The Haemophilus influenzae Type b hcsA and hcsB gene products facilitate transport of capsular polysaccharide across the outer membrane and are essential for virulence

Soila Sukupolvi-Petty  
Washington University School of Medicine in St. Louis

Susan Grass  
Washington University School of Medicine in St. Louis

Joseph W. St. Geme III  
Duke University School of Medicine

Follow this and additional works at: http://digitalcommons.wustl.edu/open_access_pubs

Recommended Citation
http://digitalcommons.wustl.edu/open_access_pubs/2611

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact engeszer@wustl.edu.
The *Haemophilus influenzae* Type b *hcsA* and *hcsB* Gene Products Facilitate Transport of Capsular Polysaccharide across the Outer Membrane and Are Essential for Virulence

Soila Sukupolvi-Petty, Susan Grass and Joseph W. StGeme III


Updated information and services can be found at:
http://jb.asm.org/content/188/11/3870

These include:

REFERENCES

This article cites 46 articles, 28 of which can be accessed free at: http://jb.asm.org/content/188/11/3870#ref-list-1

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
The Haemophilus influenzae Type b hcsA and hcsB Gene Products Facilitate Transport of Capsular Polysaccharide across the Outer Membrane and Are Essential for Virulence

Soila Sukupolvi-Petty,1† Susan Grass,1,2 and Joseph W. St. Geme III1,2*

Edward Mallinckrodt Department of Pediatrics and Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, Missouri 63110,1 and Departments of Pediatrics and Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina2

Received 22 December 2005/Accepted 14 March 2006

Haemophilus influenzae type b is a common cause of invasive bacterial disease, especially among children in underdeveloped countries. The type b polysaccharide capsule is a polymer of ribose and ribitol-5-phosphate and is a critical determinant of virulence. Expression of the type b capsule is dependent upon the cap b locus, which consists of three functionally distinct regions, designated regions 1 to 3. Region 3 contains the hcsA and hcsB genes, which share significant homology with genes that have been implicated in encapsulation in other pathogenic bacteria but have unclear functions. In this study, we inactivated hcsA alone, hcsB alone, and both hcsA and hcsB together and examined the effects of these mutations on polysaccharide transport and bacterial virulence properties. Inactivation of hcsA alone resulted in accumulation of polysaccharide in the periplasm and a partial decrease in surface-associated polysaccharide, whereas inactivation of hcsB alone or of both hcsA and hcsB together resulted in accumulation of polysaccharide in the periplasm and complete loss of surface-associated polysaccharide. All mutations eliminated serum resistance and abrogated bacteremia and mortality in neonatal rats. These results indicate that the hcsA and hcsB gene products have complementary functions involved in the transport of polysaccharide across the outer membrane and are essential for virulence.

Polysaccharide capsules are common cell surface components in bacterial pathogens that cause systemic disease. Based on in vitro studies, it is known that polysaccharide capsules mediate resistance to important host defense mechanisms, including phagocytosis and complement-mediated killing. In addition, capsules may prevent desiccation in the environment, potentially facilitating transmission between hosts. Consistent with these observations, genetically defined nonencapsulated mutants of Streptococcus spp., Actinobacillus pleuropneumoniae, Klebsiella pneumoniae, Staphylococcus aureus, Pasteurella multocida, and Haemophilus influenzae have reduced virulence in animal models (3, 11, 20, 24, 36, 39, 44, 45).

Bacterial capsules are long polysaccharide chains consisting of smaller repeating units. The composition of these repeating units varies among bacterial species and is usually distinct among different serotypes of the same species. Capsular polysaccharide biosynthesis occurs in the cytoplasm, and the resulting polysaccharide is then transported across the cytoplasmic membrane into the periplasm and then across the outer membrane to the bacterial surface. Typically, capsules are substituted with phospholipids at the reducing end of the polysaccharide chains (14, 21, 30, 33). According to the prevailing model, lipidation of capsular polysaccharide is required for transport across the inner membrane and possibly for anchoring to the outer membrane (30).

Encapsulated strains of H. influenzae are an important cause of sepsis, meningitis, epiglottitis, and septic arthritis in young children, especially in underdeveloped countries where vaccination rates are low (26). These strains are characterized by the presence of one of six structurally and serologically distinct polysaccharide capsules, referred to as serotypes a through f (28). Type b isolates are most common and express a capsule that is a polymer of ribose and ribitol-5-phosphate (polyribosylribitolphosphate [PRP]) (9). The genes responsible for the biosynthesis and surface expression of the type b capsule are located in the cap b locus, which contains three functionally distinct regions (19), similar to capsulation loci in other bacteria. Most isolates contain a partial tandem duplication of the cap b locus, with the two copies separated by a 1.2-kb bridge segment and flanked by IS1016 elements.

The three functionally distinct regions in the cap b locus are referred to as regions 1 to 3. Region 1 contains genes designated hexA, hexB, hexC, and hexD and encodes an ABC transporter system involved in the export of capsular polysaccharide to the bacterial surface (18). Region 2 contains genes currently designated orf1 to orf4 and encodes enzymes involved in biosynthesis of ribose-ribitol-5-phosphate disaccharide subunits (41). Region 3 contains genes referred to as hcsA and hcsB, which share significant homology with genes in a number of other encapsulated pathogens, including Neisseria meningitidis (lipA and lipB), Escherichia coli K1 and E. coli K5 (kpsC and kpsS), Mannheimia (Pasteurella) haemolytica (wbrA and wbrB), P. multocida A:1 (phyA and phyB), P. multocida B:2 (webA and webO), Burkholderia mallei, and Burkholderia pseudomallei (4, 7, 10, 13, 22, 29, 32, 34). Overall, the hcsA and lipA gene products are 60.4% identical, and the hcsB and lipB gene products are 55.1% identical. Based on analysis of mutations in...
the N. meningitidis lipA and lipB genes, it appears that lipA and lipB play a role in surface localization and possibly lipidation of capsular polysaccharide, although published reports are conflicting (13, 40). Whether hcsA and hcsB have functions like those of lipA and lipB remains unclear.

In this study, we inactivated the H. influenzae type b hcsA and hcsB genes and examined the resulting mutants for polysaccharide transport, polysaccharide lipidation, and virulence properties. Our results established that hcsA and hcsB have complementary functions involved in the transport of the se-

type b polysaccharide across the outer membrane and are essential for in vitro serum resistance and for virulence in neonatal rats. We found no evidence that hcsA and hcsB play a role in polysaccharide lipidation.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** H. influenzae strain Eagan is a type b clinical isolate that was originally isolated from a child with meningitis and has a partial duplication of the cap b locus, like most type b isolates (35). H. influenzae strain Rd is a long-time laboratory strain and is a spontaneous capsule-
deficient mutant of a type d isolate, containing an IS1016 element but no cap gene (12). H. influenzae strain RM135 is a transformant of strain Rd that harbors a single copy of the cap b locus and expresses the type b capsule (47). E. coli DH5α has been previously described (15).

The plasmid pUC18K carries a kanamycin resistance cassette from Enterococcus faecalis and was a generous gift from Philippe Sansonetti (Pasteur Institute, Paris, France) (23). The plasmid pUC4K carries the kanamycin resistance cassette from Trb63 and was obtained from Pharmacia (43). The plasmids pUC19 and pACYC184 were obtained from New England Biolabs and have been de-
scribed previously (6, 46).

H. influenzae strains were grown in brain heart infusion (BHI) broth supple-
mented with hemin and NAD, on BHI agar supplemented with hemin and hemin (BHI-DB agar), or on chocolate agar, as described previously (2, 37). E. coli strains were grown on Luria-Bertani (LB) agar or in LB broth. Antibiotic concentrations were as follows: for H. influenzae, kanamycin at 55 μg/ml and chlor-
amphenicol at 2 μg/ml, and for E. coli, kanamycin at 50 μg/ml and ampicillin at 100 μg/ml.

**General molecular techniques.** DNA ligations, restriction endonuclease diges-
tions, gel electrophoresis, and PCR were performed according to standard tech-
niques (31). Plasmids were introduced into E. coli by chemical transformation (31). Linearized DNA was introduced into H. influenzae made competent using the MIV method of electroporation (16).

**Construction of hcsA and hcsB mutants.** To create an hcsA null mutant without affecting the hcsB gene downstream, we generated an in-frame deletion in hcsA, leaving the remainder of region 3 intact. To prepare this construct, we cloned the hcsA and hcsB genes and flanking sequence into pUC19, ligating three separate PCR-amplified fragments. The PCR primers were designed to create BamHI sites on both sides of hcsA, making it possible to excise hcsA and preserve the correct reading frame for hcsB. To allow selection of transformants carrying this con-
struct, the kanamycin cassette from pUC4K was introduced into a unique SalI site in the IS1016 element downstream of the hcsA gene. As a control, an analogous construct containing wild-type region 3 and a kanamycin cassette in the IS1016 element was generated.

To create an hcsB null mutant, we generated PCR fragments corresponding to the 5’ and 3’ ends of hcsB and cloned these fragments into pUC19. The PCR primers were designed to create a BamHI site at the point of fusion of the 5’ and 3’ fragments, making it possible to insert the kanamycin cassette from pUC4K and interrupt the hcsB gene.

To create an hcsA hcsB double mutant, we began by generating two separate plasmids. As a first step, we cloned a 6.2-kb fragment containing hcsA and flanking sequence into pUC19 and then inserted the kanamycin cassette from pUC4K into Eco47III site in the hcsA gene. As a second step, we exploited the plasmid harboring hcsB with a unique internal BamHI site and inserted the chloramphenicol cassette from PACYC184 into this BamHI site.

Constructs were linearized and then transformed into strain RM135 made competent using the MIV method of Herriott et al. (16). Transformants were selected by plating on BHI-DB agar containing kanamycin or kanamycin plus chloramphenicol, as appropriate.

Detection and quantitation of type b polysaccharide. The presence of the type b polysaccharide capsule on the bacterial surface was detected semiquantitatively using a Wellcogen H. influenzae b kit (Alexon-Trend, Inc., Minnesota), which contains latex particles coated with antibody against PRP. Levels of agglutination were graded as nonexistent (−), weak (+), moderate (++), and strong (+++). Bacterial cellular fractions were prepared as described previously (20). In brief, bacterial cultures were incubated to exponential phase and were pelleted by centrifugation at 12,000 × g at 4°C for 15 min. Culture supernatant was saved as a source of extracellular released polysaccharide. To recover periplasm-associated polysaccharide, the bacterial pellet was resuspended in 0.03 M Tris (pH 7.4) containing 3 mM EDTA and 25% (wt/vol) sucrose, incubated for 10 min at 25°C, and pelleted by centrifugation. The pellet was rapidly resuspended in ice-cold distilled water and incubated for 10 min at 4°C and then centrifuged at 12,000 × g at 4°C for 15 min. Supernatant, containing periplasmic polysaccharide, was saved (41). Control assays measuring glucose-6-phosphate dehydrogenase, as a cytoplasmic marker and alkaline phosphatase as a periplasmic marker confirmed that the periplasmic fraction was free of cytoplasmic contents.

Polysaccharide content in samples was measured by enzyme-linked immu-
nosorbent assay (ELISA) as described previously (27). The capture antibody used for coating ELISA plates was burro anti-PRP (a generous gift from John Robbins and Rachel Schneerson, Bureau of Biologies), and bound antigen was detected with a mouse monoclonal anti-PRP antibody (a gift from Bruce Green, Wyeth) and horseradish peroxidase-conjugated anti-mouse secondary antibody. The peroxidase substrate used for supernatant and periplasm-associated polysaccharide samples was SureBlue TMB microwell peroxidase substrate (Kirkegaard & Perry, Gaithersburg, Maryland).

**Immunoelectron microscopy.** To prepare bacteria for analysis by electron microscopy, organisms initially were fixed in 4% paraformaldehyde-0.1% glutar-
aldehyde (Polysciences Inc., Warrington, PA) in 100 mM piperezine-N,N’-bis(2-
ethanesulfonic acid) (PIPES) phosphate buffer, pH 7.2, for 1 h at 4°C. Subse-
quently, samples were rinsed extensively in deionized water to remove phosphate ions and were then stained en bloc with aqueous 2% uranyl acetate for 1 h at 4°C. Samples were then dehydrated and infiltrated with LR Gold resin (Ted Pella, Inc., Redding, CA) under the following conditions: 50% ethanol for 30 min at 4°C, 70% for 30 min at −20°C, 90% ethanol for 30 min at −20°C, and 100% LR Gold ethanol for 60 min at −20°C. 3:1 LR Gold:ethanol for 60 min at −20°C, two changes of 100% LR Gold (60 min and overnight) at −20°C, and two changes of 100% LR Gold with 0.1% initiator (benzoin methyl ether) (4 h and overnight) at −20°C. Samples were then embedded in fresh LR Gold resin with initiator and polymerized at −20°C under UV light for 48 h.

Samples were sectioned with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL). Seventy- to 80-nm sections were immuno-
abeled with mouse monoclonal anti-PRP antibody and subsequently with 18-nm-diameter colloidal gold-conjugated goat anti-mouse immunoglobulin G. Sections were stained with uranyl acetate and lead citrate and viewed on a JEOL 1200EX transmission electron microscope (JEOL USA Inc., Peabody, MA). All labeling experiments were processed in parallel with controls in which the pri-
mary antibody was omitted. Controls were consistent with the absence of concen-
tration of gold-conjugated secondary antibodies used in these studies.

**Purification of H. influenzae capsular polysaccharide.** To purify capsular polysaccharide, periplasm-associated polysaccharide was recovered as described above (41), and then absolute ethanol was added to achieve a final concentration of 25% (vol/vol). After incubation for 2 h, bacterial debris was removed by centrifugation at 25,000 × g for 20 min. Polysaccharide was then precipitated by adding absolute ethanol to a final concentration to 80% (vol/vol) and was col-
lected by centrifugation at 2,000 × g for 10 min. The precipitated polysaccharide was resuspended in distilled water, and Cetavlon (hexadecyltrimethyl ammonium bromide) was added to the suspension to achieve a final concentration of 0.5%. After 1 h of mixing, the polysaccharide-Cetavlon complex was collected by centrifugation at 10,000 × g for 20 min. The polysaccharide-Cetavlon complex was resuspended in 0.3 M NaCl, and polysaccharide was recovered by centrifu-
gation at 10,000 × g for 20 min. The relative quantity of polysaccharide in each sample was determined by adding orcinol to samples and measuring the A490 using serial dilutions of ribose as a standard.

**Thin-layer chromatography.** Capsular polysaccharide preparations from 1.5 liters of late-exponential-phase cultures were chromatographed on silica gel-
coated aluminum plates (Whatman) with butanol-methanol-water (5:3:2) (42). The dried plates were stained with orcinol-ferric chloride (Bial’s reagent) (Sigma, St. Louis, MO).

**Serum resistance assay.** Serum from healthy adult donors was collected, pooled, and stored at −80°C as 100-μl aliquots. Bacterial assays were per-
formed for 30 min at 37°C using 20% serum. Percent survival was determined as the ratio of viable counts after incubation in active serum to viable counts after
incubation in heat-inactivated serum (56°C for 30 min to inactivate complement function).

**Virulence studies.** Virulence studies using the infant rat model were performed essentially as described previously (35). Sprague-Dawley albino rats used for these experiments were purchased from Taconic Farms (Germantown, New York). Infant rats that were 5 or 6 days old were randomized among litters. Intraperitoneal inoculations were carried out with 100 μl of bacterial suspension adjusted to a density of ~10⁷ CFU/ml. To evaluate the development of systemic infection, a blood sample of 10 μl was obtained from a dorsal foot vein, serially diluted in phosphate-buffered saline, and spread on chocolate agar plates to determine the number of viable bacteria in blood. Blood samples were obtained 24 h, 48 h, and 96 h after inoculation.

**RESULTS**

**Inactivation of *H. influenzae* serotype b hcsA and hcsB genes.** The genetic organization of region 3 in the *H. influenzae* cap b locus is shown in Fig. 1. This region contains the hcsA and hcsB genes, which are both transcribed away from region 2 and are separated from each other by 259 nucleotides of noncoding DNA. The IS1016 element that flanks the cap b locus begins 79 nucleotides downstream of the hcsB stop codon.

To define the functions of the hcsA and hcsB gene products, we constructed three mutants of *H. influenzae* strain RM135, including an hcsA null mutant with an in-frame deletion in hcsA (RM135hcsA), an hcsB mutant with a kanamycin cassette inserted into hcsB (RM135hcsB), and an hcsA hcsB double mutant with a kanamycin cassette inserted into hcsA and a chloramphenicol cassette inserted into hcsB (RM135hcsA-hcsB). To facilitate recovery of the hcsA in-frame deletion mutant, we introduced a kanamycin cassette into the IS1016 element downstream of the hcs region, allowing selection for transformants on the basis of kanamycin resistance. As a control, we generated a strain with wild-type hcsA and hcsB genes and a kanamycin cassette in the flanking IS1016 element (RM135-IS). All mutants were confirmed to have the appropriate deletion or insertion using PCR and Southern analysis (data not shown).

**Localization of capsular polysaccharide in the hcsA, hcsB, and hcsA hcsB mutants.** To assess the effects of inactivation of hcsA, of hcsB, and of both hcsA and hcsB on surface-associated polysaccharide capsule, we examined the abilities of the parent and mutant strains to agglutinate latex particles coated with antibody against the *H. influenzae* type b capsule in semiquantitative assays. As shown in Table 1, wild-type RM135 and RM135-IS agglutinated latex particles strongly. In contrast, RM135hcsA displayed very weak agglutination, and RM135hcsB and RM135hcsA-hcsB displayed no agglutination, results comparable to observations with noncapsulated strain Rd. These results indicate that mutations in hcsA, in hcsB, and in both hcsA and hcsB result in diminution of surface-associated polysaccharide capsule.

To address whether the decrease in surface-associated polysaccharide capsule in the RM135hcsA, RM135hcsB, and RM135hcsA-hcsB mutants was a consequence of a block in translocation across the outer membrane or a defect in anchoring to the bacterial surface, we used a sandwich ELISA to quantify polysaccharide in periplasm fractions and in culture supernatants. In performing these assays, we used wild-type RM135 and RM135-IS as positive controls and nonencapsulated strain Rd as a negative control. As shown in Fig. 2, examination of periplasmic fractions revealed similar quantities of polysaccharide in wild-type RM135 and RM135-IS and in all three mutant strains. In contrast, examination of culture supernatants revealed abundant polysaccharide in wild-type RM135 and RM135-IS, a ~60% decrease in released polysaccharide in RM135hcsA, and no detectable released polysaccharide in RM135hcsB and RM135hcsA-hcsB.

To extend these results, we used a murine monoclonal antibody against PRP and performed immunoelectron microscopy on thin sections from wild-type RM135, RM135hcsA, RM135hcsB, and Rd. As shown in Fig. 3A, wild-type strain RM135 had immunoreactive polysaccharide in the cytoplasm, in the periplasm, and on the bacterial surface (Fig. 3A), and strain Rd had no appreciable immunolabeling of any compartment (Fig. 3B), as expected. Consistent with our latex agglutination and ELISA results, RM135hcsA had wild-type levels of labeling in the cytoplasm and periplasm and a significant decrease in labeling on the bacterial surface (Fig. 3C), and RM135hcsB had appreciable labeling in the cytoplasm and periplasm but no clear labeling on the bacterial surface (Fig. 3D).

**Phospholipid modification of capsular polysaccharide in the hcsA, hcsB, and hcsA hcsB mutants.** Previous work has suggested that *N. meningitidis* homologs of hcsA and hcsB may play a role in substitution of a 1,2-diacylglycerol lipid moiety at the reducing end of the *N. meningitidis* serogroup B capsule polymer (13). To assess whether the hcsA and hcsB gene products affect lipidation of the type b polysaccharide, we purified periplasmic polysaccharide from wild-type RM135, RM135hcsA, RM135hcsB, and RM135hcsA-hcsB and then compared these polysaccharide samples by thin-layer chromatography (lipidated and nonlipidated polysaccharides migrate differently on thin-layer chromatography plates). As shown in Fig. 4, all polysaccharide samples migrated in identical fashion, arguing that no detectable released polysaccharide from the mutant strains. To confirm this result, we separated samples by using a Tris-borate-buffered polyacrylamide gel electrophoresis system and again observed no difference in migration (data not shown).
Sensitivity to serum complement of the \( hcsA, hcsB, \) and \( hcsA \) \( hcsB \) mutants. Previous studies have demonstrated the importance of surface-associated capsule in \( H. \) influenzae resistance to complement-mediated killing (17, 25, 38). With this information in mind, we compared wild-type RM135, RM135\( hcsA \), RM135\( hcsB \), and RM135\( hcsA-hcsB \) in 30-minute serum resistance assays, using RM135-IS and Rd as controls. Using the ratio of viable counts in 20% normal human serum to viable counts in 20% heat-inactivated human serum as a measure of serum resistance, wild-type RM135 and RM135-IS were ~80% to 100% resistant (Fig. 5). In contrast, the RM135\( hcsA \), RM135\( hcsB \), and RM135\( hcsA-hcsB \) mutants were completely susceptible to complement-mediated killing, analogously to strain Rd (Fig. 5). These observations are consistent with our quantitative latex agglutination and ELISA results and suggest that a certain threshold level of surface-associated capsule is required for serum resistance.

Virulence in the infant rat bacteremia model by the \( hcsA, hcsB, \) and \( hcsA \) \( hcsB \) mutants. To investigate the role of \( hcsA \) and \( hcsB \) in virulence, we compared wild-type RM135, RM135\( hcsA \), RM135\( hcsB \), and RM135\( hcsA-hcsB \) in the infant rat bacteremia model in two independent experiments using an intraperitoneal route of infection. In experiment 1, among rats infected with wild-type strain RM135, 50% were dead after 72 h and 70% were dead after 96 h (Fig. 6A). All rats infected with either RM135\( hcsA \) or RM135\( hcsB \) survived, indicating that inactivation of either \( hcsA \) or \( hcsB \) attenuates virulence. One pup infected with RM135\( hcsA-hcsB \) died, but this pup was much smaller than its littersmates, raising the possibility that factors other than infection contributed to death. In experiment 2, results were similar, although mortality among rats infected with wild-type RM135 was generally higher (Fig. 6B). In this experiment, infection with wild-type RM135 resulted in death for 70% of rats after 48 h and 100% of rats after 72 h. All rats infected with RM135\( hcsA \), RM135\( hcsB \), or RM135\( hcsA-hcsB \) survived during this experiment.

To complement our assessment of mortality, we collected blood samples at 24, 48, and 96 h after inoculation and determined the magnitude of bacteremia in surviving animals. As shown in Table 2, animals infected with wild-type RM135 had high numbers of CFU per ml of blood at all time points during both experiments. In contrast, none of the animals infected with RM135\( hcsA \), RM135\( hcsB \), or RM135\( hcsA-hcsB \) had bacterial counts higher than the limit of detection (100 CFU per ml of blood) in either experiment.

These observations are consistent with our quantitative latex agglutination and ELISA results and our serum resistance assays and suggest that a certain threshold level of surface-associated capsule is required for virulence in animals.

DISCUSSION

The \( H. \) influenzae type b \( cap \) b locus contains three functional regions, designated region 1, region 2, and region 3 (19). Recent work has established that region 3 contains genes, called \( hcsA \) and \( hcsB \), with homology to genes in a number of other encapsulated bacteria (32). In this study, we examined the function of \( hcsA \) and \( hcsB \). Based on analysis of \( hcsA, hcsB, \) and \( hcsA \) \( hcsB \) mutants, we found that both the \( hcsA \) and \( hcsB \) gene products are required for efficient transport of capsular polysaccharide across the outer membrane. Consistent with the decrease in surface-associated polysaccharide in these mutants, we observed abrogation of serum resistance and elimination of virulence in experimental rats.

The \( hcsA \) and \( hcsB \) gene products share homology with gene products in other pathogenic bacteria that express group II capsules, including \( N. \) meningitidis, \( E. \) coli K1, \( E. \) coli K5, \( M. \) haemolytica, \( P. \) multocida A:1, \( P. \) multocida B:2, \( B. \) mallei, and \( B. \) pseudomallei (4, 7, 10, 13, 22, 29, 34). In some cases, the \( hcsA \) and \( hcsB \) homologs are present as an independent unit in a distinct region of the capsule gene complex, which is analogous to the arrangement in \( H. \) influenzae (for example, \( lipA \) and \( lipB \) in region B in \( N. \) meningitidis). In other cases the \( hcsA \) and \( hcsB \) homologs are interspersed among other capsule-related genes, as illustrated by the \( kpsS \) and \( kpsC \) genes at the end of the \( kpsFEDUCS \) gene cluster in region 1 in \( E. \) coli K1 and \( E. \) coli K5.

In initial studies of the \( N. \) meningitidis \( lipA \) and \( lipB \) genes,
the cloned meningococcal capsule gene complex (cps) was examined in *E. coli* K-12 (13). In this heterologous background, deletion of the *lipA* and *lipB* genes resulted in intracellular accumulation of capsule polymers lacking a phospholipid substitution, leading to the proposal that the *lipA* and *lipB* gene products mediate polysaccharide lipidation prior to transport of capsule polymers to the bacterial surface (13). However, more recently, Tzeng et al. generated *lipA* and *lipB* mutations in *N. meningitidis* strain NMB and observed intracellular accumulation of capsule polymers that were clearly lipidated (40). Interestingly, studies of *E. coli kpsS* and *kpsC* have also yielded mixed results. In particular, in *E. coli* K-12 carrying the complete K5 capsule gene cluster on a plasmid, a *kpsS* insertion mutation and a *kpsC* deletion mutation resulted in cytoplasmic accumulation of capsule polysaccharide lacking the phosphatidic-3-deoxy-manno-2-octulosonic acid substitution (5). In contrast, Cieslewicz and Virm found that Tn10 insertion mutations in either *kpsC* or *kpsS* in the chromosome in an *E. coli* K-12-K1 hybrid strain were associated with intracellular accumulation of capsule polymers with wild-type lipidation (8). Together, these observations raise the possibility that the function of the *lipA* and *lipB* and the *kpsC* or *kpsS* genes may be altered in the K-12 genetic background. In the present study, we generated mutations in *hcsA* and *hcsB* in *H. influenzae* directly and observed accumulation of polysaccharide in the periplasm, suggesting a block in transport across the

FIG. 3. Immunoelectron micrographs of wild-type RM135, Rd, RM135*hcsA*, and RM135*hcsB*. PRP was immunolabeled with mouse monoclonal anti-PRP antibody followed by 18-nm colloidal gold-conjugated goat anti-mouse immunoglobulin G. (A) Wild-type RM135. (B) Rd. (C) RM135*hcsA*. (D) RM135*hcsB*. Bars, 0.2 μm.
outer membrane. Based on thin-layer chromatography and electrophoretic mobility of polysaccharide purified from the periplasm, the hcsA and hcsB mutations had no effect on lipidation.

Fractionation of polysaccharide in the hcsA, hcsB, and hcsA hcsB mutants revealed reduced quantities of surface-localized polysaccharide with inactivation of hcsA and a complete absence of surface-localized polysaccharide with inactivation of either hcsB alone or hcsA and hcsB together. These results suggest that the HcsA and HcsB proteins have distinct functions involved in transport of polysaccharide from the

Fractionation of polysaccharide in the hcsA, hcsB, and hcsA hcsB mutants revealed reduced quantities of surface-localized polysaccharide with inactivation of hcsA and a complete absence of surface-localized polysaccharide with inactivation of either hcsB alone or hcsA and hcsB together. These results suggest that the HcsA and HcsB proteins have distinct functions involved in transport of polysaccharide from the

### TABLE 2. Bacteremia in infant rats after intraperitoneal inoculation with wild-type RM135, RM135hcsA, RM135hcsB, or RM135hcsA-hcsB

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Strain</th>
<th>Bacteremia at a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>1</td>
<td>RM135</td>
<td>$1.5 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>RM135hcsA</td>
<td>&lt;100</td>
</tr>
<tr>
<td></td>
<td>RM135hcsB</td>
<td>&lt;100</td>
</tr>
<tr>
<td></td>
<td>RM135hcsA-hcsB</td>
<td>&lt;100</td>
</tr>
<tr>
<td>2</td>
<td>RM135</td>
<td>$8.0 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>RM135hcsA</td>
<td>&lt;100</td>
</tr>
<tr>
<td></td>
<td>RM135hcsB</td>
<td>&lt;100</td>
</tr>
<tr>
<td></td>
<td>RM135hcsA-hcsB</td>
<td>&lt;100</td>
</tr>
</tbody>
</table>

a Numbers represent geometric means of the numbers of CFU/ml of blood. Limit of detection was 100 CFU/ml. ND, not done.
periplasm to the bacterial surface. In considering possible
functions, it is notable that the predicted amino acid sequences of HcsA and HcsB lack typical signal sequences, arguing either that they are cytoplasmic proteins or that they are localized in the membrane or the periplasm via a Sec-independent mech-
anism. Future studies of HcsA and HcsB will assess their cel-
lular localization and their relationship to the ABC transporter
comprised of the *H. influenzae* bexA, bexB, bexC, and bexD gene
products.

In approximately 98% of *H. influenzae* type b isolates, the cap region contains two tandem copies of the cap b locus, including one copy that is intact and a second copy that is complete except for a partial deletion of bexA (1). In the remaining 2% of *H. influenzae* type b isolates, a single copy of the cap b locus is present (1). In earlier studies, Kroll and Moxon compared capsule production in strains with one copy or two copies of the cap b locus and observed a gene dosage effect, with twice as much polysaccharide capsule associated with two copies of the cap b locus (20). Interestingly, despite differences in capsule production, all strains were capable of producing sustained bacteremia and meningitis in the infant rat model, suggesting that one copy of the cap b locus is sufficient for virulence (20). In this context, it is noteworthy that our studies of RM1356c are revealed appreciable quantities of surface-associated polysaccharide but complete sensitivity to complement-mediated killing and avirulence in infant rats. These findings suggest that a certain threshold level of poly-
saccharide capsule on the bacterial surface is required for intravascular survival and virulence.

To summarize, our results demonstrate that the hcsA and hcsB genes in *H. influenzae* type b are essential for efficient transport of capsular polysaccharide across the bacterial outer membrane and for virulence in experimental animals. Given that homologs of hcsA and hcsB are present in many encapsu-
lated bacterial pathogens, the hcsA and hcsB gene products may be viable targets for antimicrobial development.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants RO1-AI044167, RO1-AI049322, and RO1-DC02873 to J.W.S.G.

We thank Wendy Beatty and Darcy Gill for assistance with electron microscopy and David Haslam for assistance with thin-layer chromato-

REFERENCES


