Genetic characterization of the capsulation locus of *Haemophilus influenzae* serotype e

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Running title: *Haemophilus influenzae* serotype e capsulation locus

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The capsulation (cap) locus of *Haemophilus influenzae* type e was characterized and sequenced. No IS1016 element was found to flank the locus. The 18.2-kb locus included 14 ORFs, which were grouped into three functional regions. Eight new ORFs (named *ecs1* to *ecs8*) were identified in the Hi capsule-specific region II.
In the post-Haemophilus influenzae serotype b (Hib) vaccine era, concern about the potential emergence of non-vaccine preventable strains has arisen (1, 17, 20, 23, 26). In encapsulated *H. influenzae*, the genes for the production of the polysaccharide capsules are organized in a capsulation (*cap*) locus, which consists of three different functional regions (11, 13). Regions I and III are common to all capsular types and contain genes necessary for transport and process of the capsular material, while region II contains serotype-specific biosynthesis genes (7, 10, 18, 19, 25). Invasive disease caused by *H. influenzae* serotype e (Hie) strains has recently been observed in Italy, suggesting the importance of further molecular investigations on Hie *cap* locus (4, 5). It is recognized that Hie capsule is a copolymer of repeat unit of a N-acetylgalactosamine and N-acetylmannosamine uronic acid (22, 24), but the genes involved in the polysaccharide biosynthesis have neither been identified nor characterized.

In the present study, we characterized the Hie *cap* locus for the first time. Eleven invasive Hie strains isolated in Italy during the period January 2000–December 2008 were analyzed. The strains were identified as type e by PCR capsular genotyping (6).

**Location of the Hie cap locus within the chromosome.**

PCR amplification of the 5’ and 3’ end junctions of the Hie *cap* locus was performed by using primer sets “capfSodC /bexBrev” and “hcsBfrw/HI1637”, respectively (Table 1). The resulting PCR products were sequenced and analyzed. All 11 Hie strains were found to have the *cap* locus in the identical chromosomal location as that of *H. influenzae* serotype f (Hif), associated with the same flanking genes (*sodC* at the 5’ end and *HI1637* at the 3’ end), confirming previous investigations (19). Sequence analysis of the two end junctions also revealed that they contained no sequences reminiscent of the insertion element IS1016. It is well known that this element provides the molecular substrate for amplifications of the *cap* gene sequences (11). Most Hib strains, in which the *cap* locus lies between direct repeats of the *IS1016*, possess a duplication of the capsule genes (11, 12). The finding that our Hie strains lack of *IS1016* sequences flanking the *cap* locus is remarkable, since, reasonably, the locus can not be amplified.
Sequencing of the Hie cap locus.

The complete cap locus from the strain Hie 274 (isolated from the CSF of a patient with meningitis) was sequenced. To cover the entire Hie cap locus, overlapping amplicons, ranging from 1500 bp to 9 kb, were obtained by PCR using several primer pairs based on the published sequences of the Hib and Hif cap loci (GenBank accession numbers AF549213 and AF549211, respectively) (Table 1). Each amplicon was then subcloned into pCR4-TOPO (TOPO TA cloning kit or TOPO XL PCR cloning kit, Invitrogen, Milan, Italy). Both strands of the insert from each plasmid were sequenced by the primer walking service at Eurofins MWG Operon, Ebersberg, Germany. The nucleotide sequences were assembled and analyzed using DNAMAN sequence analysis software (version 5.2; Lynnon Corp., Quebec, Canada). Nucleotide and deduced amino acid sequences were compared to other known sequences databases by using the National Center for Biotechnology Information BLAST programs. The entire Hie cap locus was shown to be 18.2-kb in length. It contained 14 ORFs which, by analogy with other capsule loci, were grouped into three distinct regions (I, II and III) (Fig. 1). Comparison of the deduced proteins from the Hie cap locus genes with the corresponding gene products from Hib and Hif as well as with proteins from other bacterial species is shown in Table 2.

Region I. Overall, region I exhibited 90% and 96% sequence identity with the previously described Hib and Hif regions I, respectively (18, 19). Region I included four ORFs, which were named bexA, bexB, bexC and bexD. Although the putative proteins of bexABCD genes were nearly identical (from 91 to 98% identity) to the region I corresponding gene products from both Hib and Hif (Table 2), some polymorphism at nucleotide sequence level was observed. The bexA gene from Hie cap locus exhibited 95% identity to bexA from Hif but only 84% identity to bexA from Hib, in agreement with a previous study demonstrating bexA nucleotide sequence diversity among different H. influenzae serotypes (27).

Region III. Overall, region III showed 91% and 93% sequence identity with the previously described Hib and Hif regions III, respectively (18, 19). Region III contained two ORFs, which
were named \textit{hcsA} and \textit{hcsB}. Their deduced amino acid sequences exhibited high identity (from 90 to 96% identity) with the corresponding products from both Hib and Hif regions III (Table 2).

Recently, both HcsA and HcsB proteins have been demonstrated to be crucial for transport of capsular polysaccharide from the periplasm to the bacterial surface across the outer membrane (21).

\textbf{Region II.} Overall, region II showed no sequence identity with the previously described specific capsular regions from other \textit{H. influenzae} serotypes (7, 18, 19). On the contrary, high overall sequence identity (67%) was found with the capsule biosynthetic specific region II from \textit{Pasteurella multocida} B:2 (accession number AF169324), indicating that the genetic organization of the whole region is similar (2, 3). The G+C content of the DNA in the Hie \textit{cap} locus region II is 31.3%, significantly different from that of both regions I and III (38% and 39.4% respectively) and from the overall background for the \textit{H. influenzae} species (38%), suggesting that region II might be more recently acquired. However, since the G+C content of DNA of \textit{P. multocida} \textit{cap} locus region II is 35%, this microorganism was probably not the direct source of the region II for Hie. Although we cannot rule out a common evolutionary origin of the two polysaccharide biosynthetic regions followed by a partial diversification of their DNA content, no data are available to support this hypothesis. Region II contained 8 ORFs, which were named \textit{ecs1} to \textit{ecs8} (for serotype e capsule specific genes) (Table 2). The deduced products of \textit{ecs1} and \textit{ecs2} had homology with putative UDP-N-acetyl-D-glucosamine 2-epimerase and UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase enzymes, respectively, which catalyze the two-step conversion of UDP-N-acetyl-D-glucosamine to N-acetyl-D-mannosaminuronic acid, as previously demonstrated in \textit{E. coli} (14). The encoded protein by \textit{ecs3} gene showed similarity to glycosyltransferases (Table 2), which is involved in polymerization of the sugar monomers in several bacterial species (8, 9). Considering that the structure of the Hie capsular polymer is composed of repeating units of N-acetylglucosamine and N-acetylmannosamine uronic acid (22, 24), it is likely that the products of \textit{ecs1}, \textit{ecs2} and \textit{ecs3} genes play an essential role in the biosynthesis of serotype e polysaccharide. No specific putative functions were assigned to the remaining 5 ORFs (\textit{ecs4} to \textit{ecs8}), although similarity with other
deduced products in the database was detected, including the predicted products of the \textit{bcbDEFGI} genes from \textit{cap} locus region II from \textit{P.multocida} (2), (Table 2). Further studies on functional activities of the Hie \textit{cap} locus region II genes are required.

Although Hie strains belong to the phylogenetic division I of the encapsulated \textit{H. influenzae} strains (15), the Hie \textit{cap} locus shares two remarkable features of the division II \textit{cap} loci: chromosomal location and lack of association with the \textit{IS1016} insertion element, confirming the previously described genetic distance of Hie from all other division I \textit{H. influenzae} (16). The availability of the Hie \textit{cap} locus sequences may be regarded as a powerful tool to be used in further investigations on molecular detection and characterization of the Hie isolates.

\textbf{Nucleotide sequence accession number.} The nucleotide sequence for the Hie \textit{cap} locus from this study has been deposited in the EMBL nucleotide sequence database under the accession number FM882247.

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REFERENCES


Figure 1. Genetic organization of the Hie capsulation locus of strain 274. The arrows indicate genes. Region I contains four genes called \textit{bexDCBA} homologous to those found in Hib and Hif (white arrows). Region II includes eight serotype-specific genes designed \textit{ecs1} to \textit{ecs8} (right-hatched arrows). Region III comprised two genes named \textit{hcsA} and \textit{hcsB} homologous to those found in Hib and Hif (gray arrows).
TABLE 1. PCR primers and products used for sequencing of the Hie capsulation locus

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Table 2. Comparison of the deduced proteins from the *H. influenzae* serotype e capsulation locus of strain 274

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<td>CP000948</td>
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<td>(Vibrio cholerae)</td>
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<td>(Vibrio cholerae)</td>
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<td>(Vibrio cholerae)</td>
<td>CP001144</td>
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BAG50482 (Vibrio parahaemolyticus) AB353134 64 78

Orf7 (Ecs7), 519 aa
Unknown function
BcbG (Pasteurella multocida) AF302466 64 78
ORF7 (Actinobacillus suis) AV253361 64 78
BcbG (Photobacterium damselae) AB074283 37 56
VC0395 (Vibrio cholerae) CP0000627 36 55
BcbG (Zymomonas mobilis) AE108692 36 54

Orf8 (Ecs8), 325 aa
Unknown function
BcbI (Pasteurella multocida) AF169324 64 78
ORF2 (Mannheimia haemolytica) AF170495 63 74
Pphi_1179 (Franciscella philomiragia) CP0000317 54 73
Neut_1976 (Nitrosomonas europaea) CP0000450 40 63
NE1334 (Nitrosomonas europaea) AL854747 43 63

Region III

HcsA, 595 aa
HcsA (H. influenzae serotype b) DQ368335 96 97
HcsA (H. influenzae serotype f) AF549211 95 97
LipA2 (Actinobacillus pleuropneumoniae) CP0000687 62 75
PhyA (Mannheimia haemolytica) AF170495 59 72
LipA (Neisseria meningitidis) AM421808 56 71

HcsR, 420 aa
HcsR (H. influenzae serotype b) DQ368335 91 95
HcsR (H. influenzae serotype f) AF549211 90 94
PhyB (Actinobacillus pleuropneumoniae) CP0000687 65 78
PhyB (Pasteurella multocida) AF067175 64 77
LipR (Neisseria meningitidis) Z13995 55 68