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Genetic Analysis of the Capsule Locus of *Haemophilus influenzae* Serotype f

Sarah W. Satola, Patricia L. Schirmer, and Monica M. Farley*

Atlanta Veterans Affairs Medical Center and Department of Medicine, Emory University School of Medicine, Decatur, Georgia 30033

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A 19-kb DNA region containing genes involved in the biosynthesis of the capsule of *Haemophilus influenzae* serotype f (Hif) has been cloned and characterized. The Hif *cap* locus organization is typical of group II capsule biosynthetic loci found in other *H. influenzae* serotype b bacteria and other gram-negative bacteria. However, the Hif *cap* locus was not associated with an IS*1016* element. Three new open reading frames, Fcs1, Fcs2, and Fcs3, were identified in the Hif capsule-specific region II. The chromosomal location of the Hif *cap* locus and the organization of the flanking sequences differed significantly from previously described division I *H. influenzae* serotypes.

Many bacterial species produce an extracellular polysaccharide capsule (CPS), which contributes to the pathogenicity of these organisms. The capsule of *Haemophilus influenzae*, a human pathogen causing disease in both adults and children, is important in establishing and maintaining invasive disease such as bacteremia and meningitis by conferring bacterial resistance to complement-mediated phagocytosis (28). *H. influenzae* is serotyped based on the presence of six antigenically distinct CPS (types a to f) (31). *H. influenzae* strains that lack CPS are classified as nontypeable or nonencapsulated. Each of the six capsule types can be identified by both serologic (31) and molecular methods (5). Nonencapsulated strains fail to react specifically with antisera against capsular serotypes a through f or to hybridize with capsule-specific genes. Encapsulated strains of *H. influenzae* can be divided further into two phylogenetic divisions, I and II, based on multilocus enzyme electrophoresis typing (30).

Prior to routine immunization of infants with conjugate *H. influenzae* serotype b (Hib) vaccine, Hib was the most common *H. influenzae* serotype causing invasive disease (>90%), and *H. influenzae* serotype f (Hif), although rare, was the second most common (44). With the postvaccine reduction in Hib disease, the proportion of invasive *H. influenzae* disease caused by Hif rose from 1% in 1989 to 17% in 1994 (40). Invasive Hif disease now contributes to a substantial proportion of all invasive *H. influenzae* disease (39, 43) and has been associated with infections such as bacteremia, endocarditis, and a mycotic aneurysm (1, 8, 35). Recently, Hif was isolated from a case of rapidly fatal sepsis in an otherwise healthy child (45).

Strong homology of the genes involved in capsule biosynthesis from a number of gram-negative bacteria suggests a common molecular origin. Although the genetic elements of *H. influenzae* capsule biosynthesis have been most extensively characterized in Hib (13, 14, 19, 34), the *H. influenzae cap* locus for all six serotypes contains the same three functionally unique regions, I, II, and III (18, 20). Regions I and III are common to all six capsular types and contain genes involved in the exportation and processing of the capsular material. Region I genes (*bexDCBA*) code for an ATP-driven capsule export apparatus (16). Region II contains serotype-specific biosynthesis genes that appear to be unique to each of the six capsule types (34, 41). Region III genes, *hcsA* and *hcsB*, recently identified in Hib, appear to be involved in postpolymerization steps (10, 25, 34). In order to further understand the role of the Hif capsule, we identified and characterized the genetic locus for the capsule biosynthetic genes of *H. influenzae* serotype f.

Cosmid sequencing and assembly of the Hif *cap* locus. A cosmid library of the American Type Culture Collection (Manassas, Va.) *H. influenzae* serotype f strain 700222 (40) *Sau*3A-partially digested chromosomal DNA was constructed in *Escherichia coli* XL1-Blue MR cells using the GigaPack III XL packaging system (Stratagene, La Jolla, Calif.) as described by the manufacturer. Cosmids pTS66 and pTS67 were found to contain DNA specific to region II of the Hif *cap* locus by PCR for the presence of Hif *cap*-specific region II DNA using oligonucleotide primers f1 and f2 as previously described (5) (Table 1). pTS66 and pTS67 were further characterized by Southern hybridization analysis (33) using a Hif *cap*-specific region II probe (f1 to f2) as well as with probes generated by PCR from the Hib *cap* locus in conserved regions I (Hi-1 to Hi-2) (5) and III (ORF6 [24] to Reg3b; 5′-GCAATGGCACATCATGCAC-3′).

Cosmid pTS66 was sent to Lark Technologies, Houston, Tex., for sequencing using randomly sheared cosmid DNA in a pUC19 shotgun library (4). DNA sequencing was performed using a BigDye Terminator Cycle Sequencing Ready Reaction kit, with AmpliTaq DNA polymerase FS (Applied Biosystems, Foster City, Calif.) and analyzed using an ABI377 apparatus from Lark Technologies. Sequencing reads provided approximately 8.8-fold coverage of the cosmid clone. Oligonucleotide primers were created to close gaps using PCR and sequencing directly from a second cosmid, pTS67, using primers f1 and f2 (5) and primer walking as needed.

Sequencing and computer assembly using the Phred-Phrap package (CodonCode Corporation, Dedham, Mass.) and

* Corresponding author. Mailing address: Atlanta VA Medical Center, Medical Research Service 151, 1670 Clairmont Rd., Decatur, GA 30033. Phone: (404) 728-7688. Fax: (404) 329-2210. E-mail: mfarley@emory.edu.

TABLE 1. Oligonucleotide primer pairs used in RT-PCR of Fcs1, Fcs2, and Fcs3, analysis of the adjacent Hif *cap* DNA, and as probes in Southern blot hybridization

Oligonucleotide name	Sequence (5′-3′)	Gene(s) (size [bp])	Reference
f1	GCTACTATCCAAGTCCAAATC	Hif *cap* specific (450)	5
f2	CGCAATTATGGAAGAAAGCT		5
HI-1	CGTTTGTATGATGTTGATCCAGAC	*bexA* (343)	5
HI-2	TGTCCATGTCTTCAAAATGATG		5
ORF6	GTTATTACTTGCGTGATCGT	*hcsB* (311)	24
Reg3b	GCAATGGCACATCATGCAC		This study
Fcs1-a	CGTGACGCATTGAATAATAAATAC	*fcs1* (720)	This study
Fcs1-b	CTGTGCCTTAGCATTTGCGGAG		This study
Fcs2-a	GTTAATGCAAAAGATCAAG	*fcs2* (1,149)	This study
Fcs2-b	CATAGTTATACCATGCTGTAACC		This study
Fcs3-a	CCGGGAATCCATAGAGTTCA	*fcs3* (992)	This study
Fcs3-b	GAGCATTCTATAATATGCAATGG		This study
Fcs1-2-a	CGCTCCGCAAATGCTAA	*fcs1-fcs2* (323)	This study
Fcs1-2-b	TTCTTCACTGTTATCAGGGCTAC		This study
Fcs2-3-a	CGATGAATTATCTCTATCTTACAAGG	*fcs2-fcs3* (417)	This study
Fcs2-3-b	CTGATGGTGTATGAACTCTATG		This study
capfSodC	CATGCGCATTTTCCACGCCAGC	*bexA-sodC* (875)	This study
capfBexA	GGGATTGAGGCGCAATGATTCG		This study
capfMerT	CCTTTTGGGTTGCCATAGCC	*merT-sodC* (774)	This study
SodC 3970-2951	CACTCAGATCATCCTGCTCC		This study
capf region III	CAGTACGAGTGGTATTTCAG	*hcsB-HI1637* (700)	This study
HI1637	AAATTTCCATTATGGGAAACG		This study

LaserGene version 5.03 (DNASTAR, Inc., Madison, Wis.) of pTS66 and pTS67 resulted in a contiguous region of 19,576 bp from the Hif chromosome. Nucleotide and amino acid homology comparisons using GenBank DNA and protein sequence databases of the National Center for Biotechnology Information BLAST network server (3) and TBLASTX analysis (3) identified a total of 15 open reading frames (ORFs), 9 of which appear to be specifically associated with the Hif *cap* locus (Fig. 1).

From analysis of the sequence data and ORFs, it is apparent that the Hif *cap* locus is composed of three distinct regions (I, II, and III), a genetic organization common to the Hib *cap* locus as well as other group II capsule biosynthetic clusters (Fig. 1). However, the capsule locus of the serotype f capsule includes genes that are not found in previously reported capsule loci of *H. influenzae* serotypes a and b (7, 34, 41), and it is not associated with an IS*1016* element. The DNA that flanks the Hif *cap* locus does not appear to be directly involved in capsule production, although functions associated with the capsule cannot be excluded.

Sequence analysis of Hif *cap* locus regions I and III. Region I contains four ORFs with homology to Hib, *bexA, bexB, bexC*, and *bexD* (Fig. 1), as well as other region I capsule genes from *E. coli*, *Neisseria meningitidis, Actinobacillus pleuropneumoniae*, and *Mannheimia haemolytica* (34). Because the encoded polypeptides from each of these four genes were nearly iden-

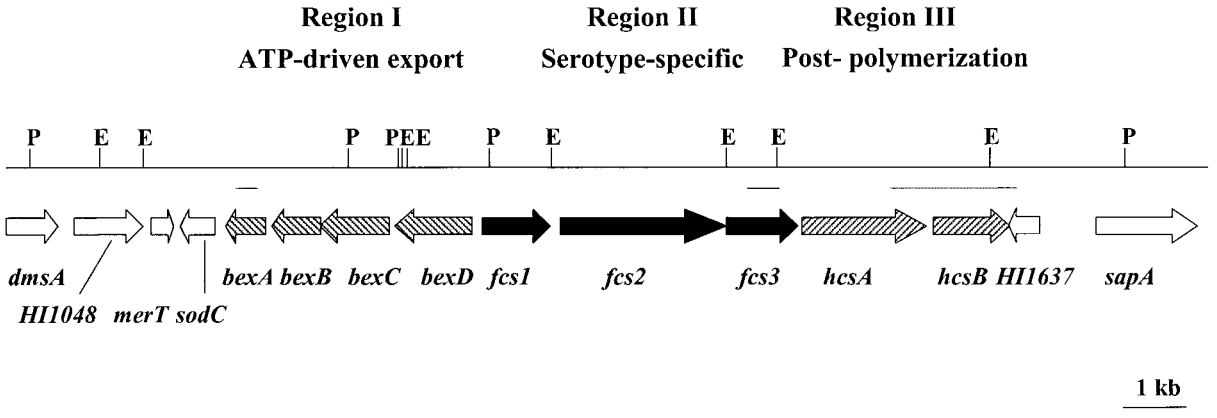

FIG. 1. Genetic organization of the Hif capsule locus and adjacent DNA. Arrows indicate genes and ORFs. Region I contains four genes, on the opposite strand, homologous to those found in Hia and Hib, *bexDCBA* (left-hatched arrows). Region II is comprised of three serotype-specific genes designated *fcs1*, *fcs2*, and *fcs3* (black arrows). Region III has two genes, *hcsA* and *hcsB*, also found in the *cap* locus of Hib (right-hatched arrows). Six additional ORFs outside of the Hif *cap* locus are noted with white arrows. Vertical lines show cleavage sites for restriction endonucleases *Eco*RI (E) and *Pst*I (P). Horizontal lines below the restriction map show the locations and sizes of the probes used to identify cosmid clones with the Hif *cap* locus.

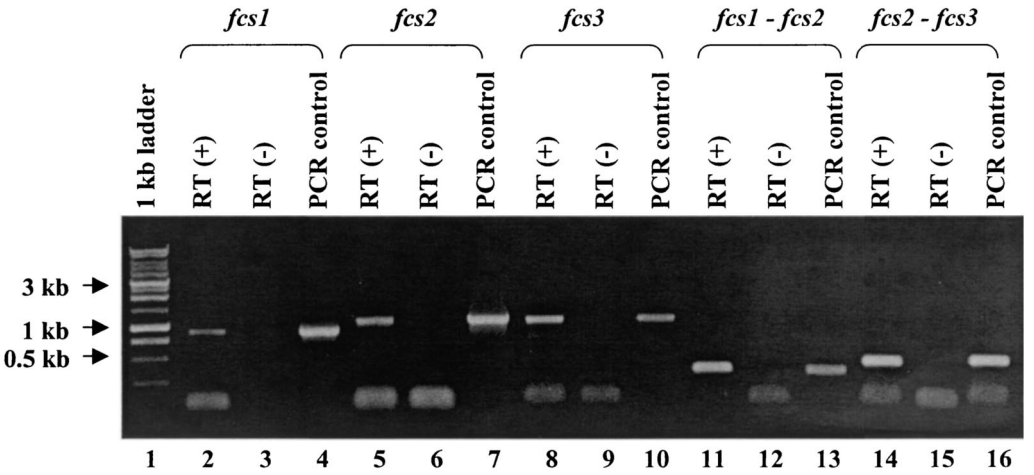

FIG. 2. *fcs1, fcs2*, and *fcs3* expression detected in log-phase Hif 700222 cells and shown to be cotranscribed by RT-PCR. Lane 1, 1-kb standard marker (Promega Corp., Madison, Wis.); lanes 3, 6, 9, 12, and 15, RT negative controls; lanes 4, 7, 10, 13, and 16, positive controls with chromosomal DNA from Hif 700222 as template; lane 2, Fcs1-a and Fcs1-b RT (+); lane 5, Fcs2-a and Fcs2-b RT (+); lane 8, Fcs3-a and Fcs3-b RT (+); lane 11, Fcs1-2-a and Fcs1-2-b RT (+); lane 12, Fcs1-2-a and Fcs1-2-b RT (-); lane 13, Fcs1-2-a and Fcs1-2-b as PCR control; lane 14, Fcs2-3-a and Fcs2-3-b RT (+). Primers and product sizes are listed in Table 1.

tical (89 to 94% identity) to the region I gene products from Hib, they were named *bexDCBA*. The BexD protein in Hif appeared to be slightly less conserved than BexA, BexB, and BexC, with only 89% identity to the Hib counterpart.

The nucleotide sequence in region III contained two genes, *hcsA* and *hcsB* (for *Haemophilus* capsule synthesis), recently sequenced from the Hib capsule locus (34). HcsA and HcsB are highly conserved between the Hib and Hif capsule loci at 97 and 91% similarity, respectively (Fig. 1).

Sequence analysis of Hif *cap* locus region II. Region II, the central, serotype-specific region of the Hif *cap* locus, included three ORFs. The G+C content of the DNA in region II was 26%, significantly lower than the 38 and 39.7% G+C content of regions I and III and the overall G+C content of *H. influenzae* DNA. This may indicate that region II DNA was more recently acquired by the bacterium. The three ORFs had no homology with Hib Bcs1 to Bsc4 and were designated Fcs1, Fcs2, and Fcs3 for f capsule-specific gene products (Fig. 1).

The deduced amino acid sequence of Fcs1 was compared to those of other known sequences using BLAST (2), and homology with other capsule polysaccharide genes was detected. Fcs1 is 68% similar to Cps1A, capsular polysaccharide synthesis A from *A. pleuropneumoniae* (GenBank accession no. AAM69355), 67% similar to LcbA, a putative *N. meningitidis* serogroup L region D capsule protein (GenBank accession no. AAF21950), and 54% similar to the *N. meningitidis* serogroup X strain M7575 putative capsule biosynthesis protein XcbA (39a). Less similarity (49%) was seen between Fcs1 and the C-terminal half of SacB, an *N. meningitidis* serogroup A capsule biosynthesis gene (GenBank accession no. AAC38286) (36).

Sequence homology search with Fcs2 for known proteins detected two putative conserved domains. The N-terminal amino acids 8 to 178 of Fcs2 were 98.8% identical to the consensus sequence from the conserved domain database (26, 27) protein family glycosyltransferase 2, Pfam00535. This is a highly diverse family of proteins with the capacity to transfer sugar from UDP-glucose, UDP-*N*-acetylgalactosamine, GDP-mannose, and CDP-abequose to a range of substrates that includes cellulose, dolichol phosphate, and teichoic acids. C-terminal amino acids 516 to 887 were 94.1% identical to the consensus sequence from COG1887, a cluster of orthologous groups (COG) (37, 38) of putative glycosl-glycerophosphate transferases involved in teichoic acid biosynthesis.

CDART (conserved domain architecture retrieval tool) (11) identified six proteins with similar domain architecture. Fcs2 is 54% similar to Cps1B and 45% similar to Cps1C, capsular polysaccharide synthesis-B and -C from *A. pleuropneumoniae* (GenBank accession no. AAM69356 and AAM69358, respectively). Fcs2 is 46% similar to EpsJ, a *Lactococcus lactis* subsp. *cremoris* glycosyltransferase involved in synthesis of the polysaccharide backbone (GenBank accession no. NP_053024) (42), 48% similar to GgaB, a *Bacillus subtilis* minor teichoic acids biosynthesis protein (GenBank accession no. P46918) (21), and 40% similar to LcbB, a putative bifunctional polymerase from *N. meningitidis* serogroup L (GenBank accession no. AAF21951). Fcs2 is also 41% similar to a hypothetical protein (Acs3), the third of four ORFs found in region II of *H. influenzae* serotype a strain RM107 (GenBank accession no. S49240). Finally, no sequence homology was found for the third ORF, Fcs3, with known proteins as detected by BLAST (2). Functional characterization of Fcs1, Fcs2, and Fcs3 remains to be established.

Region II *fcs1*, *fcs2*, and *fcs3* transcription. To determine whether *fcs1*, *fcs2*, and *fcs3* were transcribed and organized as an operon, we performed reverse transcription-PCR (RT-PCR) experiments with whole-cell RNA obtained from exponential broth cultures of Hif 700222 with RNeasy (Qiagen, Valencia, Calif.) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif.) and PCR reagents from Applied Biosystems at an annealing temperature of 55°C. Transcription of *fcs1* (Fig. 2, lane 2), *fcs2* (Fig. 2, lane 5), and *fcs3* (Fig. 2, lane 8) was confirmed by RT-PCR using primers designed from their coding sequences (Table 1). An RT-PCR product of 323 bp was amplified between *fcs1* and *fcs2* using a primer in the

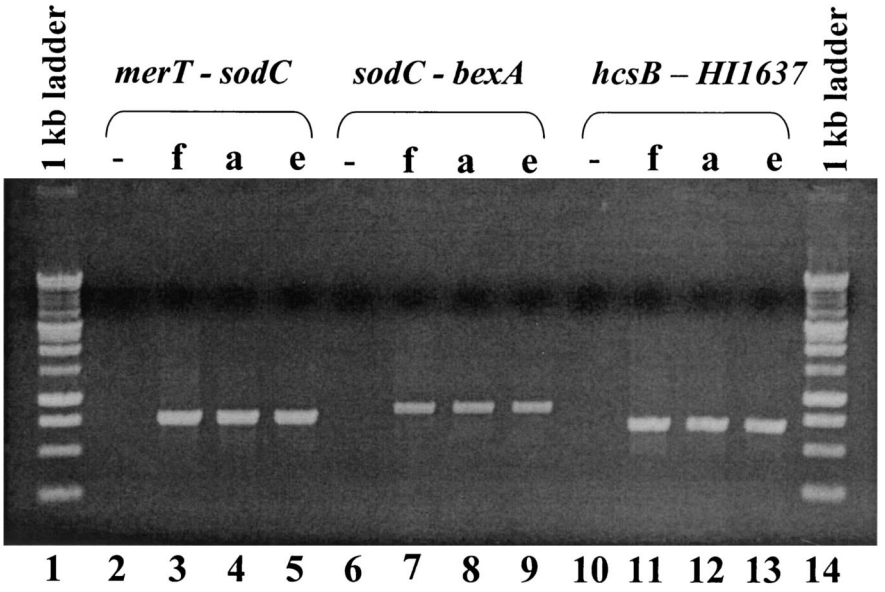

FIG. 3. PCR analysis of the end junctions and outside DNA of the Hif *cap* locus and *H. influenzae* serotypes a and e. Lanes 1 and 14, 1-kb standard marker (Promega Corp.). Hif 700222 chromosomal DNA was amplified with primer pairs capfMerT and SodC 3970-2951 (lane 3), capfSodC and capfBexA (lane7), and capf region III and *HI1637* (lane 11). Hia ATCC 9006 chromosomal DNA was amplified with primer pairs capfMerT and SodC 3970-2951 (lane 4), capfSodC and capfBexA (lane 8), and capf region III and *HI1637* (lane 12). Hie ATCC 8142 chromosomal DNA was amplified with primer pairs capfMerT and SodC 3970-2951 (lane 5), capfSodC and capfBexA (lane 9), and capf region III and HI1637 (lane 13). Lanes 2, 6, and 10 are the no-template controls. Primers and product sizes are listed in Table 1.

coding sequence of *fcs2* (Fcs1-2-b) and a second primer in *fcs1* (Fcs1-2-a) during the amplification of the cDNA (Fig. 2, lane 11). An RT-PCR product of 417 bp was amplified between *fcs2* and *fcs3* using a primer in the coding sequence of *fcs3* (Fcs2-3-b) and a second in *fcs2* (Fcs2-3-a) during the amplification of the cDNA (Fig. 2, lane 14). These results indicate that the three region II genes, *fcs1, fcs2*, and *fcs3*, are cotranscribed on the same mRNA.

Chromosomal location of Hif *cap* locus. Sequence analysis of the DNA adjacent to the Hif *cap* locus within cosmid pTS66 identified six additional ORFs, five highly homologous to previously identified ORFs from *H. influenzae* Rd (GenBank accession no. NC_000907): *merT, sapA*, *HI1048*, *HI1637*, and an incomplete copy of *dmsA*. An additional gene, *sodC*, not found in the Rd genome, is located immediately adjacent to *bexA* and the 5′ junction of the Hif *cap* locus. The layout of the flanking sequences with respect to the Hif *cap* locus is shown in Fig. 1. HI1048 and HI1637 are hypothetical proteins (GenBank accession no. AAC22710 and AAC23282, respectively); *dmsA* (*HI1047*) encodes an anaerobic dimethyl sulfoxide reductase, chain A protein (GenBank accession no. AAC22706); *merT* (*HI1049*) encodes a mercuric ion transport protein (GenBank accession no. AAC22707); *sapA* (*HI1638*) encodes a peptide ABC transporter periplasmic protein (GenBank accession no. AAC23285); and *sodC* (GenBank accession no. M84012) encodes a copper-zinc superoxide dismutase (Cu,Zn-SOD).

Unlike the DNA flanking the Hib *cap* locus, which is contiguous in the *H. influenzae* Rd genome, the DNA outside of the Hif *cap* locus is found in two widely separated sections of the Rd genome. *dmsA* (*HI1047*), *HI1048*, and *merT* (*HI1049*) are found in section 100 of the Rd genome, while *HI1637* and *sapA* (*HI1638*) are in sections 151 and 152, respectively (*sodC* is not present in the Rd genome). Sections 100 and 151 to 152 are separated by 589,419 bp in Rd (GenBank accession no. NC_000907) (6). This finding was not necessarily unexpected. Rd, formerly an encapsulated *H. influenzae* serotype d strain, is a division I *H. influenzae* strain, and all *H. influenzae* serotype f strains belong to division II. Divisions I and II have been demonstrated to be widely separated phylogenetic divisions (30). Furthermore, *sodC* is present only in division II and not present in division I strains with the exception of serotype e, which shares features of both divisions I and II (15, 22).

To determine if the layout of the Hif *cap* locus and flanking sequences found on the cosmid clones was identical in the type f chromosome, PCR analysis of the end junctions of the Hif *cap* locus was performed using primers specific to region I, *sodC*, *merT*, region III, and *HI1637* (Table 1) and chromosomal DNA from Hif strain 700222. The same layout of genes was observed in Hif strain 700222 and the sequenced cosmid pTS66 (data not shown).

We looked further at a collection of 69 Hif strains isolated from patients with invasive *H. influenzae* disease. Isolates were confirmed to be *H. influenzae* serotype f strains by serologic and molecular methods (5). All 69 Hif isolates were examined by one-colony PCR (32) using primer pairs as described above and were found to have the Hif *cap* locus in the identical location as that of Hif 700222 and associated with the same flanking genes (data not shown). This suggests that the chromosomal location of the Hif *cap* locus is highly conserved and is as presented in Fig. 1.

It has been suggested that all division I *H. influenzae* strains have the same chromosomal *cap* locus location, between direct repeats of IS*1016* (34). It is possible that all *cap* loci in *H. influenzae* division II strains are located between *sodC* and

HI1637. Previously, we noted that *H. influenzae* serotype a strain ATCC 9006 and *H. influenzae* serotype e strain 8142 did not have the same *cap* locus chromosomal location shared by most division I *H. influenzae* strains (34). Although most Hia belong to division I, some have been classified as division II. Hie, while classified as division I, has the greatest genetic distance from all other division I *H. influenzae* (29) and is the only division I *H. influenzae* reported to have *sodC* (22). We also examined the end junctions of Hia strain 9006 and Hie strain 8142 using primers specific to region I, *sodC*, *merT*, region III, and HI1637 (Table 1). Both the Hia and the Hie strains had the same PCR profile as the Hif strains, suggesting the identical chromosomal location of the *cap* loci in these strains (Fig. 3). Further studies will be necessary to determine whether the *cap* locus location is uniformly conserved among all *H. influenzae* division II strains (all Hif, and a minority of Hia and Hib) and division I Hie strains.

The location of *sodC* adjacent to *bexA* and the capsule locus of *H. influenzae* NCTC8468, a division II type b strain, have been previously reported (15). Hif *sodC* is 98.4% identical to the Hib *sodC* described in detail by Kroll et al. (15) and also located adjacent to *bexA*. The identical palindromic pairs that match the 11-bp *Haemophilus* DNA uptake motif seen 19 nucleotides downstream from the stop codon of Hib *sodC* (GenBank accession no. M84012) (17) are found at the same location downstream from the termination codon in the Hif *sodC*. The *sodC* gene encodes for Cu,Zn-SOD, a metalloenzyme catalyzing the conversion of superoxide radicals into hydrogen peroxide and oxygen (9, 12). Cu,Zn-SOD has been found in the periplasm of a wide range of gram-negative commensal and pathogenic bacteria, including *Haemophilus* species (22), and its role in virulence has been suggested for pathogenic bacteria (23). Unlike Fe-SODs and Mn-SODs, which are located in the cytosol and have a primary role of minimizing the effects from anaerobic respiration, Cu,Zn-SODs are exported from the cytosol to the periplasm or beyond. Since superoxide generated within the cytosol cannot cross the cytoplasmic membrane, this suggests that Cu,Zn-SODs provide protection from extracellular superoxide production by phagocytic host cells (23).

The possession of a polysaccharide capsule greatly enhances the virulence of *H. influenzae* and its ability to cause invasive disease. Although the Hib conjugate vaccine has been highly successful in dramatically reducing Hib invasive disease, occurrence of invasive disease due to other capsule serotypes as well as nontypeable *H. influenzae* has persisted and may be increasing in some populations. The apparent increase of Hif disease in the post-Hib vaccine era suggests the need for further characterization of *H. influenzae* serotype f. We report the genetic analysis of the Hif *cap* locus and its surrounding DNA. Future studies to better understand the functional activities of the capsule-specific genes *fcs1, fcs2*, and *fcs3* are warranted.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in GenBank under the accession no. AF549211.

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