of four differing morphologic phenotypes by electron microscopy. The data presented do not allow us to determine with certainty which of these phenotypes would describe the HA^+ pili or fimbriae discussed here.

These data show a possible paradox between the hemagglutination results and erythrocyte-binding results. The HA^+ NT strains (which, as defined in this study, hemagglutinate at a titer of ≥1:8) showed little difference in erythrocyte binding compared with HA^- NT strains. We also observed that many of our NT isolates showed low-level hemagglutination titers of ≤1:2 that did not correlate with reactivity to antipilin antisera. In addition, in both the buccal epithelial cell- and the erythrocyte-binding assays, the HA^- NT organisms showed increased binding over the HA^- Hib. These observations corroborate those of other investigators that HA^- NT strains can adhere to both erythrocytes and respiratory epithelial cells by non-pilus-mediated mechanisms (2, 21, 23). In addition, other, heretofore undefined pili may also contribute to adherence.

The data presented here show that HA^+ NT pilins appear to possess highly conserved epitopes that cross-react with those of Hib pilins. Recently published genetic analyses of pilins corroborate these immunologic findings. The pilin genes of Hib strains M43 (also called AO-2) and Eagan have been cloned, and their nucleotide sequences showed 84% homology, with 78% derived amino acid homology (9, 14, 17). Analysis of 20 N-terminal amino acids of pilin from a Brazilian purpuric fever strain of *H. influenzae* (NT) showed nearly complete homology to the N terminus of pilin from Hib strain M43 (26). Recently, Coleman et al. have cloned and sequenced the entire pilin gene from NT strain M37 (5), and this gene showed 77% DNA homology and 68% derived amino acid sequence homology with the pilin gene of their Hib strain Minn A, whose pilin gene is identical to that of strain M43. By Southern analysis, Forney et al. (9) showed that the DNA from two NT strains, M37 and 1128, hybridized to an intragenic probe from the pilin gene of Hib strain Eagan. In our study, strain 1128 did not hemagglutinate and did not express the 24-kDa protein that binds antipilin antibody. In addition, we were unable to identify a hemagglutinating variant from strain 1128 by enrichment. This observation corroborates those of other investigators that *H. influenzae* may possess the pilin gene but not express pili (9, 17).

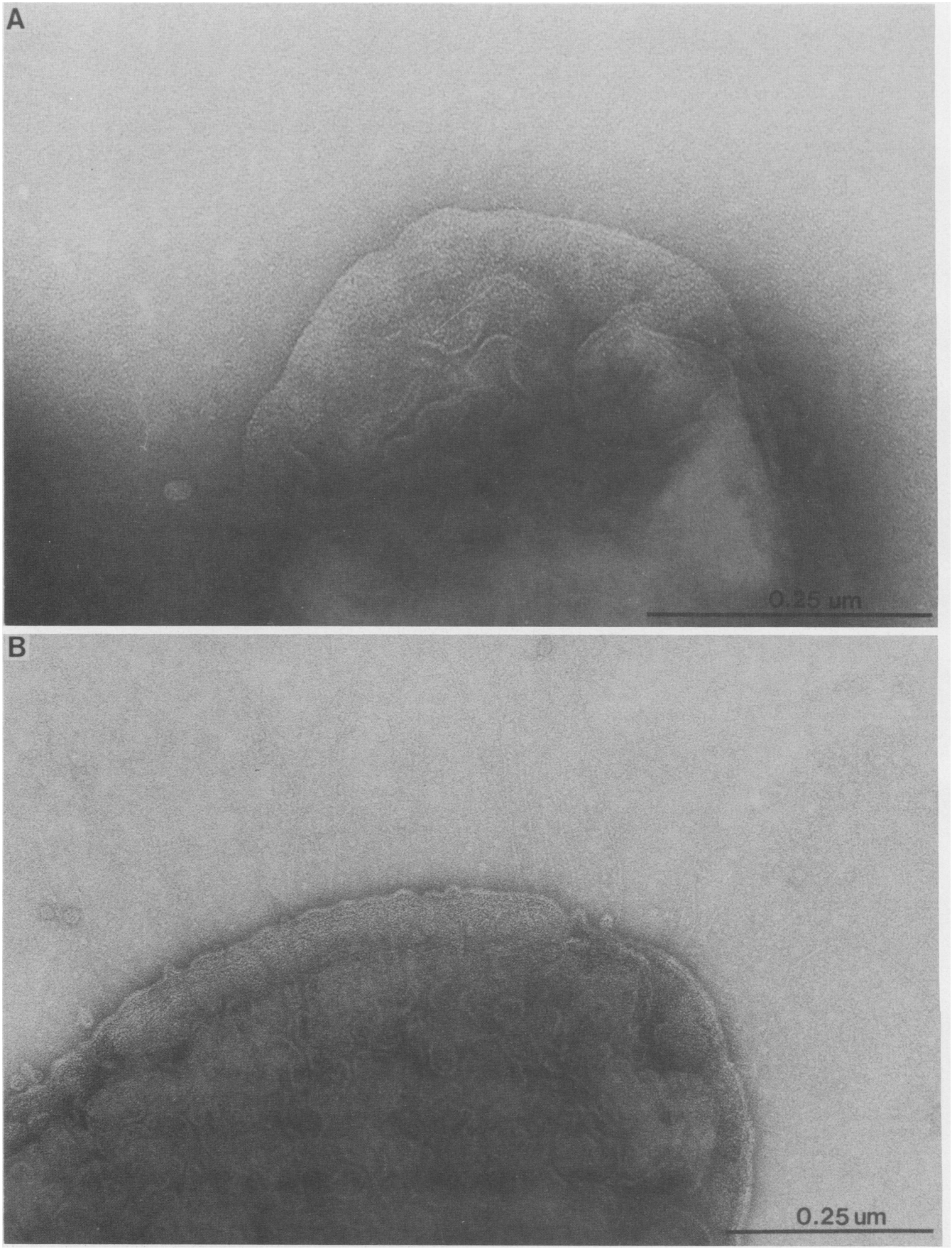

FIG. 2. Electron microscopy of nonhemagglutinating NT *H. influenzae* AAr91 (A) and its hemagglutinating variant (B).

Thus, the strong association between hemagglutination, adherence to erythrocytes and buccal epithelial cells, and presence of pili composed of pilin that exists with Hib seems less clear with NT *H. influenzae*. The relatively weak correlation between the binding of NT strains to erythrocytes and epithelial cells and the presence of pilin suggests that the adherence mechanisms of NT strains may differ from those of Hib. Additional investigation of NT *H. influenzae* adherence to respiratory epithelial cells will further define these mechanisms. Nevertheless, pilins of HA^+ NT strains appear to be conserved and immunologically similar to those of Hib, but if assembled into pili, they may be immunologically distinct from the pili of Hib. Other differences related to possible accessory pilus proteins may also exist between NT *H. influenzae* and Hib.

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