

Loss of Capsule Expression by *Haemophilus influenzae* Type b Results in Enhanced Adherence to and Invasion of Human Cells

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***Haemophilus influenzae* type b is a common cause of systemic bacterial disease in children, and the serotype b capsule is a major determinant of virulence. Nevertheless, as a consequence of the genetic configuration of the *capb* locus, type b strains become capsule deficient at a high frequency. To investigate the potential biological relevance of the predisposition to capsule loss, we compared the adherent and invasive abilities of several strains of *H. influenzae* type b and their isogenic capsule-deficient mutants by using cultured human epithelial cells. In all cases the capsule-deficient mutant demonstrated significantly greater adherence and invasion than the encapsulated parent. Transformation of one capsule-deficient mutant to restore encapsulation resulted in a marked decrease in adherence and invasion. All strains were capable of adherence and invasion by a pilus-independent mechanism. We conclude that capsule loss by *H. influenzae* type b results in enhanced in vitro adherence and invasion, properties that may be relevant to colonization of the nasopharynx and persistence within the respiratory tract. These observations suggest an explanation for the evolution of the *capb* locus as directly repeated segments of DNA with a consequent predisposition to recombination resulting in capsule loss.**

Haemophilus influenzae is a leading cause of bacterial meningitis and septicemia in infants and young children and is also a common cause of other systemic diseases in this patient population (31). In addition, this organism is a frequent cause of localized respiratory tract disease in both children and adults (31). Serotype b strains are responsible for over 95% of cases of *H. influenzae* invasive disease (30). These strains express a capsule composed of a polymer of ribose and ribitol-5-phosphate (PRP) that allows efficient intravascular survival and is an important determinant of virulence (20, 32). In contrast, nontypeable (nonencapsulated) strains account for the majority of local disease, including otitis media, sinusitis, bronchitis, and conjunctivitis (30).

It has been known for many years that serotype b strains of *H. influenzae* become nonencapsulated very readily (5, 9, 24). Hoiseith et al. examined the frequency of capsule loss in a number of independently isolated type b strains and found that nonencapsulated variants arose at frequencies of 0.1 to 0.3% (12). This instability of encapsulation is a consequence of the genetic configuration of the *capb* locus, which is organized as directly repeated 18-kb segments joined by a 1.3-kb bridge region (14, 16). Recombination between the two copies of the repeated DNA results in deletion of one copy and disruption of the bridge region (14, 15). The bridge region contains a gene encoding a protein that is required for the export of capsule (15). The high frequency and specificity with which capsule-deficient variants arise raises the question of the biological significance of this event.

In this study we compared the adherent and invasive abilities of several strains of *H. influenzae* type b and their isogenic capsule-deficient mutants by using cultured human epithelial cells. Our results support the hypothesis that capsule loss promotes persistence within the respiratory tract.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. influenzae* type b strains are listed in Table 1. Solid bacterial growth media included chocolate agar supplemented with 1% Supplement VX (Difco Laboratories, Detroit, Mich.) and translucent brain heart infusion agar supplemented with hemin and NAD (BHI-DB agar) (2). For growth in broth, bacteria were suspended in brain heart infusion broth supplemented with hemin and NAD. Strains were stored frozen at -80°C in brain heart infusion broth with 20% glycerol.

Plasmids. pSKH5 is a cosmid clone that contains the intact *capb* locus with the bridge region and both copies of the duplication (14). pSKH2 contains the central 9-kb *EcoRI* fragment of the *capb* locus subcloned into pBR328 (12). Southern analysis of *EcoRI*-digested chromosomal DNA from most wild-type *H. influenzae* type b strains, using this 9-kb fragment as a probe, yields one of three patterns of hybridization: bands of 20, 10.2, and 9 kb; bands of 10.2, 9, and 5.6 kb; or bands of 20, 10.2, 9, and 5.6 kb (1, 12). Spontaneous capsule-deficient mutants are missing the 9-kb band (12). (Both pSKH5 and pSKH2 were kindly provided by S. K. Hoiseith.)

Selection of mutants and variants. Spontaneous capsule-deficient mutants were identified after growth on BHI-DB agar. Viewing with obliquely transmitted light allowed selection of gray, noniridescent colonies as described previously (12).

A nonpilated variant of strain C54 was selected by using the hemadsorption technique of Connor and Loeb (6). Briefly, bacteria were suspended in phosphate-buffered saline (PBS) and incubated with a suspension of human O+ erythrocytes. Following centrifugation at low speed to sediment hemagglutinating (pilated) organisms, a sample of the supernatant was plated on supplemented chocolate agar and incubated overnight. A nitrocellulose disk (Schleicher & Schuell, Keene, N.H.) precut to fit a standard petri dish was moistened in PBS and applied to the surface of the agar plate. The disk was carefully removed to preserve colonies

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TABLE 1. *H. influenzae* type b strains used

Strain	Phenotype	Source (reference)
C54, wild type	Cap ⁺ P ⁺	P. Anderson (23)
C54 Cap ⁺ P ⁻	Cap ⁺ P ⁻	This study
C54 Cap ⁻ P ⁺	Cap ⁻ P ⁺	This study
C54 Cap ⁻ P ⁻	Cap ⁻ P ⁻	This study
C54 Cap ⁺ (t1)	Cap ⁺ P ⁺	This study
C54 Cap ⁺ (t2)	Cap ⁺ P ⁺	This study
1060, wild type	Cap ⁺ P ⁻	J. Musser
1060 Cap ⁻	Cap ⁻ P ⁻	This study
M42, wild type	Cap ⁺ P ^{-a}	J. Gilsdorf (10)
M42 Cap ⁻	Cap ⁻ P ^{-a}	This study
Sterm, wild type	Cap ⁺ P ⁻	S. Hoiseth (12)
Sterm Cap ⁻	Cap ⁻ P ⁻	S. Hoiseth (12)

^a Strain M42 lacks classic long filamentous hemagglutinating pili but expresses the recently described β pili (8a).

and placed contact side up in a fresh dish containing PBS with 3% bovine serum albumin. Subsequently, the disk was washed twice in PBS and finally incubated with a suspension of human O⁺ erythrocytes. The disk was removed and examined for hemagglutination-negative (nonpiliated) colonies.

Transformation. The piliated capsule-deficient mutant of strain C54 had encapsulation restored by transformation with pSKH5. The recipient strain was made competent by growth in supplemented heart infusion broth followed by incubation in the MIV medium of Herriott et al. (11). The plasmid was first digested with *Bam*HI to create two pieces of linear DNA, both containing an entire copy of the duplication. Transformation mixtures were plated on supplemented brain heart infusion agar plates containing 2% (vol/vol) burro antiserum to *H. influenzae* type b capsular polysaccharide (obtained from J. Robbins, National Institutes of Health, Bethesda, Md.). Encapsulated transformants were detected by the presence of large precipitin halos (4).

DNA preparation and analysis. High-molecular-weight chromosomal DNA was prepared from 3 ml of an overnight broth culture (19). Plasmid DNA was isolated by the alkaline lysis method (3). Restriction enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and were used according to the recommendations of the supplier. Gel-purified restriction fragments were prepared by separation on 1.0% low-melting-point agarose-Tris acetate gels, followed by excision, sequential extraction with phenol and chloroform, and ethanol precipitation.

Southern analysis of genomic DNA. DNA was digested to completion with *Eco*RI and electrophoresed on 0.7% agarose-Tris acetate gels at 50 V. The DNA was transferred to nitrocellulose filters by the method of Smith and Summers (29). Nick translation and Southern hybridization were performed by standard techniques (27). Autoradiograms were made by using Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and an intensifying screen.

Tissue culture cells. Chang epithelial cells, Wong-Kilbourne derivative, clone 1-5c-4 (human conjunctiva), were obtained from the American Type Culture Collection (ATCC CCL20.2). Cells were maintained in modified Eagle's medium with Earle's salts and nonessential amino acids (Irvine Scientific) supplemented with 10% heat-inactivated fetal calf

serum (GIBCO, Grand Island, N.Y.) and 2.0 mM L-glutamine. For adherence and invasion assays, approximately 1.5×10^5 cells suspended in 0.5 ml of tissue culture medium were seeded into each well of 24-well tissue culture plates (Becton Dickinson Labware), which were incubated overnight at 37°C in 5% CO₂. By the beginning of an assay, the cell number had increased to approximately 2.0×10^5 cells per well.

Adherence and invasion assays. For adherence assays, bacteria were grown to log phase in supplemented brain heart infusion broth. Approximately 1×10^7 to 2×10^7 CFU was inoculated directly from the broth culture onto monolayers, and tissue culture plates were incubated at 37°C in 5% CO₂. Following incubation for 4 h, monolayers were rinsed four times with PBS and then treated with trypsin-EDTA (0.05% trypsin, 0.5 mM EDTA) in PBS to release them from the plastic support. Well contents were agitated, and dilutions were plated on supplemented chocolate agar, yielding the number of adherent CFU per monolayer. The average number of adherent bacteria per epithelial cell was calculated based on the assumption that there was no epithelial cell division during the course of the assay. Monolayers appeared intact at the conclusion of the 4-h incubation, showing no evidence of cytopathic effect.

Invasion assays were performed identically except that after the initial 4-h incubation, monolayers were rinsed three times with PBS and fresh tissue culture medium containing gentamicin (100 μ g/ml) was added. Tissue culture plates were incubated for another 2 h, rinsed twice with PBS, and then treated with trypsin-EDTA in PBS. Dilutions were plated to quantitate the number of internalized bacteria per monolayer. Gentamicin at a concentration of 100 μ g/ml was sufficient to kill 100% of an inoculum of 2×10^8 CFU for all strains examined.

Transmission electron microscopy. (i) Thin section. Chang epithelial cells were seeded into Contour Permanox tissue culture dishes (35 by 10 mm), and broth-grown bacteria were inoculated onto monolayers. Following the appropriate incubation period, monolayers were rinsed four times with PBS and fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C overnight. After a wash with 0.1 M sodium phosphate buffer, samples were postfixed with 1% OsO₄ in sodium phosphate buffer for 90 min. Samples were next washed with distilled water and stained with 0.25% aqueous uranyl acetate overnight. They were dehydrated with a series of graded ethanol solutions and embedded in a firm Spurr resin. Samples were sectioned, stained with uranyl acetate and lead citrate, and examined in a Phillips 201c electron microscope.

(ii) Negative stain. A 20- μ l drop of bacterial suspension in PBS was applied to a carbon-coated grid and incubated for 2 min. Excess fluid was removed, and the specimen was rinsed with distilled water. Staining was performed with 0.5% (wt/vol) aqueous uranyl acetate for 1 min.

Light microscopy. (i) Phase contrast. Cells were seeded onto 12-mm glass coverslips placed on the bottom of each well of tissue culture plates. After inoculation of bacteria and incubation for 4 h, monolayers were rinsed four times with PBS. Samples were fixed with high-performance liquid chromatography grade methanol for 15 min and then stained with Giemsa stain (EM Diagnostic Systems, Gibbstown, N.J.) diluted 1:20 for 30 min. They were rinsed four times with distilled water and were mounted on glass slides for viewing.

(ii) Fluorescence. Monolayers were prepared, and bacteria were incubated as for phase-contrast microscopy. Following rinsing with PBS, samples were fixed for 2 min with metha-

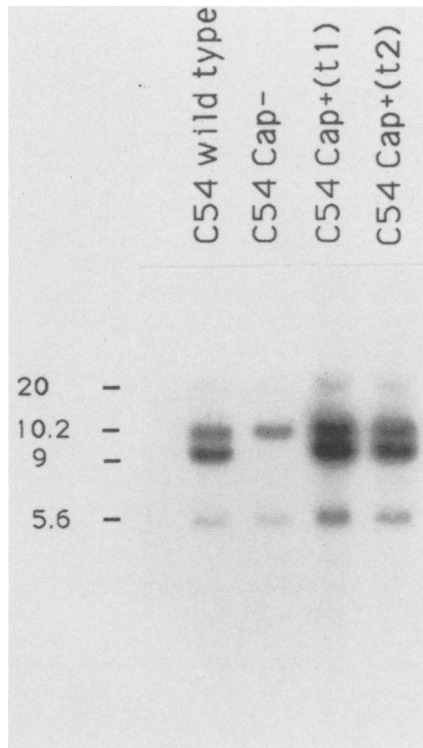


FIG. 1. Southern hybridization analysis of chromosomal DNA from wild-type strain C54 and its derivatives. Chromosomal DNA was digested with *Eco*RI and probed with the 9-kb *Eco*RI fragment of pSKH2. Lane 1, Wild-type strain C54; lane 2, capsule-deficient mutant of strain C54 (C54 Cap⁻ P⁺); lane 3, encapsulated transformant of C54 Cap⁻ P⁺ [C54 Cap⁺(t1)]; lane 4, encapsulated transformant of C54 Cap⁻ P⁺ [C54 Cap⁺(t2)]. Molecular size in kilobases is indicated next to each band. Slightly less DNA was unintentionally loaded into lanes 1 and 2, so that the 20- and 5.6-kb bands are faint.

nol. After being rinsed, they were incubated for 20 min with a 1:500 dilution of high-titer antiserum directed against PRP (gift from Bruce Green, Praxis Biologics, Rochester, N.Y.). Samples were rinsed again and incubated for 20 min with a 1:50 dilution of fluorescein isothiocyanate-conjugated goat antibody to rabbit immunoglobulin G (Bethesda Research Laboratories). Bacteria were counterstained with Hoechst dye (5 µg/ml; Molecular Probes) for 20 min and were mounted for viewing with a Zeiss Axiophot microscope.

Statistical analysis. Mean values were compared by using the two-tailed *t* test. Significance was defined as $P < 0.05$.

RESULTS

Adherence and invasion by strain C54 and an isogenic capsule-deficient mutant. Strain C54 is a serotype b isolate that constitutively expresses pili and adheres to buccal epithelial cells (23). In initial experiments, we plated C54 on BHI-DB agar and identified a gray, noniridescent colony that was subsequently streaked for pure growth. The resulting growth failed to react with antiserum against the type b capsule (Difco) in a slide agglutination assay, suggesting the loss of capsule expression. Southern analysis of this mutant (C54 Cap⁻ P⁺) demonstrated the loss of a 9-kb *Eco*RI fragment at the *capb* locus, consistent with deletion of one copy of the duplication at this locus (Fig. 1, lanes 1 and 2).

As shown in Table 2 and Fig. 2, wild-type strain C54 displayed negligible adherence and virtually no invasion. In contrast, the capsule-deficient mutant of C54 demonstrated high levels of adherence and appreciable invasion. The loss of capsule expression resulted in a 50-fold increase in adherence and a nearly 300-fold increase in invasion ($P < 0.05$).

Analysis of encapsulated transformants of capsule-deficient mutant of strain C54. To confirm that the increase in adherence and invasion by C54 Cap⁻ P⁺ compared with the C54 wild-type strain was the result of a loss of capsule expression and not some other mutation occurring simultaneously, we examined the effect of restoration of encapsulation on adherence and invasion by C54 Cap⁻ P⁺. Encapsulation was restored by transformation with a plasmid containing the entire *capb* locus (pSKH5). Two transformants were selected for further study, C54 Cap⁺(t1) (transformant 1) and C54 Cap⁺(t2) (transformant 2). Southern analysis demonstrated that both of these transformants had regained the copy of the duplication at the *capb* locus that had been deleted in C54 Cap⁻ P⁺ (Fig. 1, lanes 3 and 4).

As shown in Table 2, the numbers of adherent and internalized bacteria for transformant 1 were nearly identical to those seen with the wild-type strain. Interestingly, in the case of transformant 2, adherence and invasion were decreased compared with C54 Cap⁻ P⁺ but were still greater than observed for the C54 wild type. Further analysis of this transformant demonstrated evidence of instability of encapsulation, with capsule-deficient variants arising at a frequency of approximately 2% (10-fold greater than observed for wild-type C54 or transformant 1). Upon closer inspection of colonies on adherence and invasion plates, we noted two

TABLE 2. Adherence and invasion by *H. influenzae* type b strain C54 and derivatives

Strain	Adherence		Invasion (CFU/monolayer)	P value ^a
	Bacteria/cell	CFU/monolayer		
C54, wild type	1.0 (±0.1) ^b	2.0 × 10 ⁵ (±0.2 × 10 ⁵)	4.5 × 10 ¹ (±1.7 × 10 ¹)	
C54 Cap ⁺ P ⁻	1.7 (±0.2)	3.4 × 10 ⁵ (±0.4 × 10 ⁵)	9.5 × 10 ¹ (±2.4 × 10 ¹)	<0.05
C54 Cap ⁻ P ⁺	55 (±10)	1.1 × 10 ⁷ (±0.2 × 10 ⁷)	1.7 × 10 ⁴ (±0.4 × 10 ⁴)	<0.05
C54 Cap ⁻ P ⁻	80 (±5)	1.6 × 10 ⁷ (±0.1 × 10 ⁷)	3.7 × 10 ⁴ (±0.7 × 10 ⁴)	<0.05
C54 Cap ⁺ (t1)	0.9 (±0.2)	1.7 × 10 ⁵ (±0.2 × 10 ⁵)	2.7 × 10 ¹ (±1.2 × 10 ¹)	NS
C54 Cap ⁺ (t2)	7.5 (±1.0)	1.5 × 10 ⁶ (±0.2 × 10 ⁶) ^c	4.7 × 10 ³ (±1.4 × 10 ³) ^d	<0.05

^a Compared with values for wild-type C54. NS, Not significant.

^b Numbers represent mean (±standard error of the mean) of measurements in triplicate or quadruplicate.

^c Adherent CFU = 20% Cap⁺ and 80% Cap⁻.

^d Intracellular CFU = 1% Cap⁺ and 99% Cap⁻.

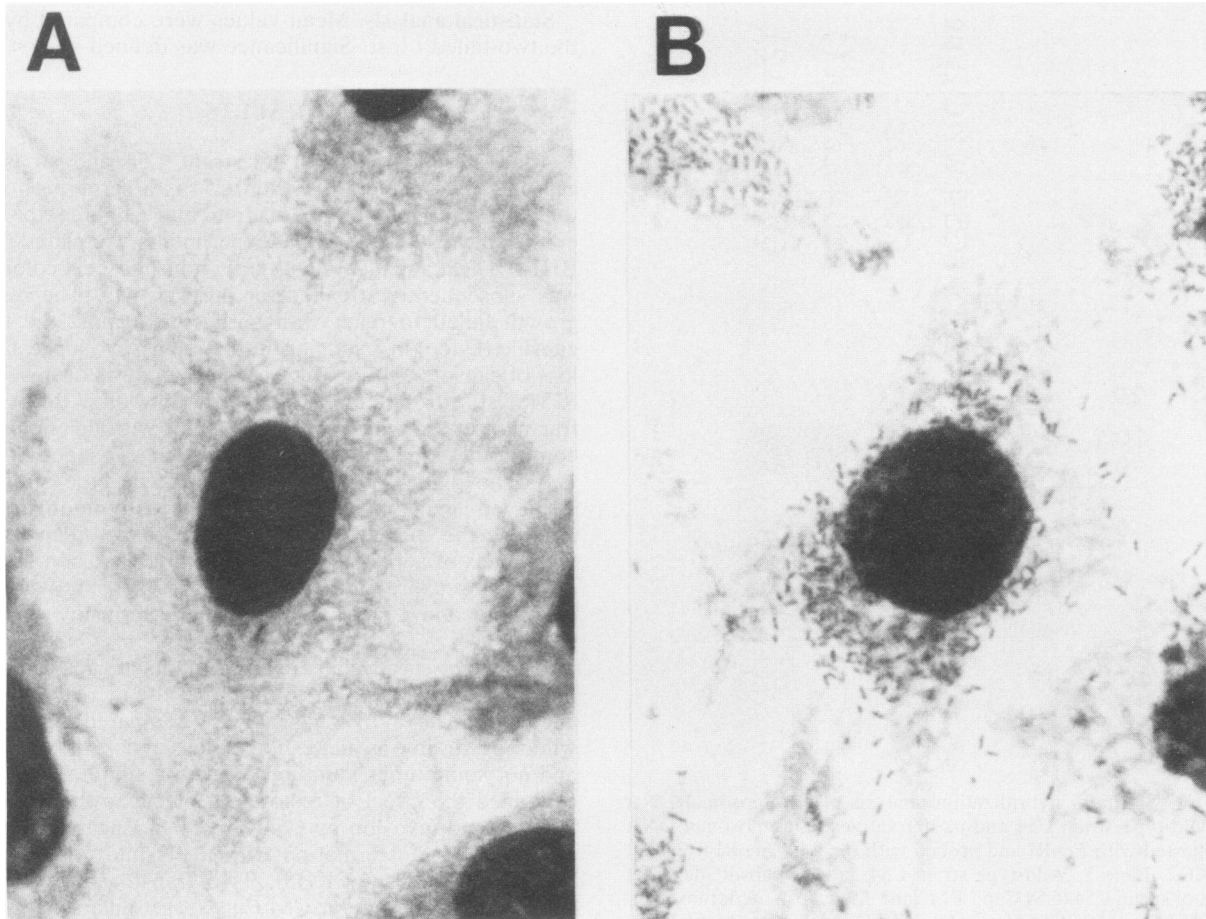


FIG. 2. Light micrographs of strain C54 incubated with Chang epithelial cells. Bacteria were incubated with a monolayer of epithelial cells for 4 h, and samples were then stained with Geimsa stain as described in Materials and Methods. (A) Wild-type strain C54 ($\text{Cap}^+ \text{P}^+$); (B) capsule-deficient mutant of strain C54 ($\text{Cap}^- \text{P}^+$).

morphologies, representing encapsulated and capsule-deficient colonies. Among adherent colonies, capsule-deficient forms predominated, in a ratio of approximately 4 to 1. The ratio of capsule-deficient to encapsulated internalized colonies was even greater, nearly 100 to 1. Examination of bacteria in the supernatants of wells inoculated with transformant 2 revealed that about 2% of these organisms were capsule deficient. Thus, while it appeared that encapsulation of transformant 2 was unstable, there was evidence for a selection at the epithelial cell surface favoring capsule-deficient bacteria. We confirmed this conclusion by immunofluorescence microscopy using an antiserum directed against the serotype b capsule. As shown in Fig. 3, very few of the cell-associated bacteria reacted with the antiserum, indicating that the majority of adherent and internalized bacteria expressed no capsule.

Role of pili in adherence and invasion. Our observation that the C54 wild-type strain (encapsulated and piliated) demonstrated minimal adherence and invasion suggested that interaction by the nonencapsulated mutant might be occurring independently of pili. To examine the possibility of nonpilus-mediated adherence and invasion, we isolated a nonpiliated variant of C54 $\text{Cap}^- \text{P}^+$. Because *H. influenzae* type b pili mediate hemagglutination, we were able to identify nonpiliated variants by screening for nonhemagglutinating colonies. We selected one such colony and streaked it for purity.

Microscopic analysis of this variant (C54 $\text{Cap}^- \text{P}^-$) demonstrated that there were no cell surface appendages (Fig. 4). Comparison of outer membrane profiles for the piliated and nonpiliated forms demonstrated identical patterns except for the absence of the 25-kDa pilin monomer in the nonpiliated variant (data not shown). Adherence and invasion values for this variant were slightly greater than those noted for the piliated form from which it was derived (Table 2). To exclude the possibility that adherent and intracellular bacteria had undergone a phase switch and were actually piliated, we examined their ability to agglutinate erythrocytes (6). Fewer than 1% of all colonies on agar plates from adherence and invasion assays demonstrated hemagglutination, substantiating the presence of a nonpilus mechanism of interaction with epithelial cells.

In a related set of experiments, we isolated a nonpiliated variant of the encapsulated piliated wild-type strain. Adherence and invasion levels for this variant (C54 $\text{Cap}^+ \text{P}^-$) were consistently slightly greater than those observed for the wild-type strain but were still minimal (Table 2).

Thin-section transmission electron microscopy. We used thin-section transmission electron microscopy to confirm the ability of capsule-deficient strain C54 to enter Chang epithelial cells. Both the piliated and the nonpiliated forms exhibited the same general sequence of events following inoculation onto a monolayer. As shown in Fig. 5, bacteria appeared

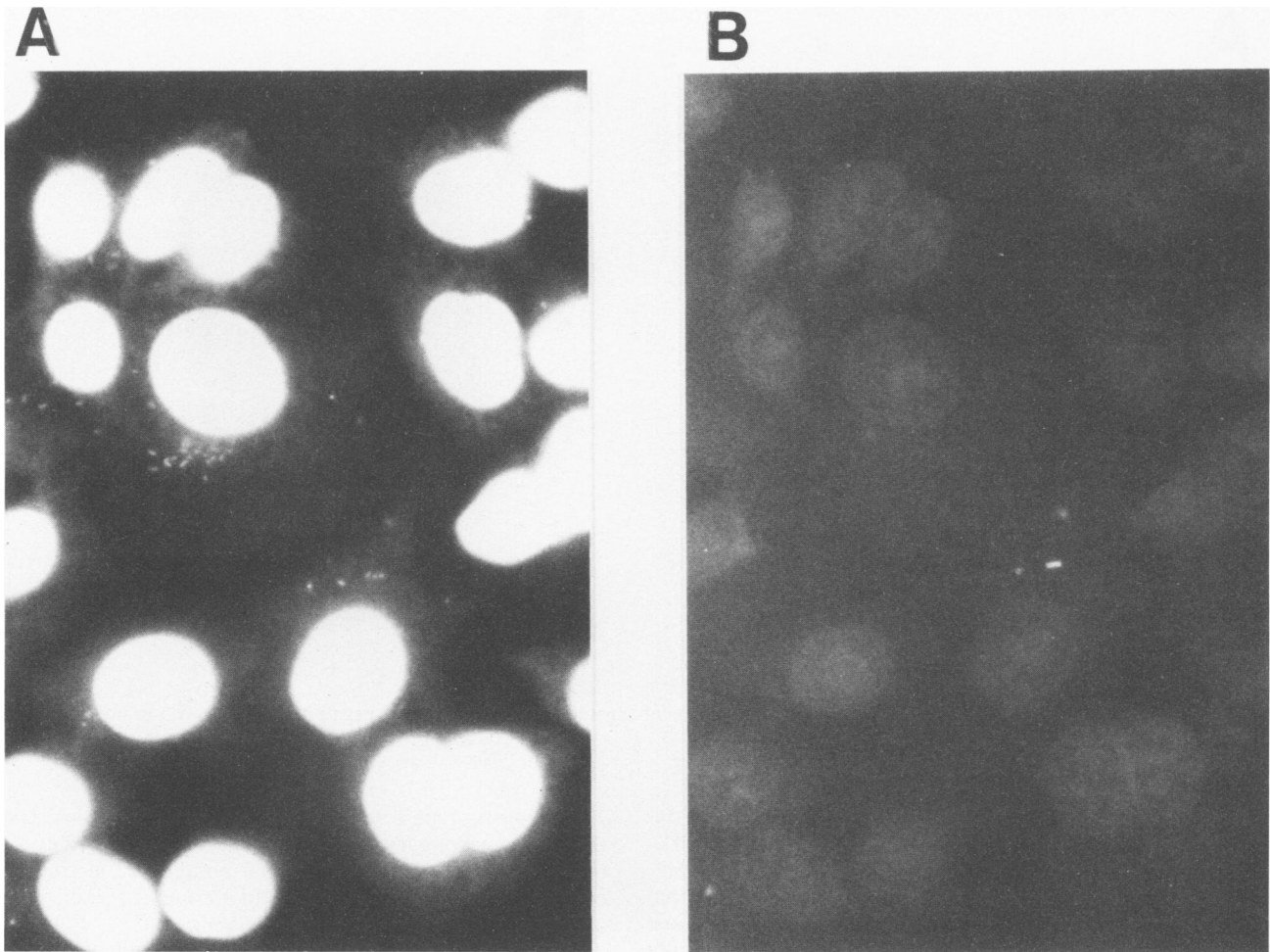


FIG. 3. Fluorescent micrographs of C54 transformant 2 incubated with Chang epithelial cells. C54 transformant 2 is an unstable encapsulated transformant of strain C54 $\text{Cap}^- \text{P}^+$. This strain was incubated with a monolayer of epithelial cells for 4 h, and samples were then prepared for fluorescence microscopy as described in Materials and Methods. (A) Hoechst stain (DNA stain) highlighting all bacteria associated with epithelial cells. Note clusters of bacteria associated with the two center cells. (B) Fluorescein labeling of bacteria reacting with anticapsular antibody, highlighting only encapsulated bacteria associated with epithelial cells. Note three bacteria associated with the center cell.

to make initial contact with the epithelial cell surface at the level of the microvilli. Subsequently, they moved into direct apposition with the plasma membrane of the eukaryotic cell, often appearing to line up along the cell surface. Internalized organisms were usually observed in isolation but were sometimes noted in pairs. Most invaded cells contained one or a few internalized bacteria in a given thin section; however, on occasion a single cell was noted to harbor many intracellular organisms.

Relationship of encapsulation with adherence and invasion for other serotype b strains. To determine the prevalence of the phenomenon observed for strain C54, we compared adherence and invasion for three additional strains of *H. influenzae* type b and their capsule-deficient mutants. Strain 1060 is a nonpiliated isolate that represents a clone of *H. influenzae* type b responsible for a sizeable fraction of invasive disease in the United States and Europe (electrophoretic type 12.5, outer membrane protein subtype 3L) (21). Strain M42 lacks classic *H. influenzae* pili but expresses the recently described β pili (8a). For both of these strains

spontaneous capsule-deficient mutants were isolated after the strains were plated on BHI-DB agar. Strain Sterm was used for rat colonization studies by Hoiseith et al. (12). The capsule-deficient mutant of this strain was isolated from the nasopharynx of a rat 4 weeks after intranasal inoculation with the encapsulated parent, at a time when capsule-deficient forms were beginning to predominate. Electron microscopy of negatively stained bacteria confirmed the absence of pili for both Sterm strains (results not shown). Southern analysis demonstrated loss of the 9-kb fragment at the *capb* locus for all three of these capsule-deficient mutants (1060 Cap^- , M42 Cap^- , and Sterm Cap^-) (results not shown).

As shown in Table 3, for all three of these pairs of strains the capsule-deficient mutant demonstrated a marked increase in adherence and invasion compared with the encapsulated parent. The encapsulated forms of strains 1060 and Sterm demonstrated minimal adherence and virtually no invasion, similar to encapsulated strain C54. Interestingly, encapsulated strain M42 demonstrated higher levels of ad-

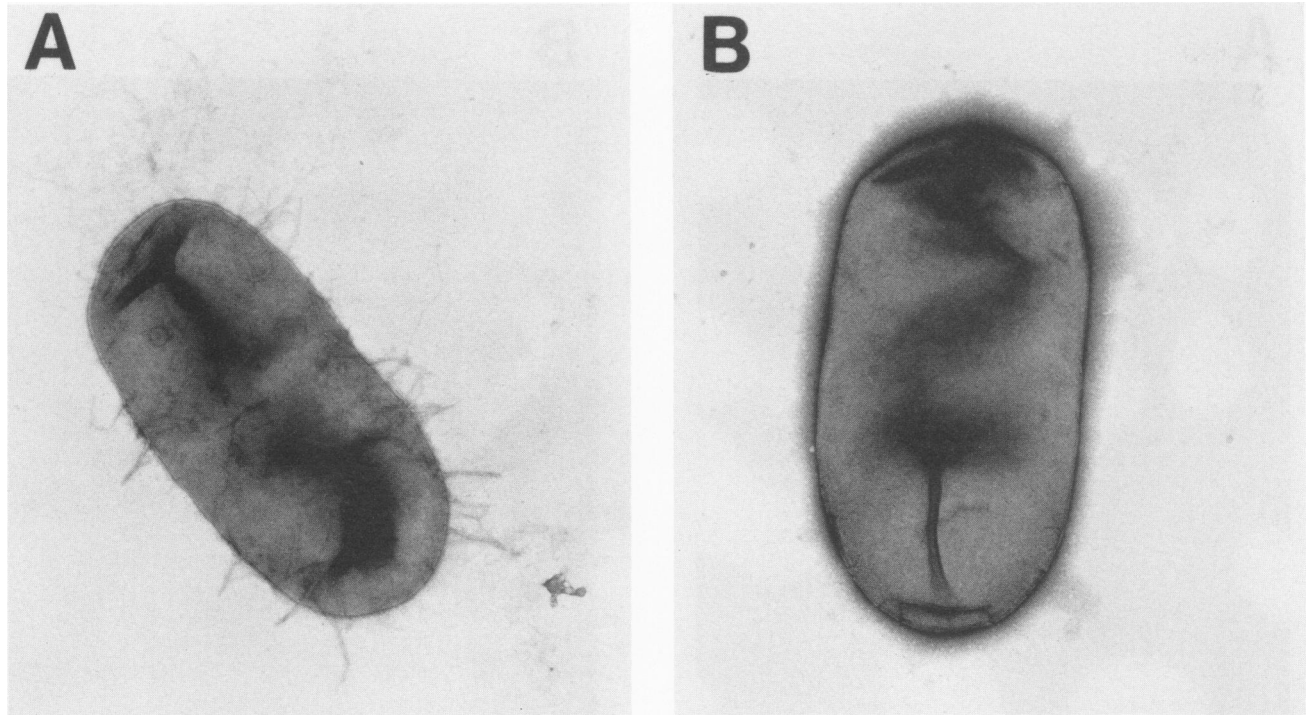


FIG. 4. Electron micrographs of piliated and nonpiliated forms of capsule-deficient strain C54 negatively stained with uranyl acetate. (A) Piliated form (C54 Cap⁻ P⁺); (B) nonpiliated form (C54 Cap⁻ P⁻).

herence and invasion. However, as noted with C54 Cap⁺(t2), close inspection revealed that approximately 50% of adherent colonies were capsule deficient and nearly 95% of internalized colonies were nonencapsulated. Examination of organisms in the supernatant at the time adherence was measured indicated that only a small minority were capsule deficient (<1%), again suggesting a selection at the eukaryotic cell surface favoring nonencapsulated bacteria.

DISCUSSION

In this study we compared the adherent and invasive abilities of several strains of *H. influenzae* type b and their isogenic capsule-deficient mutants by using a human epithelial cell line. In all cases the capsule-deficient mutant demonstrated significantly greater adherence and invasion than the encapsulated parent. Transformation of one capsule-deficient mutant to restore encapsulation resulted in a marked decrease in adherence and invasion, confirming the conclusion that the serotype b capsule interferes with these functions. Moreover, all strains were capable of adherence and invasion by a pilus-independent mechanism.

Interestingly, there are other examples of bacteria that express a capsule that interferes with interactions with host epithelial cells. Runnels and Moon recently examined K99-mediated adherence by enterotoxigenic *Escherichia coli* to isolated intestinal epithelial cells from neonatal pigs and noted an inhibitory effect of capsule (25). In the case of *Neisseria meningitidis* there is an inverse correlation between the quantity of capsular polysaccharide and in vitro adherence to human oropharyngeal cells (7). Similarly, when pneumococci are passaged through mice, capsule size is increased and attachment to human posterior pharyngeal cells is decreased (28).

Recently a number of groups have presented evidence that

H. influenzae type b may possess nonpilus adhesins. Using the human nasopharyngeal organ culture model, Farley et al. (8) and Loeb et al. (18) noted that nonpiliated strains were capable of mucosal attachment. In a comparison of piliated and nonpiliated forms of *H. influenzae* C54, Sable and coworkers found that adherence to HEP-2 cells was greater by nonpiliated organisms (26). Our results provide further support for nonpilus mechanisms of interaction with human epithelial cells and indicate an important influence of the capsule.

Several other investigators have also compared in vitro adherence to human cells by isogenic encapsulated and capsule-deficient strains of *H. influenzae* type b. Pichichero examined adherence to human oropharyngeal epithelial cells by strain Eagan and a capsule-deficient mutant (22). While the capsule-deficient mutant consistently adhered in higher numbers, adherence by both strains was poor (range, 0.02 to 0.66 bacterium per epithelial cell). Realizing the role of pili in mediating attachment to oropharyngeal epithelial cells, Li-Puma and Gilsdorf used the same model to study adherence by piliated variants of five different *H. influenzae* type b strains and their respective capsule-deficient mutants (17). They observed no significant difference between the Cap⁺ parents and the Cap⁻ mutants (mean, 7.32 ± 1.19 versus 9.34 ± 0.66 bacteria per epithelial cell). These results suggest that the capsule has a minimal effect on pilus-mediated attachment, perhaps because the *H. influenzae* type b capsule is not dense enough to mask the long filamentous pilus structure. Interestingly, in our model using an epithelial cell line derived from normal human conjunctiva, even the heavily piliated wild-type strain C54 showed minimal attachment, suggesting that these cells may lack the receptor for pili. Our observations indicate that the capsule effectively blocks the interactions we are studying, possibly by masking a nonpilus adhesin. It is also possible that the capsule

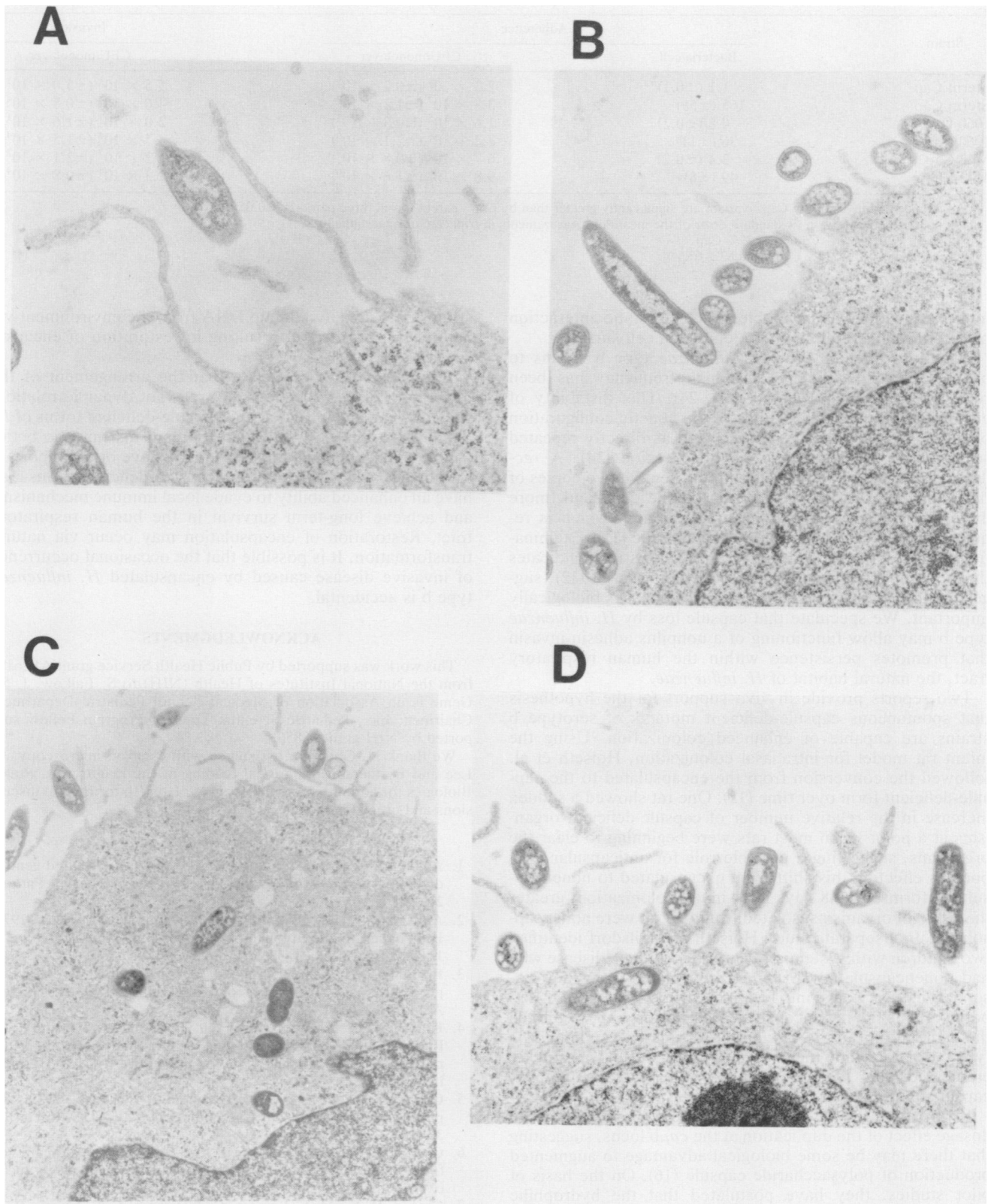


FIG. 5. Interaction of capsule-deficient strain C54 with Chang epithelial cells as viewed by transmission electron microscopy. (A) Bacterium apparently making initial contact with epithelial cell, enveloped by microvilli (1 h); (B) multiple bacteria aligned along plasma membrane of cell (4 h); (C) multiple intracellular bacteria (4 h); (D) single intracellular organism in cell with multiple bacteria in various stages of attachment (4 h) (note some adherent organisms enmeshed in microvilli and others in closer proximity to plasma membrane).

TABLE 3. Adherence and invasion by encapsulated and capsule-deficient isogenic pairs of *H. influenzae* type b^a

Strain	Adherence		Invasion
	Bacteria/cell	CFU/monolayer	CFU/monolayer
Sterm Cap ⁺	1.1 (±0.1) ^b	2.3 × 10 ⁵ (±0.2 × 10 ⁵)	5.5 × 10 ¹ (±5.0 × 10 ¹)
Sterm Cap ⁻	165 (±55)	3.3 × 10 ⁷ (±1.1 × 10 ⁷)	4.6 × 10 ⁴ (±0.8 × 10 ⁴)
1060 Cap ⁺	0.8 (±0.2)	1.6 × 10 ⁵ (±0.3 × 10 ⁵)	2.0 × 10 ¹ (±1.6 × 10 ¹)
1060 Cap ⁻	36 (±11)	7.2 × 10 ⁶ (±2.1 × 10 ⁶)	4.7 × 10 ⁴ (±1.5 × 10 ⁴)
M42 Cap ⁺	3.4 (±0.2)	6.7 × 10 ⁵ (±0.8 × 10 ⁵) ^c	5.2 × 10 ² (±2.1 × 10 ²) ^d
M42 Cap ⁻	49 (±8)	9.8 × 10 ⁶ (±1.6 × 10 ⁶)	2.3 × 10 ⁴ (±0.8 × 10 ⁴)

^a Adherence and invasion by Cap⁻ variant are significantly greater than by Cap⁺ parent for all three pairs ($P < 0.05$).

^b Numbers represent mean (±standard error of the mean) of measurements in triplicate or quadruplicate.

^c Adherent CFU = 63% Cap⁺ and 37% Cap⁻.

^d Intracellular CFU = 13% Cap⁺ and 87% Cap⁻.

interferes with a hydrophobic or electrostatic interaction between the bacterium and the epithelial cell surface.

A natural tendency for *H. influenzae* type b strains to become capsule deficient at a high frequency has been recognized for many years (5, 9, 24). This instability of encapsulation is a consequence of the genetic configuration of the *capb* locus, which is organized as directly repeated segments of DNA flanking a bridge region (14). A *rec*-dependent recombinational event between the two copies of the duplication results in deletion of one copy and, more importantly, disruption of the bridge region, which is required for export of capsular polysaccharide (15). Examination of many independently isolated type b strains indicates that the duplication is extremely well conserved (12), suggesting that the predisposition to capsule loss is biologically important. We speculate that capsule loss by *H. influenzae* type b may allow functioning of a nonpilus adhesin-invasin that promotes persistence within the human respiratory tract, the natural habitat of *H. influenzae*.

Two reports provide *in vivo* support for the hypothesis that spontaneous capsule-deficient mutants of serotype b strains are capable of enhanced colonization. Using the infant rat model for intranasal colonization, Hoiseth et al. followed the conversion from the encapsulated to the capsule-deficient form over time (12). One rat showed a sudden increase in the relative number of capsule-deficient organisms at a point when most rats were beginning to clear the organisms, suggesting a possible role for anticapsular antibody in effecting this shift from encapsulated to nonencapsulated forms. By 48 days after initial colonization, greater than 90% of organisms isolated from this rat were nonencapsulated. In a separate study Hoiseth and Gilsdorf identified two children with systemic *H. influenzae* type b disease who had nonencapsulated *H. influenzae* isolated from their nasopharynx 8 or 9 days into treatment with antibiotics (13). In both patients the nonencapsulated organisms appeared to be the same strain as the original encapsulated *H. influenzae* type b isolate. While the encapsulated organism was quickly eradicated from the nasopharynx with antibiotic therapy, capsule-deficient derivatives persisted.

Kroll and Moxon have recently demonstrated a gene dosage effect of the duplication at the *capb* locus, suggesting that there may be some biological advantage to augmented production of polysaccharide capsule (16). On the basis of pilot studies, they have postulated that the hydrophilic capsule promotes survival in the external environment, preventing dehydration and thereby enhancing dissemination between hosts (16). While spontaneous reversion of capsule-deficient strains to Cap⁺ has not been demonstrated, it is possible that capsule-deficient mutants of type b

strains occasionally take up DNA from the environment via natural transformation, resulting in restoration of encapsulation.

In summary, we speculate that the arrangement of the *capb* locus allows a biologically relevant dynamic relationship between encapsulated and capsule-deficient forms of *H. influenzae* type b. Encapsulated organisms may be better able to persist between hosts and may have other important advantages as well. In contrast, capsule-deficient forms may have an enhanced ability to evade local immune mechanisms and achieve long-term survival in the human respiratory tract. Restoration of encapsulation may occur via natural transformation. It is possible that the occasional occurrence of invasive disease caused by encapsulated *H. influenzae* type b is accidental.

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REFERENCES

- Allan, I., M. R. Loeb, and E. R. Moxon. 1987. Limited genetic diversity of *Haemophilus influenzae* (type b). *Microb. Pathog.* 2:139-145.
- Anderson, P., R. B. Johnston, Jr., and D. H. Smith. 1972. Human serum activity against *Haemophilus influenzae* type b. *J. Clin. Invest.* 51:31-38.
- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.
- Bradshaw, M. W., R. Schneerson, J. C. Parke, Jr., and J. B. Robbins. 1971. Bacterial antigens cross-reactive with capsular polysaccharide of *Haemophilus influenzae* type b. *Lancet* i:1095-1097.
- Chandler, C. A., L. D. Fothergill, and J. H. Dingle. 1939. The pattern of dissociation in *Hemophilus influenzae*. *J. Bacteriol.* 37:415-427.
- Connor, E. M., and M. R. Loeb. 1983. A hemadsorption method for detection of colonies of *Haemophilus influenzae* type b expressing fimbriae. *J. Infect. Dis.* 148:855-860.
- Craven, D. E., M. S. Pepler, C. E. Frasch, L. F. Mocca, P. P. McGrath, and G. Washington. 1980. Adherence of isolates of *Neisseria meningitidis* from patients and carriers to human buccal epithelial cells. *J. Infect. Dis.* 142:556-568.
- Farley, M. M., D. S. Stephens, L. L. Kaplan, and E. O. Mason. 1990. Pilus- and non-pilus-mediated interactions of *Haemophi-*

- lus influenzae* type b with human erythrocytes and human nasopharyngeal mucosa. *J. Infect. Dis.* **154**:752-759.
- 8a. Forney, L. J., J. Gilsdorf, T. Giddings, T. Hoepf, and L. Bakaletz. 1990. Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, B-60, p. 36.
 9. Fothergill, L. D., and C. A. Chandler. 1936. Observations on the dissociation of meningitic strains of *H. influenzae*. *J. Immunol.* **31**:401-415.
 10. Guerina, N. G., S. Langermann, H. W. Clegg, H. W. Kessler, D. A. Goldmann, and J. R. Gilsdorf. 1982. Adherence of piliated *Haemophilus influenzae* type b to human oropharyngeal cells. *J. Infect. Dis.* **146**:564.
 11. Herriott, R. M., E. M. Meyer, and M. Vogt. 1970. Defined nongrowth media for stage II competence in *Haemophilus influenzae*. *J. Bacteriol.* **101**:517-524.
 12. Hoiseth, S. K., C. J. Connelly, and E. R. Moxon. 1985. Genetics of spontaneous, high-frequency loss of b capsule expression in *Haemophilus influenzae*. *Infect. Immun.* **49**:389-395.
 13. Hoiseth, S. K., and J. R. Gilsdorf. 1988. The relationship between type b and nontypable *Haemophilus influenzae* isolated from the same patient. *J. Infect. Dis.* **158**:643-645.
 14. Hoiseth, S. K., E. R. Moxon, and R. P. Silver. 1986. Genes involved in *Haemophilus influenzae* type b capsule expression are part of an 18-kilobase tandem duplication. *Proc. Natl. Acad. Sci. USA* **83**:1106-1110.
 15. Kroll, J. S., I. Hopkins, and E. R. Moxon. 1988. Capsule loss in *H. influenzae* type b occurs by recombination-mediated disruption of a gene essential for polysaccharide export. *Cell* **53**:347-356.
 16. Kroll, J. S., and E. R. Moxon. 1988. Capsulation and gene copy number at the *cap* locus of *Haemophilus influenzae* type b. *J. Bacteriol.* **170**:859-864.
 17. LiPuma, J. J., and J. R. Gilsdorf. 1987. Role of capsule in adherence of *Haemophilus influenzae* type b to human buccal epithelial cells. *Infect. Immun.* **55**:2308-2310.
 18. Loeb, M. R., E. Connor, and D. Penney. 1988. A comparison of the adherence of fimbriated and nonfimbriated *Haemophilus influenzae* type b to human adenoids in organ culture. *Infect. Immun.* **56**:484-489.
 19. Mekalanos, J. J. 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. *Cell* **35**:253-263.
 20. Moxon, E. R., and K. A. Vaughn. 1981. The type b capsular polysaccharide as a virulence determinant of *Haemophilus influenzae*: studies using clinical isolates and laboratory transformants. *J. Infect. Dis.* **14**:517-524.
 21. Musser, J. M., J. S. Kroll, D. M. Granoff, E. R. Moxon, B. R. Brodeur, J. Campos, H. Dabernat, W. Frederiksen, J. Hamel, G. Hammond, E. A. Hoiby, K. E. Jonsdottir, M. Kabeer, I. Kallings, W. N. Khan, M. Kilian, K. Knowles, H. J. Koornhof, B. Law, K. I. Li, J. Montgomery, P. E. Pattison, J.-C. Piffaretti, A. K. Takala, M. L. Thong, R. A. Wall, J. I. Ward, and R. K. Selander. 1990. Global genetic structure and molecular epidemiology of encapsulated *Haemophilus influenzae*. *Rev. Infect. Dis.* **12**:75-111.
 22. Pichichero, M. E. 1984. Adherence of *Haemophilus influenzae* to human buccal and pharyngeal epithelial cells: relationship to piliation. *J. Med. Microbiol.* **18**:107-116.
 23. Pichichero, M. E., P. Anderson, M. Loeb, and D. H. Smith. 1982. Do pili play a role in pathogenicity of *Haemophilus influenzae* type b? *Lancet* **ii**:960-962.
 24. Pittman, M. 1931. Variation and type specificity in the bacterial species *Haemophilus influenzae* b: a preliminary report. *J. Pediatr.* **97**:233-237.
 25. Runnels, P. L., and H. W. Moon. 1984. Capsule reduces adherence of enterotoxigenic *Escherichia coli* to isolated intestinal epithelial cells of pigs. *Infect. Immun.* **45**:737-740.
 26. Sable, N. S., E. M. Connor, C. B. Hall, and M. R. Loeb. 1985. Variable adherence of fimbriated *Haemophilus influenzae* type b to human cells. *Infect. Immun.* **48**:119-123.
 27. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 28. Selinger, D. S., and W. P. Reed. 1979. Pneumococcal adherence to human epithelial cells. *Infect. Immun.* **23**:545-548.
 29. Smith, G. E., and M. D. Summers. 1980. The biochemical transfer of DNA and RNA to nitrocellulose or diazobenzoyloxymethyl-paper. *Anal. Biochem.* **109**:123-129.
 30. Turk, D. C. 1982. Clinical importance of *Haemophilus influenzae*, p. 3-9. In S. H. Sell and P. F. Wright (ed.), *Haemophilus influenzae* epidemiology, immunology, and prevention of disease. Elsevier/North-Holland Publishing Co., New York.
 31. Turk, D. C. 1984. The pathogenicity of *Haemophilus influenzae*. *J. Med. Microbiol.* **18**:1-16.
 32. Zwahlen, A., J. S. Kroll, L. G. Rubin, and E. R. Moxon. 1989. The molecular basis of pathogenicity in *Haemophilus influenzae*: comparative virulence of genetically-related capsular transformants and correlation with changes at the capsulation locus *cap*. *Microb. Pathog.* **7**:225-235.