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The *Haemophilus influenzae* Type b *hcsA* and *hcsB* Gene Products Facilitate Transport of Capsular Polysaccharide across the Outer Membrane and Are Essential for Virulence

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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).

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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 IS*1016* elements.

The three functionally distinct regions in the *cap* b locus are referred to as regions 1 to 3. Region 1 contains genes designated *bexA*, *bexB*, *bexC*, and *bexD* 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 (*wcbA* and *wcbO*), *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 serotype 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 longtime laboratory strain and is a spontaneous capsule-deficient mutant of a type d isolate, containing an IS*1016* 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 Tn*903* and was obtained from Pharmacia (43). The plasmids pUC19 and pACYC184 were obtained from New England Biolabs and have been described previously (6, 46).

H. influenzae strains were grown in brain heart infusion (BHI) broth supplemented with hemin and NAD, on BHI agar supplemented with NAD 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 chloramphenicol 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 digestions, gel electrophoresis, and PCR were performed according to standard techniques (31). Plasmids were introduced into *E. coli* by chemical transformation (31). Linearized DNA was introduced into *H. influenzae* made competent using the M-IV method of transformation (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 construct, the kanamycin cassette from pUC4K was introduced into a unique SalI site in the IS*1016* element downstream of the *hcsB* gene. As a control, an analogous construct containing wild-type region 3 and a kanamycin cassette in the IS*1016* 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 pUC18K into the 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 M-IV 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 immunosorbent 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 Biologics), 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% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) phosphate buffer, pH 7.2, for 1 h at 4°C. Subsequently, 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% ethanol for 30 min at −20°C, 90% ethanol for 30 min at −20°C, 1:1 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 immunolabeled 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 primary antibody was omitted. Controls were consistently negative at the concentration 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 collected 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 centrifugation 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 A_{665}, 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. Bactericidal assays were performed 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

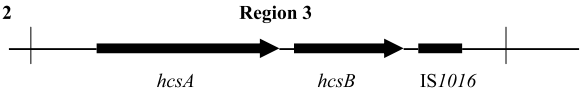

FIG. 1. Diagram of region 3 of the *H. influenzae* type b *cap* b locus, showing the locations of *hcsA* and *hcsB* relative to region 2 and the flanking IS*1016* element.

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 $\sim 10^3$ 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 IS*1016* 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* (RM135*hcsA*), an *hcsB* mutant with a kanamycin cassette inserted into *hcsB* (RM135*hcsB*), and an *hcsA hcsB* double mutant with a kanamycin cassette inserted into *hcsA* and a chloramphenicol cassette inserted into *hcsB* (RM135*hcsA-hcsB*). To facilitate recovery of the *hcsA* in-frame deletion mutant, we introduced a kanamycin cassette into the IS*1016* 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 IS*1016* 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, RM135*hcsA* displayed very weak agglutination, and RM135*hcsB* and RM135*hcsA-hcsB* displayed no agglutination, results comparable to observations with nonencapsulated 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 RM135*hcsA*, RM135*hcsB*, and RM135*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 RM135*hcsA*, and no detectable released polysaccharide in RM135*hcsB* and RM135*hcsA-hcsB*.

To extend these results, we used a murine monoclonal antibody against PRP and performed immunoelectron microscopy on thin sections from wild-type RM135, RM135*hcsA*, RM135*hcsB*, 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, RM135*hcsA* had wild-type levels of labeling in the cytoplasm and periplasm and a significant decrease in labeling on the bacterial surface (Fig. 3C), and RM135*hcsB* 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, RM135*hcsA*, RM135*hcsB*, and RM135*hcsA-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 against any defect in lipid modification of 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).

TABLE 1. Latex agglutination of wild-type RM135, RM135*hcsA*, RM135*hcsB*, and RM135*hcsA-hcsB* with latex particles coated with antibody against PRP[a]

Strain	Latex agglutination[b]
RM135	+++
RM135*hcsA*	+
RM135*hcsB*	−
RM135*hcsA-hcsB*	−
Rd	−

[a] PRP is the *H. influenzae* type b polysaccharide.
[b] Levels of agglutination were graded as nonexistent (−), weak (+), moderate (++), and strong (+++).

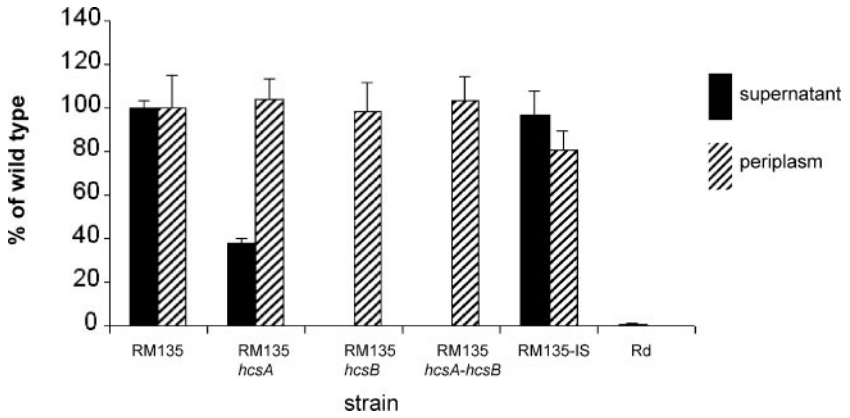

FIG. 2. Quantity of PRP (the *H. influenzae* type b polysaccharide capsule) in the periplasms and culture supernatants of wild-type RM135, RM135*hcsA*, and RM135*hcsB* and of RM135*hcsA-hcsB*, RM135-IS, and Rd. PRP content was measured by ELISA, and values were compared to values for wild-type RM135, which were set at 100%. RM135-IS is a control strain that contains intact *hcsA* and *hcsB* genes and harbors a kanamycin cassette in the IS*1016* element downstream of *hcsB* (analogous to the kanamycin cassette in the IS*1016* element in strain RM135*hcsA*). Rd is a control strain that lacks capsule genes.

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 littermates, 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 the *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,

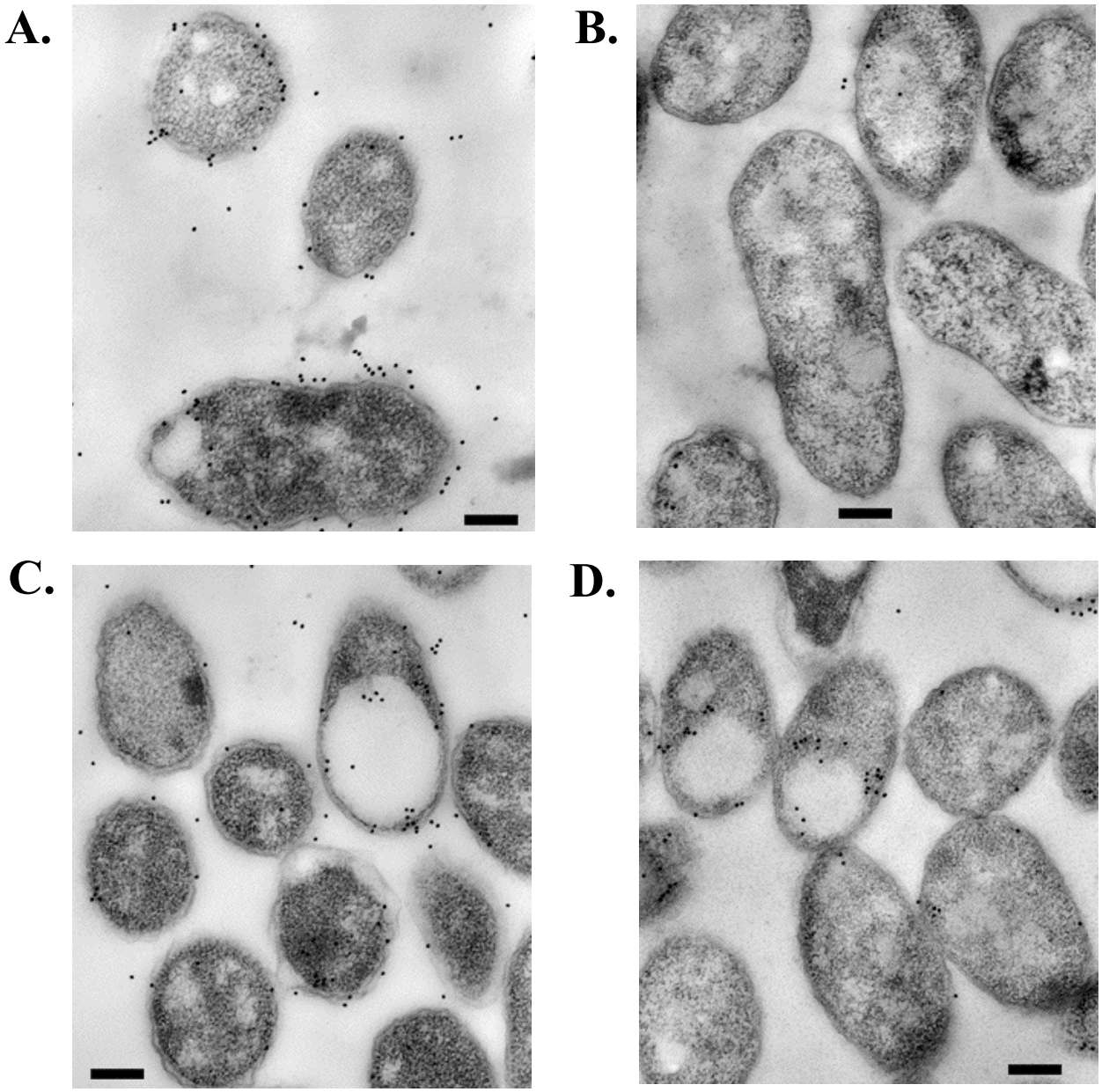

FIG. 3. Immunoelectron micrographs of wild-type RM135, Rd, RM135*hcsA*, and RM135*hcsB*. PRP was immunolabeled with mouse monoclonal anti-PRP antibody followed by 18-nm colloidal gold-conjugated goat anti-mouse immunoglobulin G. (A) Wild-type RM135. (B) Rd. (C) RM135*hcsA*. (D) RM135*hcsB*. Bars, 0.2 μm.

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 Vimr found that Tn*10* 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

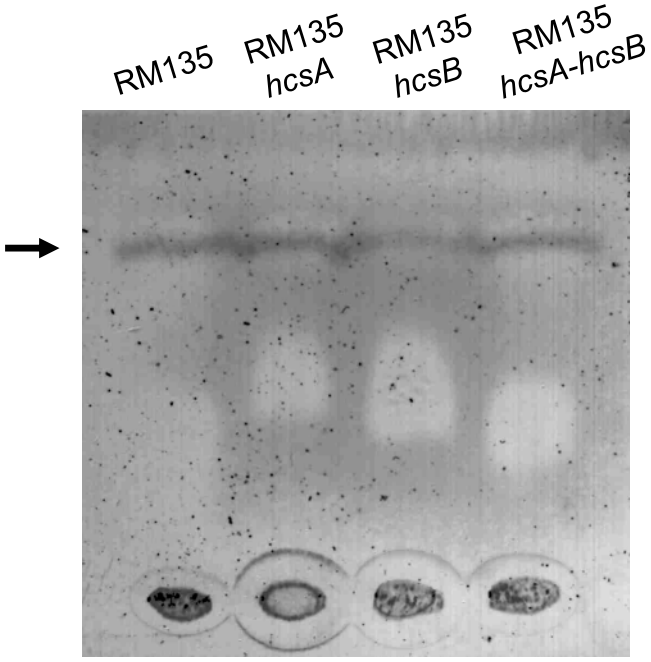

FIG. 4. Thin-layer chromatography of polysaccharide samples purified from the periplasms of wild-type RM135, RM135*hcsA*, RM135*hcsB*, and RM135*hcsA-hcsB*. For each sample, similar quantities of polysaccharide were spotted onto a gel-coated aluminum plate, and chromatography was performed with butanol-methanol-water (5:3:2). Polysaccharide was visualized by staining with orcinol-ferric chloride. The arrow indicates the polysaccharide.

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.

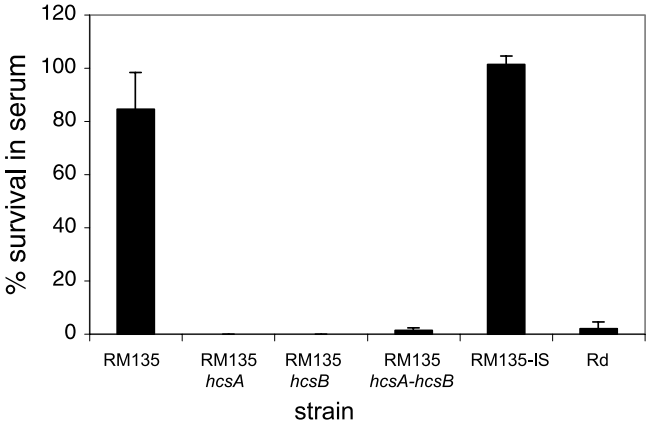

FIG. 5. Serum resistance of wild-type RM135, RM135*hcsA*, and RM135*hcsB* and of RM135*hcsA-hcsB*, RM135-IS, and Rd. Samples were incubated in 20% human serum for 30 min at 37°C. Percent survival was determined as the ratio of viable counts after incubation in active serum to viable counts after incubation in heat-inactivated serum. RM135-IS is a control strain that contains intact *hcsA* and *hcsB* genes and harbors a kanamycin cassette in the IS*1016* element downstream of *hcsB* (analogous to the kanamycin cassette in the IS*1016* element in strain RM135*hcsA*). Rd is a control strain that lacks capsule genes.

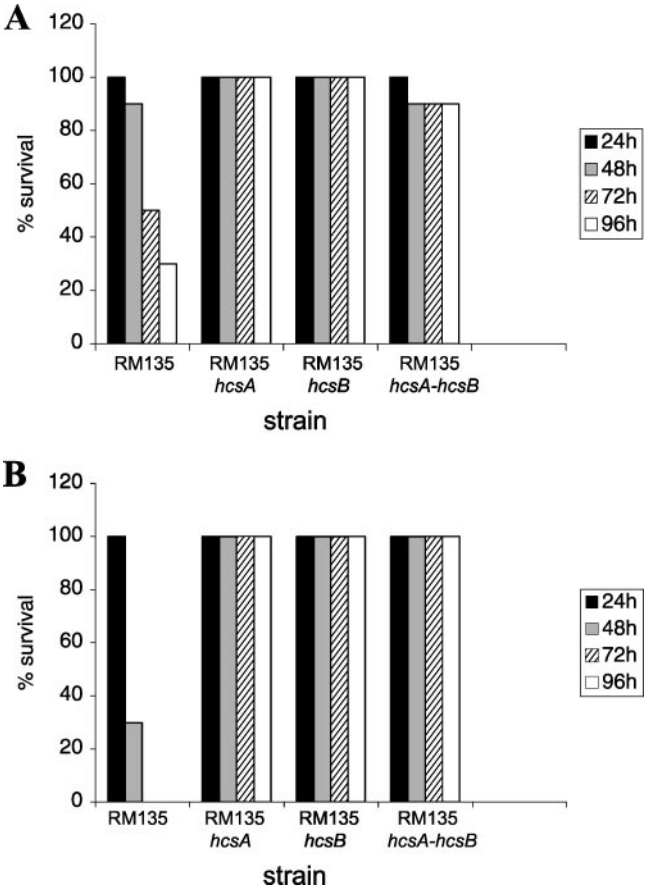

FIG. 6. Virulence of wild-type RM135, RM135*hcsA*, RM135*hcsB*, and RM135*hcsA-hcsB* in infant rats after intraperitoneal inoculation. Survival was measured at 24, 48, 72, and 96 h after inoculation. (A) Plot of the results of experiment 1. (B) Plot of the results of experiment 2.

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, RM135*hcsA*, RM135*hcsB*, or RM135*hcsA-hcsB*

Expt no.	Strain	Bacteremia at[a]:		
		24 h	48 h	96 h
1	RM135	1.5×10^7	3.6×10^8	7.0×10^4
	RM135*hcsA*	<100	<100	<100
	RM135*hcsB*	<100	<100	<100
	RM135*hcsA-hcsB*	<100	<100	<100
2	RM135	8.0×10^7	9.0×10^8	ND
	RM135*hcsA*	<100	<100	<100
	RM135*hcsB*	<100	<100	<100
	RM135*hcsA-hcsB*	<100	<100	<100

[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 mechanism. Future studies of HcsA and HcsB will assess their cellular 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 RM135*hcsA* 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 polysaccharide 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 encapsulated bacterial pathogens, the *hcsA* and *hcsB* gene products may be viable targets for antimicrobial development.

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