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Differences in Genetic and Transcriptional Organization of the glpTQ Operons between *Haemophilus influenzae* Type b and Nontypeable Strains

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The glpTQ operon of *Haemophilus influenzae* type b (Hib) and nontypeable *H. influenzae* (NTHi) strains is highly conserved, except for a 1.4-kb glpTQ intergenic region that was found in most Hib strains. The presence of this intergenic region results in divergent glpTQ transcriptional profiles for Hib and NTHi where Hib strains appear to have evolved an alternative promoter for glpQ expression. Based on the intergenic region’s low G+C content, we speculate that this DNA fragment was acquired by lateral transfer.

*Haemophilus influenzae* is a common pathogen, especially among children, but the clinical manifestations are largely type specific. The encapsulated *H. influenzae* serotype b (Hib) usually causes invasive infections, such as meningitis and septicemia (2), whereas the much more common nonencapsulated, or nontypeable, *H. influenzae* (NTHi) is a major cause of otitis media, sinusitis, and pneumonia (8). General vaccination against Hib has reduced the incidence of Hib infection to a near minimum (10), while attempts to construct a vaccine against the costly NTHi infections have as yet been unsuccessful due to a high genetic heterogeneity among NTHi strains (20). An extensively studied virulence factor and potential vaccine candidate in *H. influenzae* is protein D, a 42-kDa conserved lipoprotein expressed on the bacterial surface (1, 13, 21, 24). An isogenic protein D-negative mutant has been shown to be less effective than its wild-type parental strain in its ability to (i) cause experimental otitis media in rats (14), (ii) cause damage to ciliated human respiratory epithelium (15), and (iii) promote internalization into human monocytic cells (17). The mechanism behind the virulence properties of protein D is unknown but may involve choline decoration of *H. influenzae* lipoooligosaccharides (LOS), since protein D expression allows *H. influenzae* to obtain choline from cocultured host cells and subsequently incorporate this molecule into its LOS (6).

The ability of protein D to promote the incorporation of choline into LOS comes from its glycerophosphodiester phosphodiesterase activity, catalyzing the hydrolysis of glycerophosphodiester into glycerol-3-phosphate (G3P) and an alcohol (14, 18). The gene encoding protein D (*hpd*) is homologous to *glpQ* of *Escherichia coli* (16) and other bacteria. The *glpQ* gene belongs to the *glp* regulon that is involved in the utilization of glycerol and of G3P and its precursors as energy sources and to supply precursors for phospholipid biosynthesis. In *E. coli* and *Bacillus subtilis* (19), *glpQ* is transcribed together with *glpT*, which is located upstream of *glpQ* and encodes a G3P permease that acts as a G3P-inorganic phosphate antiporter (5).

The *E. coli* glpTQ operon is induced by G3P and repressed by the catabolic repressor glucose as well as a *glp*-specific repressor protein, GlpR (28). Available restriction fragment length polymorphism (RFLP) data and DNA sequences of the glpTQ region of *H. influenzae* suggest that its organization differs between strains (7, 13, 24, 25). RFLP analysis based on a combination of two restriction endonucleases and two probes complementary to the *glpQ* gene of *H. influenzae* (hpd) showed that the majority of Hib strains (34 of 39) carry the *glpQ* gene on a 3.3-kb fragment between two conserved *Pst*I sites (Fig. 1), whereas 24 of 35 NTHi strains display only a 1.9-kb fragment between the same *Pst*I sites (13). As in the case of *E. coli* and *B. subtilis*, *glpT* in most NTHi strains is located immediately upstream of *glpQ* (12, 13, 24). By contrast, *glpT* and *glpQ* are separated by a 1.4-kb fragment of unknown function in the majority of Hib strains and some other encapsulated *H. influenzae* strains, including strain Rd (Fig. 1) (7, 13, 24, 25). The present study was designed to characterize and compare the genetic structures and RNA transcriptional profiles of the glpTQ region in *H. influenzae* strains and to elucidate the potential function of the 1.4-kb glpTQ intergenic region that is present only in encapsulated *H. influenzae* strains.

**DNA sequence analysis.** In addition to using previously reported sequences (12, 24, 25), we used sequences determined in the course of this study, namely, the entire DNA sequences of the glpTQ operons of Hib Eagan and NTHi 772, as well as the 1.4-kb glpTQ intergenic region of Hib HK695 (26). The 1.4-kb glpTQ intergenic regions of Hib Eagan and HK695 were cloned into pBluescript II KS by excising a 2.2-kb *Pst*I fragment that was used to clone the *hpd* genes (24) and subsequently transformed into *E. coli* XL1-Blue. Nested deletions with exonuclease III and mung bean nuclease (22) were performed after a SacI-XbaI digestion of the resulting plasmids, pE1.4pel and pHK1.4pel, and on *Kpn*I-*Bam*HI-cleaved pXME10 and pXMHK10, respectively. The DNA sequences of the 1.4-kb intergenic regions of Eagan and HK695 were determined in both orientations from the constructed nested deletion plasmids with vector primers.
The glpT gene was also identical to the corresponding region of Hib Eagan and NTHi 772. They include two potential transcriptional termination loop sites are indicated. RNA transcripts detected by Northern blotting or transcriptional orientations. Potential transcriptional termination loop structures are marked with circles. Conserved restriction endonuclease sites are indicated. RNA transcripts detected by Northern blotting or RT-PCR are marked under the respective genes or ORFs with thick and fine lines, respectively.

FIG. 1. Schematic genetic map of the glpABC, glpTQ, and glpFK operons in H. influenzae Rd (7) and genetic map and RNA transcript analysis results of the glpTQ operons of Hib HK695, Hib Eagan, Eagan-derived 1.4-kb glpTQ intergenic region mutant Ekm1, and NTHi 772. Arrows labeled with gene names and ORFs denote transcriptional orientations. Potential transcriptional termination loop structures are marked with circles. Conserved restriction endonuclease sites are indicated. RNA transcripts detected by Northern blotting or RT-PCR are marked under the respective genes or ORFs with thick and fine lines, respectively.

M13-u and M13-r (Table 1) by using an Applied Biosystems 377 automatic sequencer. The DNA sequences of glpT and the glpTQ intergenic region of NTHi 772 were determined from a 2.0-kb PCR product amplified with primers glpA-1 and hpd-10 (Table 1) and sequenced with primers specific for the glpT gene of Hib Eagan (25). The DNA sequence upstream of glpT of Hib Eagan was obtained from a PCR amplicon by using primers glpA-1 and glpT-9 (Table 1).

The glpT sequences of Hib Eagan and NTHi 772 were almost as highly conserved as the previously sequenced glpQ gene (13, 24). The glpT genes from the two strains differed in 15 bp and displayed identical deduced amino acid sequences of the same length. The regulatory region upstream of the NTHi 772 glpT gene was also identical to the corresponding region of Eagan, except that 772 contained one extra base pair located 91 bp upstream of the glpT start codon (Fig. 2). Potential catabolic and transcriptional regulation sites with homology to consensus sequences upstream of glpT in E. coli were also found upstream of glpT in Eagan and 772. They include two catabolic activator protein binding sites (4), two operator sites with the potential of binding to the E. coli GlpR repressor protein (29), and a sequence homologous to the integration host factor (IHF) (9) (Fig. 2). The binding of IHF to this site may bend the DNA to facilitate the formation of a complex structure that is involved in the inhibition of GlpT expression, mediated by the GlpR repressor in E. coli (28). However, despite the existence of two GlpR homologues, no homologues of ihfA and ihfB (R. A. Weisberg, M. Freundlich, D. Friedman, J. Gardner, N. Goosen, H. Nash, A. Oppenheim, and J. Rouvière-Yaniv, Letter, Mol. Microbiol. 19:642, 1996) have been identified in the H. influenzae Rd genome (7).

In the glpTQ intergenic region, both Hib strains contained practically identical 1,428-bp sequences, except that HK695 contained one more tetrameric repeat (TTTA) in addition to the five repeats present in Eagan. The repeats were localized in a putative open reading frame (ORF) of 414 bp in HK695, designated ORF1 for these Hib strains (Fig. 1). The lack of one tetramer in Eagan caused a frameshift that led to a stop codon after 228 bp and resulted in two smaller ORFs (Fig. 1). Most tetrameric DNA repeats in H. influenzae have been associated with the regulation of potential virulence factors such as iron acquisition factors, adhesion molecules, and LOS biosynthesis (11, 27), but the function of ORF1 is unknown. Another putative 390-bp ORF (ORF2) of the Hib glpTQ intergenic region was found immediately upstream of glpQ (Fig. 1). The glpTQ intergenic region of the two Hib strains was highly homologous to the corresponding region of H. influenzae Rd (7). Two ORFs with unknown functions, HI0687 and HI0688, correspond to ORF1 and ORF2 of the Hib strains with the exception that HI0687 of H. influenzae Rd exhibits a larger size than ORF1 (Fig. 1). The G+C contents of the 1.4-kb glpTQ intergenic region of the Hib strains was approximately 26%, which is much lower than the average of 38% for the glpT and glpQ genes and 39% for the H. influenzae Rd genome in general. Although no typical insertion-like sequences were found in this region, the 1.4-kb fragment might have been acquired by lateral transfer from an organism with a lower G+C content. The glpTQ intergenic region of NTHi 772 was only 103 bp long, out of which the first 52 bp was homologous to the glpTQ intergenic regions of the Hib strains Eagan and HK695. This homologous part contains an inverted repeat sequence with the ability to form a typical hairpin loop structure exhibiting a lowest free energy value of −9.8 kcal/mol (23) that may function as a potential transcription terminator for glpT expression. A similar conserved inverted repeat sequence was also identified immediately downstream of the glpQ stop codon in both Hib and NTHi strains (Fig. 1). This potential glpQ transcriptional terminator exhibited a lowest free energy value of −16.8 kcal/mol (23).

Construction of a 1.4-kb mutant in H. influenzae. Reverse PCR with a Pwo PCR kit (Roche Diagnostics) was used to amplify the fragment flanking the 1.4-kb intergenic region of pXME10 with primers Hibup-3 and Hibup-4 (Table 1), resulting in a 1.2-kb deletion of the 1.4-kb intergenic region. The PCR product was ligated to the 1.3-kb kanamycin resistance (Km') cassette excised from pUC4K (Amersham Pharmacia Biotech) with HincII, and the ligation mixture was transformed into E. coli DH5α. Following the excision of the vector backbone by BamHI-SphI cleavage, the 3.5-kb linear DNA fragment was transformed into Eagan for homologous recombination (14). The chromosomal DNA of five Km' colonies was examined by Southern hybridization with digoxigenin (Roche Diagnostics)-labeled probes. The glpT and glpQ probes were labeled by PCR using a pXME10-derived nested deletion and
pHIC348 (12) as templates with M13-u-glpTx1 and hpd-6B-hpd-4 (Table 1) (24) as primer pairs, respectively. The kanamycin probe was labeled by random priming from the gel-purified Km" cassette that had been excised from pUC4K with HincII. Southern blot analysis (data not shown) of EcoRI- or HaeIII-digested chromosomal Hib DNA revealed that the Km" cassette had been inserted just downstream of the glpT coding region (Fig. 1) in all tested Km" clones. The growth rate of one cassette had been inserted just downstream of the glpT resc-1 (12) as templates with M13-u–glpA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>glpA-1</td>
<td>5′-TTGCGGatCCACCGATAATGATCAC-3′</td>
<td>Reverse primer annealing to nt 85–60 of Rd glpA, used for amplification and sequencing</td>
</tr>
<tr>
<td>glpT-9</td>
<td>5′-ATGCGetGCaGTTTTGAATGTCCA-3′</td>
<td>Reverse primer annealing to nt 29–6 of Eagan glpT, used for amplification, sequencing, and primer extension</td>
</tr>
<tr>
<td>Hibup-1</td>
<td>5′-AAAAGGCGGcaGGAAATTGGCTTAGTC-3′</td>
<td>Forward primer annealing to nt 8–35 of downstream glpT stop codon of Eagan, used for RT-PCR</td>
</tr>
<tr>
<td>Hibup-2</td>
<td>5′-CCTATCAagaTCCAAATGCCAACATTTG-3′</td>
<td>Reverse primer annealing to nt 264–234 of downstream ORF1 stop codon of HK695, used for RT-PCR</td>
</tr>
<tr>
<td>Hibup-3</td>
<td>5′-AAAGGCACAAAGCAATTTGCCCCACCGCCTTTTAAAG-3′</td>
<td>Reverse primer annealing to nt 41–5 downstream glpT stop codon of Eagan, used for construction of 1.4-kb mutant</td>
</tr>
<tr>
<td>Hibup-4</td>
<td>5′-TGCTGGAATAGATTATAAAT-3′</td>
<td>Forward primer annealing to nt 98–117 of Eagan ORF2, used for construction of 1.4-kb mutant and RT-PCR</td>
</tr>
<tr>
<td>Hibup-5</td>
<td>5′-AATGGGTTAGATTCTATTA-3′</td>
<td>Forward primer annealing to nt 41–63 of Eagan ORF1, used for RT-PCR</td>
</tr>
<tr>
<td>Hibup-6</td>
<td>5′-AATCCCTCAAATATACTAACA-3′</td>
<td>Reverse primer annealing to nt 130–107 of Eagan ORF1, used for primer extension</td>
</tr>
<tr>
<td>Hibup-7</td>
<td>5′-GCGATAGCAAGTATTCCAAAGATAC-3′</td>
<td>Reverse primer annealing to nt 328–303 of HK695 ORF1, used for RT-PCR</td>
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<tr>
<td>Hibup-8</td>
<td>5′-TTCCGTGAATAATTTTAATGAT-3′</td>
<td>Reverse primer annealing to nt 267–246 of Eagan ORF2, used for RT-PCR</td>
</tr>
<tr>
<td>Hibup-10</td>
<td>5′-CTCAaGCTTTCCCAAAATCTAATT-3′</td>
<td>Reverse primer annealing to nt 35–8 of Eagan ORF2, used for primer extension</td>
</tr>
<tr>
<td>Hibup-11</td>
<td>5′-ATTTTggATccCTTTAATCCTTCTGA-3′</td>
<td>Reverse primer annealing to nt 185–156 of Eagan ORF2, used for primer extension</td>
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<tr>
<td>hpd-10</td>
<td>5′-CCTGGAATGCCAGCTGTCAATA-3′</td>
<td>Reverse primer annealing to nt 52–28 of glpQ, used for amplification and primer extension</td>
</tr>
<tr>
<td>M13-u</td>
<td>5′-GTTTTCCGATCAGAC-3′</td>
<td>M13 universal primer for pBluescript II vectors, used for sequencing</td>
</tr>
<tr>
<td>M13-r</td>
<td>5′-AACAGCTATGACCATG-3′</td>
<td>M13 reverse primer for pBluescript II vectors, used for sequencing</td>
</tr>
</tbody>
</table>

a Lowercase letters indicate nucleotides substituted for the creation of restriction sites.

b nt, nucleotide.

Characterization of promoter positions of the glpTQ operons. Primer extension analysis (22) was applied to identify potential transcriptional start positions by using antisense DNA primers (Table 1) that bound just downstream of the respective start codons. Primers were labeled with [γ-32P]ATP by T4 polynucleotide kinase (Amersham Pharmacia Biotech) and were used for the extension of 40 μg of total RNA, prepared with an RNasy kit (QIAGEN) from cultures grown in supplemented brain heart infusion broth. Labeled products were analyzed on a 6% polyacrylamide gel containing 7 M urea in a Sequi-Gen sequencing cell (Bio-Rad Laboratories). The signal in the gel was measured either by exposure to X-ray film or by a PhosphorImager and analyzed with ImageQuant software (Molecular Dynamics). A total of four bands, designated P1 to P4, corresponding to potential transcriptional start positions of glpT were detected in NTHi 772, whereas only three bands were detected in Hib Eagan (P1, P2, and P4) (Fig. 2). The lack of P3 in Hib Eagan was unexpected since potential transcriptional start positions of glpT might differ for NTHi and Hib strains. This hypothesis was supported by the observation that glpT of NTHi 772 that was cloned into the pACYC184-based vector pSU18 (3) failed to complement the G3P uptake levels in the Hib Eagan glpT rec-1 double mutant ETM22r2 (unpublished data), although both strains contain almost identical glpT genes. We further analyzed the total RNA of ETM22r2 that had been transformed with pXMS1 (25), carrying Eagan glpT plus a partial regulatory region ending 156 bp upstream of the glpT stop codon cloned into pSU18. Only one band corresponding to position P1 was found (data not shown), although the P2 site and its putative −10 promoter sequence plus the region where a −35 consensus sequence normally is found (Fig. 2) was included in pXMS1. This result suggests that the P1 site may be essential for glpT transcription in Eagan since pXMS1 is able to complement the glpT mutant ETM22r2 to a higher level of GlpT expression than that of the wild-type strain, Eagan (25).
It also indicates that the signal at the P2 site in primer extension analysis may be a degradation product of a glpT mRNA initiated from the upstream promoter site P4. Since ETM22r2 is a glpT mutant that was created by the selection of spontaneous fosfomycin-resistant Hib Eagan clones (25), it is likely that the P4 promoter site probably exists in ETM22r2. No signal from P4 could be detected in ETM22r2(pXMS1), which may be due to the fact that this potential signal is too weak in comparison to the P1 signal from the medium-copy-number plasmid pXMS1. When potential promoter sites for glpQ were analyzed, only one band was identified 28 bp upstream of the glpQ start codon in Hib Eagan as well as in E. coli (pXME10), which contains glpQ from Eagan. A primer extension product was found 64 bp upstream of the glpQ start codon in NTHi 772 and from E. coli (pHIC348), which contains glpQ from 772 (data not shown). The transcriptional start of Hib Eagan ORF1 in the glpTQ intergenic region was mapped to 59 bp upstream of the glpQ start codon in NTHi 772 and from E. coli (pXME10), which contains glpQ from 772 (data not shown). The transcriptional start of Hib Eagan ORF1 in the glpTQ intergenic region was mapped to 59 bp upstream of the ORF1 start codon in Hib Eagan and E. coli harboring pXME10 (data not shown). The transcriptional start of Hib Eagan ORF1 in the glpTQ intergenic region was mapped to 59 bp upstream of the glpQ start codon in NTHi 772 and from E. coli (pXME10), which contains glpQ from 772 (data not shown). The transcriptional start of Hib Eagan ORF1 in the glpTQ intergenic region was mapped to 59 bp upstream of the ORF1 start codon in Hib Eagan and E. coli harboring pXME10 (data not shown). Since the glpTQ intergenic region of NTHi 772 is only 103 bp, the potential transcriptional starts of NTHi 772 glpQ and Hib Eagan ORF1 must be located within 1 bp of each other in relation to the ends of their respective glpQ genes. Both promoters share the same putative −10 and −35 sequences, which are situated within the loop structure downstream of glpT. Furthermore, in spite of the divergent sequences immediately upstream of the glpQ start codons of Eagan and 772 (24), the two strains appear to share the same putative ribosomal binding site. Attempts to locate a transcriptional start for ORF2 of the 1.4-kb region were unsuccessful, but two bands were found within the ORF2 coding region for both Eagan and E. coli harboring pXME10 (Fig. 3). These were located 85 and 89 bp, respectively, downstream of the ORF2 start codon. Shared putative −10 and −35 sequences were also found upstream of these sites.

**RNA transcript analysis.** glpT (1.4 kb) and glpQ (1.1 kb) transcripts from the total RNAs of NTHi 772, Hib Eagan, and ETM22r2(pXMS1) were visualized by using Northern hybridization (25) (Fig. 4). Hib Eagan and ETM22r2(pXMS1) displayed a single band for glpT and glpQ, respectively. NTHi 772 exhibited a band of approximately 2.6 kb that hybridized to both glpT and glpQ probes in addition to the respective smaller bands that were detected in Eagan. The possibility that monomeric glpT- and glpQ-specific bands are a result of the mRNA processing of the larger 2.6-kb transcript cannot be ruled out, but since E. coli harboring pHIC348 expresses GlpQ from its endogenous NTHi 772 promoter (12), and since we successfully mapped the glpQ promoter with primer extension analysis, it is concluded that the glpQ gene of NTHi 772 is partially cotranscribed with glpT. No variation in mRNA lengths due to alternative transcriptional start points was detected by Northern blot analysis. Based on the assumption that the hairpin loop structure immediately downstream of the glpT stop codon terminates the glpT transcription in Hib Eagan, we examined the glpTQ transcription patterns of the Eagan 1.4-kb mutant Ekm1, in which the loop structure was destroyed during mutant construction (Fig. 1 and 4). Two Ekm1 transcripts (2.7 and 3.9 kb) were found hybridizing to the glpT probe. It is likely that the 2.7-kb band is a combination of the 1.4-kb glpT and the 1.3-kb Km' cassette and that the 3.9-kb fragment is the 2.7-kb band plus the downstream 1.1-kb glpQ transcript. In addition...
to the 1.1-kb \textit{glpQ} transcript, the 3.9-kb fragment was also found when the same blot was reprobed with a \textit{glpQ} probe, suggesting that \textit{glpT} transcription could continue downstream once the loop structure was removed. However, the practically identical loop structure downstream of 772 \textit{glpT} only partially terminated \textit{glpT} transcript because a cotranscribed \textit{glpTQ} transcript was found in this strain. We therefore conclude that a complete interruption of the \textit{glpTQ} cotranscript requires the existence of the 1.4-kb \textit{glpTQ} intergenic region.

As we failed to detect any signals by using ORF1 or ORF2 probes in Northern hybridization (data not shown), DNA transcripts of the 1.4-kb intergenic region of Eagan were further characterized with reverse transcription (RT)-PCR by using Ready-To-Go beads (Amersham Pharmacia Biotech) and different combinations of RT-PCR primers (Table 1). An RNA transcript that covered the entire ORF1 and the ORF1-ORF2 intergenic region was found in this strain. We therefore conclude that a complete interruption of the \textit{glpTQ} cotranscript requires the existence of the 1.4-kb \textit{glpTQ} intergenic region.

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mease, and in part transcribed by a promoter that is situated within a hairpin loop structure. In Hib strains, the same promoter appears to transcribe at least one unique ORF (ORF1) that may have the ability to phase vary its expression due to the variation of the number of tetramer repeats situated within the ORF. It would be very interesting to further study and determine how the regulation of genes in the glpTQ region of NTHi strains differs from that in Hib strains and to investigate what role the ORFs in the 1.4-kb intergenic region of Hib strains have in pathogenesis.

Nucleotide sequence accession numbers. The entire DNA sequences of the glpT gene of NTHi 772, as well as the 1.4-kb glpTQ intergenic region of Hib Eagan and HK695 (26), were determined in this study and submitted to GenBank under the following accession numbers: for the 1.4-kb intergenic region of Hib Eagan, accession no. AF132899; for the 1.4-kb intergenic region of HK695, accession no. AF132900; and for glpT of NTHi 772, accession no. AF132901.

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