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Complete Sequence of the *cap* Locus of *Haemophilus influenzae* Serotype b and Nonencapsulated b Capsule-Negative Variants

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The complete capsule (*cap*) loci from three *Haemophilus influenzae* strains, one serotype b (Hib) and two nonencapsulated b capsule-negative variants, were sequenced. Two new open reading frames, *hcsA* and *hcsB*, were identified in region III and thought to be involved in postpolymerization modification of the capsule. The location of the *cap* locus in the *Haemophilus influenzae* chromosome was identified within section 97 of the Rd genome (chromosomal coordinates 1074542 to 1086327) and found to be the same for the Hib and two Hib$^-$ strains as well as some other encapsulated division I *H. influenzae* strains.

Haemophilus influenzae is a pathogenic gram-negative bacterium responsible for a wide variety of infections in both children and adults, ranging from bronchitis to meningitis. *H. influenzae* is classified into two main groups: typeable (encapsulated) and nontypeable (nonencapsulated) strains. Six capsule serotypes have been described (a to f) (30). Prior to the introduction of *H. influenzae* serotype b (Hib) conjugate vaccines into routine use in infants in 1991, Hib caused more than 95% of all invasive *H. influenzae* infections in children in the United States (1, 31, 36). Nontypeable *H. influenzae* is most often responsible for localized respiratory tract infections (32).

Based on genetic analysis, two major phylogenetic divisions of *H. influenzae*, divisions I and II, have been described (27, 28). Division I consists of the majority of serotype a and b strains and all of serotype c, d, and e strains. Division II includes all of serotype f strains and some serotype a and b strains. All encapsulated *H. influenzae* strains, whether division I or II, contain common genes for the production of their respective polysaccharide capsules (*cap* genes) found within the *cap* locus (21). However, in division I strains, the *cap* locus is flanked by direct repeats of insertion element IS*1016* and is frequently amplified (5, 13, 17). Although often present elsewhere in the chromosome, IS*1016* does not appear to be physically associated with the *cap* genes in division II strains (17).

The *cap* loci for all serotypes consist of functionally unique regions I, II, and III (Fig. 1A) (17, 21). Regions I and III are common to all six capsular types and contain genes necessary for the processing and exportation of the capsular material. Homologs for the genes in these regions can be found in a number of other gram-negative bacteria including *Escherichia coli*, *Neisseria meningitidis*, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, and *Mannheimia haemolytica* (24). 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). Region III genes appear to be involved in capsule postpolymerization steps (10, 24).

The genetic elements of *H. influenzae* capsule production have been characterized most extensively in Hib (12, 13, 18). The majority of *cap* loci in Hib contain a partial duplication that results in two complete copies of regions II and III, one complete copy of region I, and a truncated copy of region I with a 1.2-kb deletion within the *bexA* gene and IS*1016* (Fig. 1B) (18, 19). The presence of an incomplete IS*1016-bexA* on the end of the duplicated Hib *cap* locus is thought to stabilize capsule production by reducing chances of recombination events that could result in loss of *cap* genes. However, if during a recombination event, a complete copy of the *cap* locus were lost, a truncated copy would remain and would contain a partially deleted *bexA* gene, the ATP-binding component which is essential for the exportation of the capsule material to the surface of the bacteria. The truncated copy results in a capsule-negative phenotype known as Hib$^-$ or b$^-$ (Fig. 1C) (12, 15).

Two Hib$^-$ strains were identified, one from a collection of clinical isolates from invasive *H. influenzae* disease (strain GA834) (Active Bacterial Core Surveillance of the Georgia Emerging Infections Program) (M. M. Farley, unpublished data) and the other from a survey of nasopharyngeal carriage (strain Hi 373) (C. Whitney, J. Elliott, and Y.-H. Yang, unpublished data). Both isolates were found by Southern blot hybridization analysis to have 2.1- and 2.7-kb *Eco*RI fragments when probed with a region II capsule b-specific probe and to lack a 9-kb *Eco*RI fragment (normally present in the wild-type Hib *cap* locus) when probed with a region I (*bexA*-specific) probe (Fig. 1B; data not shown) (5, 15).

We sequenced the entire *cap* locus from clinical Hib isolate Hi 1007 (7, 25) and the Hib$^-$ variants GA834 and Hi 373, described above. Hi 1007 was isolated from cerebrospinal fluid of a child with bacterial meningitis and was confirmed as serotype b by both serologic and molecular methods in our laboratory. PCR and primers based on the known sequence of the Hib *cap* locus were used to generate overlapping PCR products ranging from 500 bp to 6 kb to cover the entire *cap* locus. Primers and the region of the *cap* locus amplified are shown in Table 1. Each amplicon was subcloned into pCR4-TOPO (Invitrogen, Carlsbad, Calif.), and both strands of the insert from

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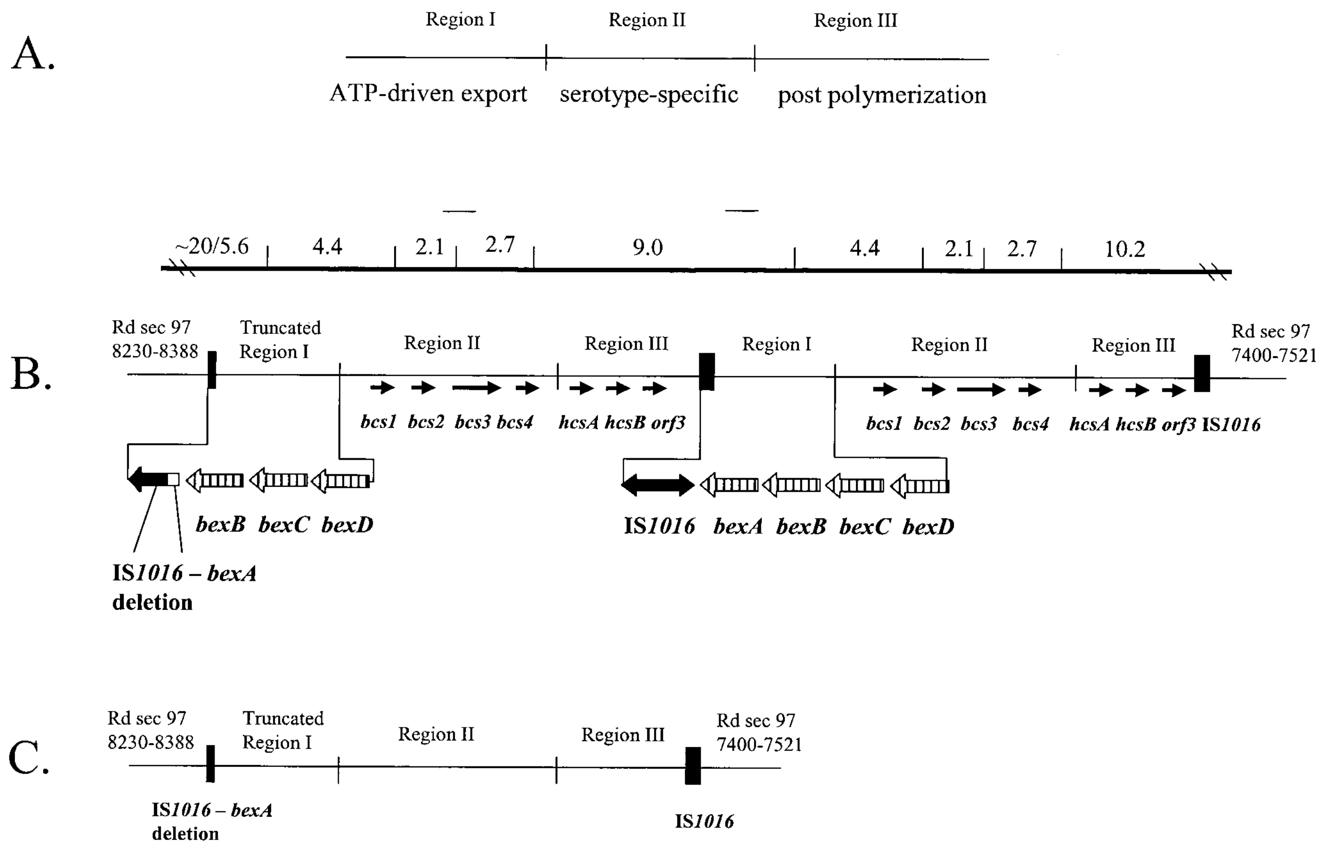

FIG. 1. (A) Common organization of *H. influenzae cap* locus. Regions I and III are shared among all encapsulated *H. influenzae* strains. Region II contains serotype-specific genes. (B) Partially duplicated *cap* locus of Hib Hi 1007 showing the truncated region I with the 1.2-kb deletion between IS*1016* and *bexA*. The top line represents an *Eco*RI restriction map with fragment sizes (in kilobases) shown; the line segments above the restriction map indicate the locations of probes used in Southern blot hybridization analysis to distinguish a duplicated Hib *cap* locus from a Hib$^-$ variant. (C) Organization of the *cap* locus in Hib$^-$ variants GA834 and Hi 373. Rd section 97 bp 7400 to 7521 and 8230 to 8388 correspond to Rd chromosomal coordinates 1081723 to 1081844 and 1082530 to 1082711, respectively.

each plasmid were sequenced by the Atlanta Veterans Affairs Medical Center/Emory University School of Medicine DNA core facility with an ABI 377 version 3.0 with D-rhodamine chemistry. The nucleotide sequences were analyzed with LaserGene, version 5.03, software programs SeqMan II, MapDraw II, and MegAlign II (DNASTAR, Inc., Madison, Wis.). Nucleotide and amino acid sequence homology comparisons were carried out with GenBank DNA and protein sequence databases by using the National Center for Biotechnology Information BLAST network server (3, 4).

Hib *cap* locus of Hi 1007. Although there is some nucleotide sequence from the Hib *cap* locus (accession no. X54987,

TABLE 1. PCR primers and products used for sequencing

Primer set[a]	Region amplified	Template(s)	Size (bp)
Rd97(8388)-bexB[b]	3′ end junction–*bexB*	Hi 373, GA834, Hi 1007	621
ISLOUT[b]-bexB[b]	IS*1016–bexB*	Hi 373, GA834, Hi 1007	345
bexBrev[a]-bexD1	*bexB–bexD*	Hi 373, GA834, Hi 1007	3,113
bexC2-b5	*bexC*–region II *orf3*	Hi 373, GA834, Hi 1007	7,487
b3[c]-orf6rev	Region II *orf3*–region III *orf2*	Hi 373, GA834, Hi 1007	5,985
ISLOUT[b]-bexB[b]	IS*1016-bexB* (bridge region)	Hi 1007	1,541
orf6[b]-capHI[c]	Region III–*bexA*	Hi 1007	3,381
orf6[b]-Rd97(7400)	Region III *orf2*–5′ end junction	Hi 373, GA834, Hi 1007	2,943
endregIII-Rd97(7400)	End region III–5′ end junction	Hi 373, GA834, Hi 1007	906

[a] Sequences of primers not described elsewhere are as follows: Rd97(8388), 5′-TTCCTAGTTTCCTACGTCAG-3′ (8388 corresponds to Rd chromosomal coordinate 1082711); bexBrev, 5′-CGTAAGTAACCACTGTATCGCC-3′; bexD1, 5′-CGCATAGAGGTGTGGTGGATTG-3′; bexC2, 5′-GTTGATAATCCGCAGTTTGTGTCG-3′; b5, 5′-CGTTTTTCAGCGGCGATCGC-3′; orf6rev, 5′-ACGATCACGCAAGTAATAAC-3′; endregIII, 5′-GGCCCTGTCTGCTTAATATC-3′; Rd97(7400), 5′-GCTTGGGTTCCTGTCTTGAG-3′ (7400 corresponds to Rd chromosomal coordinate 1081723).

[b] Previously described by Leaves et al. (23).

[c] Previously described by Falla et al. (6).

TABLE 2. Comparison of the proteins from the *cap* locus of *H. influenzae* to CPS proteins from other organisms

H. influenzae Hi 1007 region, protein	Similar protein (source)	Accession no.	% Identity	% Similarity
I, BexA	CpxA (*A. pleuropneumoniae*)	AAB64445	83	91
	Wzt (*M. haemolytica*)	AAF08240	81	92
	CtrD (*N. meningitidis*)	P32016	82	91
	KpsT (*E. coli* K5)	P24586	43	66
I, BexB	CpxB (*A. pleuropneumoniae*)	AAB64444	73	84
	Wzm (*M. haemolytica*)	AAF08241	74	86
	CtrC (*N. meningitidis*)	P32015	65	79
	KpsM (*E. coli* K5)	P24584	25	43
I, BexC	CpxC (*A. pleuropneumoniae*)	AAB64443	67	77
	Wzf (*M. haemolytica*)	AAF08242	70	80
	CtrB (*N. meningitidis*)	P32014	55	72
	KpsE (*E. coli* K5)	CAA52655	27	47
I, BexD	CpxD (*A. pleuropneumoniae*)	AAB64442	74	86
	Wza (*M. haemolytica*)	AAF08243	74	87
	CtrA (*N. meningitidis*)	P32013	55	72
	Wza (*E. coli* K-12)	NP_416566	26	42
II, Orf1 (Bcs1)	Acs1 (Hia RM107)	CAA85750	95	96
II, Orf2 (Bcs2)	Asc2 (Hia RM107)	CAA85751	71	67
II, Orf3 (Bcs3)	Cps19bR (*S. pneumoniae*)	AAB66523	19	39
	Cj1432c (*C. jejuni*)	NP_282573	17	38
II, Orf4 (Bcs4)	Cps14K (*S. pneumoniae*)	CAA59783	19	40
III, HcsA	PhyA (*P. multocida*)	AF067175	54	70
	WbrA (*M. haemolytica*)	AAF08250	56	69
	LipA (*N. meningitidis*)	Q05013	57	71
	KpsC (*E. coli* K5)	P42217	46	61
III, HcsB	PhyB (*P. multocida*)	AF067175	65	80
	WbrB (*M. haemolytica*)	AAF08251	60	73
	LipB (*N. meningitidis*)	Q05014	55	68
	KpsS (*E. coli* K5)	P42218	39	58

X78559, and S62752), the sequence for the entire partially duplicated Hib *cap* locus, from end to end, particularly region III and the end junctions within the chromosome, has not been previously described. Hi 1007 contains a complete copy of region I with four genes, *bexDCBA*, that are 99% identical to the previously described Hib region I genes (Fig. 1B) (16) and a second, truncated copy of region I with *bexDCB* and *bexA* with the deletion previously described (19). IS*1016*-V5 (accession no. X58177), a truncated copy of IS*1016*, was found at the end adjacent to the partially deleted *bexA*. IS*1016*-V5 is one of six IS*1016* variants (V1 to V6) previously described by Kroll et al. (17). Table 2 shows a comparison of Hi 1007 proteins from region I with other capsular polysaccharide (CPS) biosynthesis proteins.

Immediately adjacent to both copies of region I is region II, containing four serotype b-associated open reading frames (ORFs) previously identified by van Eldere et al. (34) (Fig. 1B). The nucleotide sequences for region II of Hib strain RM135 (accession no. X78559) and *H. influenzae* type a (Hia) strain Hia RM107 (accession no. Z37516) have been reported. Only the protein products from Hib RM135 o*rf1* and Hia *acs1* have been assigned a function, that of bifunctional ribulose 5-phosphate reductase/CDP-ribitol pyrophoshorylase (9, 34). The predicted protein encoded by Hi 1007 region II *orf1* was 99% similar to the previously described Hib region II *orf1* protein and 96% similar to Acs1 (Table 2). The predicted protein encoded by Hi 1007 region II *orf2* is 67% similar to Acs2 from Hia. We therefore suggest that Hib region II *orf1* and *orf2* be named *bcs1* and *bcs2,* for b capsule specific, based on the nomenclature proposed for the serotype a capsule-specific genes *acs1* and *acs2* (9).

Hi 1007 region II *orf3* is 100% identical to the previously sequenced Hib region II *orf3* but showed no nucleotide similarity to *acs3*, from region II of Hia, and their products showed no amino acid similarity. There was low-level similarity between the predicted protein of Hi 1007 *orf3* and a probable sugar transferase from C*ampylobacter jejuni* and a protein of unknown function from the *cap* locus of *Streptococcus pneumoniae* type 19B (Table 2). Hi 1007 region II *orf4* is 100% identical to the previously sequenced Hib region II *orf4* but showed no nucleotide similarity to *acs4*, from region II of Hia, and their products showed no amino acid similarity. There was low-level similarity between the predicted protein of Hi 1007 *orf4* and Cps14K, a CPS synthesis protein from *Streptococcus pneumoniae* type 14 (Table 2). We propose that capsule-specific region II ORFs *orf3* and *orf4* be named *bcs3* and *bcs4.*

Although region III of Hib is common to all serotypes, little is known about it. No sequence data from any *H. influenzae*

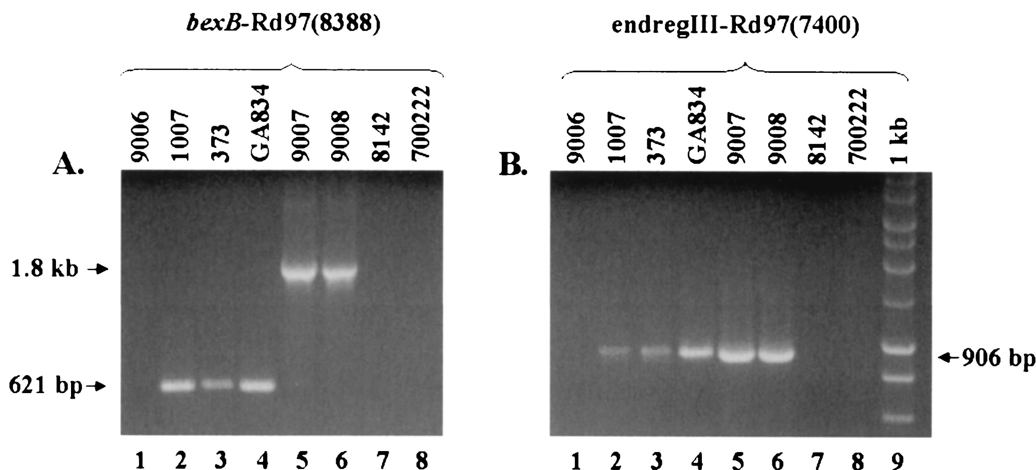

FIG. 2. PCR analysis of the end junctions of the *H. influenzae cap* locus. Lanes 1, H. *influenzae* serotype a strain ATCC 9006; lanes 2, serotype b strain Hi 1007; lanes 3, Hib$^-$ variant Hi 373; lanes 4, Hib$^-$ variant GA834; lanes 5, serotype c strain ATCC 9007; lanes 6, serotype d strain ATCC 9008; lanes 7, serotype e strain ATCC 8142; lanes 8, serotype f strain ATCC 700222; lane 9, 1-kb standard marker (Promega Corp., Madison, Wis.). (A) Samples were amplified with primer set bexB-Rd97(8388) (Table 1). The truncated end junctions of Hib (lane 2) and the Hib$^-$ variants (lanes 3 and 4) yielded a product of 621 bp due to the 1.2-kb deletion within the IS*1016-bexA* region. Strains ATCC 9007 (lane 5) and ATCC 9008 (lane 6), serotypes c and d, respectively, yielded a product of 1.8 kb, consistent with an intact IS*1016* and *bexA*. (B) Samples were amplified with primer set endregIII-Rd97(7400) (Table 1). The end junctions adjacent to region III for Hi 1007, Hi 373, GA834, ATCC 9007, and ATCC 9008 are the same size (906 bp) (lanes 2 to 6). No product is seen for serotype a (lane 1), e (lane 7), or f (lane 8).

serotype had been published for region III. We have identified two ORFs in region III of Hi 1007. Table 2 shows the strong similarity of both genes to *cap* genes involved in postpolymerization of capsules from *E. coli*, *N. meningitidis*, *A. pleuropneumoniae*, *P. multocida*, and *M. haemolytica.* The function of these genes in *H. influenzae* has yet to be determined. It has been proposed that LipA and LipB from *N. meningitidis* are involved in phospholipid substitution, necessary for translocation of the capsule (10). The strong consensus among the various genes and proteins involved in CPS biosynthesis suggests a shared function. In many cases the genes and proteins from region III, as well as those from region I, have been shown to be functionally interchangeable with other region III and region I genes, respectively, by complementation studies (10, 24). Furthermore, evidence strongly suggests that a phospholipid moiety is covalently associated with the Hib CPS (22). We propose naming *orf1* and *orf2* from region III *hcsA* and *hcsB* for *Haemophilus* capsule synthesis.

Hib$^-$ GA834 and Hi 373 *cap* loci. The two Hib$^-$ strains identified did not contain a central 9-kb *Eco*RI fragment, suggesting that there was only one copy of regions I, II, and III and that the region I present was the site of the previously described IS*1016-bexA* deletion (19). We confirmed this finding and sequenced a single truncated copy of region I and only one copy of region II and III in both strains as described above. The sequence and arrangement of region I from GA834 and Hi 373 were found to be >99% identical to the those of the truncated copy of region I in Hi 1007. Furthermore, regions II and III from GA834 and Hi 373 were nearly identical to regions II and III from Hi 1007 with one exception. Hi 373 contained a 24-bp direct repeat found at the 3′ end of *orf4* (now *bcs4*), an in-frame insertion that results in a slightly larger protein.

Location of the *cap* locus within the *H. influenzae* chromosome. The *cap* locus from division I *H. influenzae* strains is flanked by direct repeats of IS*1016* (17). However, it has not been previously determined whether there is a common location for the *cap* locus within the chromosome. The nonencapsulated *H. influenzae* Rd strain, for which the first completed *H. influenzae* genomic sequence is available, contains copies of IS*1016* at three different locations within its chromosome. HI1018, a complete, putative copy of IS*1016*, is found in section 97 at chromosomal coordinates 1082514 to 1081941 on the negative strand of the Rd genome (8) (accession no. NC_000907). Two incomplete copies of IS*1016*, HI1329 and HI1577 (chromosomal coordinates 1407406 to 1407095, negative strand, and 1646128 to 1646346, respectively), are found in sections 127 and 143 of the Rd genome (8). Rd, a division I *H. influenzae* strain, was formerly an encapsulated serotype d strain. Therefore, if Rd lost its capsule in a recombination event between the IS*1016* repeats flanking the ends of the *cap* locus, the predicted result would be a complete copy of IS*1016* remaining, as found in section 97 (HI1018; 1082514 to 1081941 on the negative strand of the complete Rd genome). In addition, Herbert et al. suggest that the site of insertion of the *cap* locus in an Rd b$^+$ transformant was at HI1018 (11).

Primers based on the region of DNA just outside of IS*1016* from section 97 in Rd (Table 1) were generated and used in combination with region I (bexB) or region III (endregIII) primers to amplify DNA at the ends of the *cap* locus from Hi 1007, GA834, and Hi 373, as well as American Type Culture Collection (Manassas, Va.) strains ATCC 9006 (9), ATCC 9007 (14), ATCC 9008, ATCC 8142, and ATCC 700222 (33) (serotypes a, c, d, e, and f, respectively). All PCRs were performed by using an annealing temperature 5°C less than the melting temperature of the primer as calculated by the manufacturer (Sigma-Genosys, The Woodlands, Tex.).

PCR amplification of the end junctions adjacent to region I

[primers bexB and Rd 97(8388)] gave a 621-bp product for Hi 1007, GA834, and Hi 373, consistent with a partial deletion of IS*1016-bexA* at the junction in section 97 (Fig. 2A). When the same primers were used with ATCC 9007 and ATCC 9008 (serotypes c and d), a product of 1.8 kb was seen (Fig. 2A). The increase of 1.2 kb indicates the presence of complete copies of IS*1016* and *bexA* at the left junction in these strains. Each PCR product was sequenced, confirming the presence of the truncated IS*1016*-V5 and partially deleted *bexA* in Hi 1007, GA834, and Hi 373 and a complete IS*1016* (IS*1016*-V2, accession no. X58174) and a complete *bexA* in the serotype c and d strains at Rd section 97 bp 8230. PCR analysis of the end adjacent to region III [primers endregIII and Rd97(7400)] demonstrated a 906-bp product for Hi 1007, GA834, Hi 373, and the serotype c and d strains (Fig. 2B). These products were sequenced, and region III was found to contain a complete copy of IS*1016*-V2 adjacent to the sequence found at Rd section 97 bp 7521. We conclude that the *cap* loci from serotypes b, c, and d (all division I *H. influenzae*) appear to be located within the same chromosomal site between bp 7520 and 8230 of Rd in section 97 (Fig. 1). The only variation noted was the IS*1016-bexA* deletion at the region I junction in duplicated Hib strains and Hib$^-$ variants compared with a complete IS*1016* and *bexA* forming the region I junction in serotypes c and d.

No PCR product was amplified with either primer pair for serotypes a, e, and f (Fig. 2), suggesting the possibility of an alternative location for the capsule genes in some *H. influenzae* strains. Capsule serotype f strains and some serotype a strains are classified as division II, distinguished by the lack of an IS*1016* element associated with the *cap* locus (17). Division II *cap* loci may therefore not be linked to the IS*1016* site located in section 97 of Rd. Although serotype e strains are division I, the failure to amplify *cap*-specific material from the serotype e strain tested suggests that it may also contain capsule genes in a different location. However, given the greater genetic distance between serotype e and other capsule serotypes within division I (27), failure to amplify a PCR product may reflect sequence variation rather than a different *cap* location. Further studies will be necessary to document *cap* locus sequence variation among non-b serotypes and to identify alternative chromosomal sites for capsule genes.

The possession of a polysaccharide capsule greatly enhances the ability of *H. influenzae* to cause invasive disease (26). The Hib conjugate vaccine has resulted in the near elimination of invasive serotype b disease (1). However, the success of the Hib conjugate vaccines should not bring about complacency. The occurrence of invasive *Haemophilus* disease due to other capsular serotypes as well as nontypeable *H. influenzae* during the postvaccine era (2, 29, 33, 35) suggests that greater understanding of non-b capsules and events leading to acquisition and/or loss of capsule genes may be relevant in the future. The IS*1016-bexA* deletion has been demonstrated in some serotype a strains, and it potentially stabilizes encapsulation and enhances virulence in these strains (2, 20). The natural transformability of *H. influenzae* and the evidence that exchange of *cap* genes has occurred between strains in the past (20) suggest that further investigation of the *cap* genes from all six capsular serotypes is warranted.

The nucleotide sequences reported in this paper have been deposited in GenBank under the accession numbers AF549213 (Hi 1007), AF549210 (GA834), and AF549212 (Hi 373).

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