Lipoprotein e(P4) Is Essential for Hemin Uptake by *Haemophilus influenzae*

By Joachim Reidl and John J. Mekalanos

From the Department of Microbiology and Molecular Genetics, Shipley Institute of Medicine, Harvard Medical School, Boston, Massachusetts 02115

**Summary**

Heme uptake is a common means of iron and porphyrin acquisition by many pathogenic bacteria. The genus *Haemophilus* includes several important pathogenic bacterial species that characteristically require hemin-, protoporphyrin-, or heme-substituted proteins as essential growth factors under aerobic conditions. However, the mechanism of heme transport is not understood for *Haemophilus*. We have cloned a DNA fragment from *H. influenzae* that allows an *Escherichia coli* hemA mutant to employ exogenous hemin or protoporphyrin IX as sole sources of porphyrin. DNA sequencing of the cloned DNA fragment suggested that a previously characterized gene (hel) encoding an antigenic, outer membrane lipoprotein e(P4) was responsible for the complementation activity. Construction of *hel* insertion mutations in strain *H. influenzae* Rd demonstrated that *hel* is essential for growth under aerobic conditions but not under anaerobic conditions. The aerobic growth defect of *hel* mutants could be reversed by providing exogenous hemin in the presence of outer membrane perturbants suggesting that these *hel* mutants are defective in transport of heme through the outer membrane. The analysis of hybrids between e(P4) and β-lactamase demonstrated that a domain of e(P4) near its NH₂-terminus was required for its function in heme use. Within this domain is a short amino acid sequence that displays similarity to *H. influenzae* heme-activated protein, heroin-binding motifs present in eukaryotic transcription activator heme-activated protein, and the heme containing proteins hemoglobin (α-chain) and cytochrome C3, suggesting that this region may be involved in heme binding and/or transport.

*Haemophilus influenzae* type b is a Gram-negative coc-cobacillus, responsible for significant morbidity and mortality in young children (1, 2). Under aerobic conditions, *H. influenzae* requires two essential growth factors: nicotinamide adenine dinucleotide (NAD)¹ and hemin (3). Hemin can serve as a source of both iron and porphyrin for this microorganism. Protoporphyrin IX (PPIX) can substitute for the hemin requirement if an exogenous iron source is also available (4, 5). Sanders et al. (6), recently characterized a periplasmic binding protein–dependent iron transport system, encoded by the HitA,B,C components in *H. influenzae* which was required for uptake of iron citrate or ferric ions. Although *H. influenzae* does not synthesize siderophores, it can utilize host iron-binding proteins as sources of iron (7). For example, two *H. influenzae* proteins encoded by *tbpA,B* have been shown to be essential for utilization of transferrin–derived iron (8).

Hemin or PPIX are taken up by *H. influenzae* if present in growth medium but both *H. influenzae* and *H. ducreyi* can also scavenge heme from certain host proteins that contain heme. Some of the bacterial components required for utilization of various heme-containing host proteins have recently been characterized. For *H. influenzae* type b, a hemopexin-binding complex has been characterized and consists of three gene products Hxu A, B, and C (9, 10). Another hemopexin receptor complex is thought to consist of three different outer membrane proteins with molecular masses of ~29, 38, and 57 kD (11). Outer membrane proteins that bind hemoglobin have also been characterized for *H. influenzae* (12) and *H. ducreyi* (13, 14).

Although *H. influenzae* can grow on laboratory media containing free heme or PPIX, there has been less progress in identifying the key components necessary for their utilization. Two heme-binding proteins have been reported. A 39.5-kD outer membrane protein (15) was shown to bind to heme–agarose and this interaction was inhibited by heme or heme-binding proteins such as hemoglobin, but not by PPIX. A 60.6-kD heme-binding lipoprotein (HbpA), also isolated from *H. influenzae* by affinity chromatography, was found to display a high degree of homology to the periplasmic dipeptide transport protein DppA of *Escherichia coli* (16). The effects of mutations in the genes

---

¹Abbreviations used in this paper: ALA, 5-aminolevulinic acid; BH1, brain heart infusion; HAP-1, heme-activated protein; LB, Luria broth; NAD, nicotinic adenine dinucleotide; PPIX, protoporphyrin.
for either of these two hemin-binding proteins have not been reported.

Given that no \(H.\ influenzae\) mutants have been identified that are deficient in the utilization of hemin or PPIX as a porphyrin source, we attempted to clone genes encoding components in this pathway using a genetic complementation approach. In this report we present results that indicate that the \(H.\ influenzae\) outer membrane lipoprotein e(P4), encoded by the hel gene, is an essential component for utilization of hemin, PPIX, or hemoglobin as exclusive sources of porphyrin. The hel gene was identified previously by Green et al. (17) as encoding the immunodominant outer membrane protein e(P4). Although antigenically highly conserved among both typable and nontypable \(H.\ influenzae\) isolates, e(P4) had not previously been assigned any biological function.

Materials and Methods

Bacterial Strains and Growth Condition. \(E.\ coli\) strain MC4100 \(hemA\) was obtained from G. Jander (Dept. of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA), strain \(H.\ influenzae\) Rd was obtained from A. Wright (Dept. of Microbiology, Tufts Medical School, Boston, MA), and \(H.\ influenzae\) type b Eagan was provided by G. Siber (Harvard Medical School, Boston, MA) (18). The \(hemA\) derivatives of \(E.\ coli\) K12 strain MC4100 (F\(^{−}\) and D\(Δ\)(argF-la)U69 rpsL150 relA1 deoC1 ptsF25 hjB5301 rhsR) (19) were grown on Luria broth (LB) medium supplemented with 50 \(\mu\)g/ml 5-amino-levulinic acid (ALA) at 37°C, under aerobic conditions. \(H.\ influenzae\) type b Eagan and \(H.\ influenzae\) Rd were grown on 3.8% brain heart infusion (BHI) agar (Difco Laboratories, Inc., Detroit, MI) supplemented with 10 \(\mu\)g/ml each of NAD, and hemin-chloride or PPIX (all from Sigma Chemical Co., St. Louis, MO) (20). \(Haemophilus\) strains were grown under anaerobic conditions using GasPak 150™, in a BBL GasPak Plus generator with catalyst (Baxter Healthcare Corporation, Medford, MA).

Genetic Methods, Plasmids and Transposon Mutagenesis. Chromosomal DNA of \(H.\ influenzae\) strains was prepared by the method of Barack et al. (20). Plasmid DNA preparation was carried out according to the Qiagen kit protocol (Qiagen, Inc., Studio City, CA). Cloning and restriction analyses were done according to Maniatis et al. (21). Plasmid pACYC184 (22, 23) was used in the construction of a genomic DNA library of \(H.\ influenzae\) type b. Briefly, chromosomal DNA from strain \(H.\ influenzae\) type b Eagan was partially digested with restriction enzyme Sau3AI, purified by gel electrophoresis and ligated into BamHI-digested pACYC184. Plasmid pACYC177 (22, 24) was used as control plasmid for the expression of native \(β\)-lactamase in immunoblot analyses (see below).

PCR amplification of the hel gene DNA fragment was performed using the GeneAmp DNA amplification kit, according to the Perkin-Elmer/Cetus Corp. (Emeryville, CA) thermal DNA cycler protocol, based on Mullis and Faloona (25). The following specific primers were used for the amplification of the hel gene DNA fragment: AR15 (26′ mer): 5′-ATTGGATCCGAATCTTTAAAAAGGAAT-3′; and AR16 (30′ mer): 5′-ATTAAAATATGGATCCGAATCTTTAAAACTGAGC-3′. These oligonucleotides were designed to anneal to the flanking DNA sequences of the hel gene at bp position 1–18 for AR15, and 1032–1047 for AR16, according to the DNA sequence published by Green et al. (17). BamHI restriction sites were designed into the 5′ ends of primers AR15 and AR16.

Southern blot analysis (26) employed the enhanced chemiluminescent detection system (ECL; Amersham Corp., Arlington Heights, IL) as described by the manufacturer. Electroporation or transformation of plasmid or linear DNA into \(H.\ influenzae\) Rd was accomplished using the method of Mitchell et al. (27) and Tomb et al. (28).

Mini transposon Tn10d-bla (29) was used to construct hybrid fusion proteins between e(P4) and \(β\)-lactamase. Plasmid pJR207 was used to mutagenize plasmid pJR4 as described earlier (29). The insertions of the Tn10d-bla elements were determined by the dideoxy nucleotide chain termination method of Sanger et al. (30), and performed with the automatic sequencing method of Applied Biosystems Inc. (Foster City, CA). Synthetic oligonucleotide AR6 is an antiparallel 16′ mer primer of the 5′ end of the blaM gene, and was used as DNA-sequencing primer to detect fusion joints of Tn10d-bla insertions as described earlier (29).

Western Blot Analysis. Derivatives of \(E.\ coli\) strain MC4100 carrying either plasmid pACYC177, pJR121, or pJR4 were grown in 30 ml LB at 37°C for 18 h. Cells were harvested by centrifugation, washed twice, and resuspended in NaPO₄ buffer (100 mM, pH 7.4). Fivefold concentrated suspensions were disrupted by sonication, and the extracts obtained were analyzed by electrophoresis in 11% polyacrylamide gels containing sodium dodecylsulfate (31). Separated proteins were transferred to nylon membranes (32), and subsequently probed with antibody directed against BlaM as described in Reidl and Mekalanos (29).

Results

Isolation and Identification of the \(H.\ influenzae\) e(P4) Gene Product. To identify specific hemin uptake components of \(H.\ influenzae\), we used complementation of an \(E.\ coli\) K12 strain MC4100 \(hemA\) mutant. The \(hemA\) gene encodes Glu-tRNA-reductase, the enzyme that initiates porphyrin synthesis by the alternative C5-pathway (33) and is responsible for the synthesis of ALA from Glu-tRNA (34, 35). \(E.\ coli\) \(hemA\) mutants are unable to grow aerobically unless ALA is provided in the growth medium. Medium supplemented with hemin does not support growth of \(hemA\) mutants because the \(E.\ coli\) K12 outer membrane is impermeable to extracellular hemin or PPIX (36). Accordingly, we expected \(H.\ influenzae\) genes encoding hemin utilization to complement \(hemA\) mutants of \(E.\ coli\) for growth on hemin or PPIX as sole porphyrin sources. A similar approach for the cloning of hemin utilization genes has recently been described by Stojiljkovic and Hanke (37).

Competent cells of strain MC4100 \(hemA\) were transformed with an \(H.\ influenzae\) genomic plasmid library. Transformed cells were plated onto LB agar plates containing ALA (50 \(\mu\)g/ml), and kanamycin (20 \(\mu\)g/ml). After overnight incubation at 37°C, the colonies were replica plated onto LB agar plates containing hemin (10 \(\mu\)g/ml) and kanamycin (20 \(\mu\)g/ml). Several colonies that grew on the latter medium were purified and their plasmid DNA isolated. Retransformation into MC4100 \(hemA\) showed that plasmid pJR121 was responsible for a hemin utilization phenotype.

Restriction and Southern blot analysis of plasmid pJR121 showed that the insert in pJR121 was a \(H.\ influenzae\) genomic DNA fragment approximately 6.2 kb in size. Southern blot hybridization of MC4100 \(hemA\) genomic DNA with Bal3I-digested pJR121 revealed a 6.2 kb fragment, and the same fragment was present in the \(hemA\) strain MC4100::miniTn10d-bla (39) and a Tn10d-bla insertion into strain MC4100::miniTn10d-bla::pJR121. Therefore, the 6.2 kb fragment most likely consists of a \(H.\ influenzae\) genomic DNA fragment containing the hemin utilization gene necessary for growth on hemin or PPIX.

Restriction and Southern blot analysis of plasmid pJR121 showed that the insert in pJR121 was a \(H.\ influenzae\) genomic DNA fragment approximately 6.2 kb in size. Southern blot hybridization of MC4100 \(hemA\) genomic DNA with Bal3I-digested pJR121 revealed a 6.2 kb fragment, and the same fragment was present in the \(hemA\) strain MC4100::miniTn10d-bla (39) and a Tn10d-bla insertion into strain MC4100::miniTn10d-bla::pJR121. Therefore, the 6.2 kb fragment most likely consists of a \(H.\ influenzae\) genomic DNA fragment containing the hemin utilization gene necessary for growth on hemin or PPIX.
showed that it carried a 1-kb insert containing *H. influenzae* DNA. Sequence analysis of the entire 1-kb insert revealed the presence of ~400 bp of DNA that was identical to the 5' end of the *hel* gene previously reported to encode outer membrane protein e(P4) (17). The sequence analysis revealed that the coding sequence for the NH2-terminal 69 amino acid residues of e(P4) were fused to a coding sequence for a *H. influenzae* homolog of the *E. coli* gene *tgt* (tRNA guanine transglycosylase) (38, 39). Given that a Sau3AI site was present at the fusion junction, we concluded that this hybrid gene was the result of a ligation event between different chromosomal Sau3AI DNA fragments that had occurred during the construction of the genomic library. Nonetheless, as shown in Fig. 1, pJR1 does allow strain MC4100 hemA to utilize hemin and PPIX, suggesting that the e(P4) portion of the fusion protein encoded by pJR1 was responsible for this phenotype.

To investigate whether the full-length e(P4) protein was also capable of complementing strain MC4100 hemA for hemin utilization, we subcloned the complete *hel* gene from the chromosome of *H. influenzae* type b Eagan using PCR. Synthetic oligonucleotides AR15 and AR16 were designed based on the published sequence of the *hel* gene (17); a subsequent PCR reaction using *H. influenzae* chromosomal DNA as the template produced a 900-bp product. This PCR product was digested with BamHI and subcloned into the BamHI restriction site of plasmid pACYC184 producing plasmid pJRP4. DNA sequence analysis confirmed that pJRP4 encoded the entire *hel* gene (data not shown).

As shown in Fig. 1, pJRP4 was also capable of complementing MC4100 hemA for utilization of hemin and PPIX, although growth was somewhat less than that seen for pJRH1. However, *E. coli* strains carrying pJRP4 produce smaller colonies on LB agar plates containing ALA (50 μg/ml) as well, suggesting that the expression of the entire e(P4) gene product is deleterious to *E. coli* and thus reduces its growth rate and yield regardless of the source of porphyrin.

**Isolation and Characterization of Tn10d-bla Insertions in the *hel* Gene.** To obtain further evidence for the role of the *hel* gene in hemin utilization, we performed transposon mutagenesis of *hel* with the mini transposable element Tn10d-bla (29). Insertion of this transposon into the correct reading frame of a gene encoding an exported protein can produce a hybrid protein that encodes resistance to ampicillin. Accordingly, plasmid pJR207 was used as a source of Tn10d-bla in mutagenesis protocol of pJRP4 similar to that previously described (29). After selection for ampicillin-resistant colonies in a strain carrying both pJR207 and pJRP4, plasmids were prepared and retransformed to confirm linkage of β-lactamase activity to the pJRP4-linked marker, chloramphenicol resistance.

The locations of the Tn10d-bla insertions on pJRP4 were determined by DNA sequence analyses. Seven Tn10d-bla insertions were identified within the *hel* gene (data not shown). Fig. 2 shows the positions of insertions number 9 and 34, encoded by plasmids pJRP4/9 and pJRP4/34. Western blot analysis showed that plasmids pJRP4/9 and pJRP4/34 encoded β-lactamase fusion proteins of 30 and 36 kD, respectively (data not shown). Given that native β-lactamase migrates at ~28 kD, the sizes of these β-lactamase fusion proteins correspond to the defined position of Tn10d-bla insertions within the *hel* gene as shown in Fig. 2.

Growth studies of strain MC4100 hemA transformed with the recombinant plasmids pJRP4, pJRH1, pJRP4/9, and pJRP4/34 were conducted. The ability to utilize hemin (10 μg/ml) or PPIX (10 μg/ml) for growth was observed for cells harboring plasmids pJRP4, pJRH1, or pJRP4/34, but not for plasmid pJRP4/9, which encodes 50 amino acids of the original e(P4) protein (Fig. 2). All transformants were able to grow in the presence of ALA (50 μg/ml), suggesting that the plasmid-linked defects in hemin and PPIX utilization were related to the position of the Tn10d-bla insertion within the *hel* gene rather than a nonspecific growth inhibition. Thus, only plasmids pJRH1 and pJRP4/34 allowed utilization of hemin and PPIX and these two plasmids maintained the coding sequence for the first 49 and 91 amino acids of e(P4), respectively (Fig. 2). It is interesting to note that homology searches of the GenBank database for the first 90 amino acids of e(P4) resulted in the identification of a short amino acid motif, KVAFDH, which was found to be present in degenerate form in several characterized hemin-binding or hemin-associated proteins. As shown in Fig. 2, the KVAFDH motif is located at amino acid position 65 in e(P4), and is thus largely contained within the e(P4)-specific sequences remaining in the two functional fusion proteins encoded by plasmids pJRP4/34 and pJRH1.

**Construction and Characterization of hel Insertion Mutations in *H. influenzae*.** To confirm that e(P4) is involved in
hemin uptake, we constructed insertion mutations in the hel gene of strain H. influenzae Rd. Plasmids pJR4/9 and pJR4/34 DNA were prepared and then linearized with restriction endonuclease BamHI to produce linear molecules in which the corresponding Tn10d-bla insertions were flanked by H. influenzae chromosomal DNA sequences. These DNA fragments were introduced into strain H. influenzae Rd by electroporation. After 3 h of phenotypic expression, cells were plated on BHI agar plates, containing NAD (10 μg/ml), and ampicillin (5 μg/ml). Critically, the plates were incubated at 37°C under anaerobic conditions to circumvent the presumed hemin requirement of the desired recombinants. Ampicillin-resistant colonies were observed within 2 d, and one colony from each electroporation was purified for Southern blot analysis.

Chromosomal DNA was prepared from isolated amp' H. influenzae Rd colonies, REI1009 (hel(9)::Tn10d-bla) and REI1034 (hel(34)::Tn10d-bla), and also from wild-type strains H. influenzae type b Eagan, and H. influenzae Rd.

After digestion with BamHI, DNA fragments were separated by gel electrophoresis, transferred to membranes, and then hybridized against hel- or blaM-specific gene probes. As shown in Fig. 3, the hel gene probe hybridized to a 9-kb fragment derived from the two wild-type strains (lanes 1 and 2) whereas the two amp' strains displayed hybridizing fragments of ~10 kb in size (lanes 3 and 4). The increase in size of these 10-kb fragments corresponds to the presence of Tn10d-bla insertions (Tn10d-bla, 850 bp) in the hel loci of strains REI1009 and REI1034, as indicated by hybridization of the same fragments to the blaM-specific probe (Fig. 3). From these data, we conclude that hel::Tn10d-bla insertions 9 and 34 have been successfully recombined onto the chromosome of strains H. influenzae REI1009 and REI1034.

In contrast to anaerobic conditions, strains REI1009 and REI1034 do not grow on BHI agar plates containing NAD (10 μg/ml), and hemin (10 μg/ml), when incubated at 37°C under aerobic conditions (Fig. 4 A). These two strains also do not form single colonies under aerobic conditions when hemoglobin (200 μg/ml) or PPIX (10 μg/ml) are used as sole sources of heme (Fig. 4 A). By comparison, wild-type strain H. influenzae Rd grows rapidly under these same aerobic conditions and forms single colonies within 2 d. When strains REI1009, REI1034, and H. influenzae Rd were inoculated in BHI liquid growth medium containing NAD (10 μg/ml) and hemin (10 μg/ml), growth was observed for the wild-type strain under aerobic conditions but not for the hel insertion mutant strains REI1009 or REI1034 (data not shown). To test directly whether e(P4) could complement the heme-dependent growth phenotype of a hel::Tn10d-bla insertion mutant, we transformed REI1009 with plasmid pJR4. Transformants were then tested for their ability to grow aerobically in BHI liquid growth medium, containing NAD (10 μg/ml), and hemin (10 μg/ml). As shown in Fig. 4 B, H. influenzae strain REI1009 transformed with pJR4 was able to grow under aerobic conditions, however no growth was observed for strain REI1009 when transformed with control plasmid pACYC184. These results indicated that the hel gene product, e(P4), is specifically required for growth of strain Rd in the presence of oxygen, a growth condition under which H. influenzae is known to require an exogenous source of heme. Given the ability of the hel gene to
Figure 4. Growth phenotypes of *H. influenzae* Rd hel mutant strains. (A) Cells were grown on 3.8% BHI agar plates containing hemin (10 μg/ml), PPIX (10 μg/ml), or hemoglobin (200 μg/ml and previously dialyzed overnight in 10 mM NaPO₄ buffer, pH 7.4) at 37°C under anaerobic and aerobic conditions. Strains: (WT) *H. influenzae* Rd, (9) REI1009, and (34) REI1034. (B) *H. influenzae* strains Rd and strain REI1009 carrying either pJRP4 or pACYC184 were grown in BHI-medium containing hemin (10 μg/ml) and NAD (10 μg/ml) at 37°C under aerobic conditions. Growth was monitored by optical density at 600 nm.
complement E. coli hemA mutants for utilization of hemin or PPIX, these data strongly suggest that e(P4) is involved in heme uptake. Because e(P4) is known to be an outer membrane protein (17), we reasoned that it was facilitating transport of heme-related compounds across the outer membrane. To test whether the aerobic growth defect associated with hel mutations was specifically associated with a defect in hemin or PPIX uptake, we attempted to complement the growth defect of strain REI1009 with outer membrane perturbants. As shown in Table 1, very low levels of EDTA (0.001-0.1 mM) substantially stimulated the growth of strain REI1009 under aerobic conditions. EDTA-stimulatory effect of EDTA was completely dependent on exogenous hemin, and furthermore, other outer membrane perturbants (e.g., polymyxin B) could substitute for EDTA in this assay (data not shown). Thus, we conclude that the inability of the hel mutant strain REI1009 to grow under aerobic conditions is specifically related to its inability to transport hemin across its outer membrane.

### Discussion

A number of different pathogenic microorganisms are known to utilize hemin as a source of iron or porphyrin. For example, *Vibrio cholerae* can utilize iron or porphyrin derived from hemin by expressing two specialized iron-regulated components, a 26-kD inner membrane protein and a 77-kD outer membrane protein (41). Similarly, the hemin uptake system of *Yersinia enterocolitica* consists of an iron-regulated outer membrane protein (37), and a specific periplasmic binding protein–dependent transport system (42). Most recently, a cloned gene from *Neisseria meningitidis* has been shown to be involved in the uptake of hemin and hemoglobin by this invasive pathogen (43).

Under aerobic conditions, members of the genus *Haemophilus* such as *H. influenzae* classically show a growth requirement for the two essential supplements, factors V and X, corresponding to NAD and hemin, respectively (3). Despite the fact that hemin requirement of *H. influenzae* must be considered the most well-known example of heme dependence, the genes involved in this process have largely evaded genetic analysis. In this report, we established that the product of the hel gene (17), the outer membrane protein e(P4), is one of the key components involved in the utilization by *H. influenzae* of hemin, PPIX, and hemoglobin as sources of porphyrin.

Previous work supports the roles of several other gene products in mediating binding to and utilization of hemin, hemopexin, or hemoglobin as heme sources in *H. influenzae*. For example, Cope et al. (9) reported that mutants defective in hxuC are at least partially defective in heme uptake given that they require higher levels of heme (in excess of 0.1 μg/ml) to grow in liquid culture. In addition, if hemoglobin is used as porphyrin source, hxuC mutants are still able to grow, suggesting that hemoglobin-derived hemin can be utilized. In contrast, the strains described here carrying insertion mutations in the hel gene show virtually no growth under aerobic conditions even in the presence of hemin (10 μg/ml). It is interesting to note that Wong et al. (11) described the isolation of a hemopexin-binding receptor complex that included a component of 29 kD that apparently had a blocked NH2-terminal amino acid. Given that e(P4) is about this size and is known to be a lipoprotein (17), it is tempting to suggest that e(P4) is the 29-kD component observed by Wong et al. Thus, to assemble a high affinity heme uptake system capable of recognizing several different heme sources, e(P4) may need to interact with several different outer membrane components, including those associated with the hemopexin receptor complexes (9, 11). The fact that the hel gene can complement an E. coli hemA mutant for growth on hemin and PPIX but not hemoglobin suggests that additional factors besides e(P4) are needed to "strip" heme from heme-associated proteins such as hemoglobin.

The hel gene product was shown by Green et al. (17) to be an outer membrane lipoprotein that is synthesized as a 274-amino acid residue precursor. Surprisingly, the first clone we obtained that complemented the E. coli hemA mutant for growth on hemin expressed only the first 69 amino acids of e(P4). Subsequently, Tn10d- bla insertion analysis confirmed that as little as the first 90 amino acids of this protein could complement E. coli for heme utilization. If one assumes normal processing and maturation of the e(P4) fusion proteins, plasmid pJRHI should encode a lipoprotein composed of only amino acids 21-69 of e(P4), yet still be capable of complementation of the hemA E. coli mutant.

In an attempt to further characterize the apparently functional NH2-terminus domain of e(P4), we compared the first 80 amino acids of e(P4) to entries in the GenBank data-

### Table 1. Effect of EDTA on Growth of H. influenzae Strains Rd and hel Mutant REI1009

<table>
<thead>
<tr>
<th>[EDTA] (mM)</th>
<th>OD600 H. influenzae Rd*</th>
<th>OD600 REI1009*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.6</td>
<td>0.08</td>
</tr>
<tr>
<td>0.001</td>
<td>1.7</td>
<td>0.35</td>
</tr>
<tr>
<td>0.01</td>
<td>1.7</td>
<td>1.9</td>
</tr>
<tr>
<td>0.1</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>0.5</td>
<td>0.9</td>
<td>0.06</td>
</tr>
<tr>
<td>1</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>3</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Strain H. influenzae Rd and REI1009 were grown in liquid BHI medium supplemented with hemin (10 μg/ml), NAD (10 μg/ml), and various concentrations of EDTA (0.001-3 mM). After overnight incubation at 37°C under aerobic conditions, growth was monitored by optical density at 600 nm.
tabase using the "fasta" program (44). This search resulted in the identification of known hemin-associated proteins that shared limited similarities with the NH$_2$-terminal region of e(P4). One homology cluster was located around amino acid position 56–69—the same region that we found to be apparently critical for hemin utilization as measured by growth complementation assays. Among those proteins that displayed some similarity to e(P4) was the $H$. influenzae HbpA protein, a hemin-binding lipoprotein characterized by Hanson et al. (16). It is interesting that HbpA is highly homologous to the dipeptide transport protein DppA of E. coli (45). Recently, DppA has been implicated in the uptake of ALA in E. coli (46). Two other hemin-associated proteins, hemoglobin (47) and cytchrome C3 (48), also showed some homology to e(P4) in this region as did a euukaryotic transcription factor heme-activated protein (HAP)-1 known to bind heme (49). In the latter case, the homology noted corresponded to a hexa-peptide motif KVAFDH of e(P4) (amino acid residues 64–69), which is similar to the "heme regulatory motifs" (KCPVDH) present in multiple copies within HAP-1 and as well as other heme-binding proteins (49). It is tempting to speculate that the KVAFDH motif is essential for e(P4) function, perhaps acting as a hemin- or PPIX-binding site.

It is clear from our data that e(P4) undoubtedly acts by facilitating the transport or diffusion of hemin-related compounds through the outer membrane. Nonlethal concentrations of the chelator EDTA and the antibiotic polymyxin B can substitute for the loss of e(P4) in $H$. influenzae hel insertion mutants when they are grown aerobically in the presence of heme. Given that the effect of EDTA and polymyxin B on these mutants most likely involves their ability to disrupt the outer membrane permeability barrier (40), these results suggest that e(P4) functions specifically by facilitating the transport or diffusion of heme across the cell's outer membrane. It is interesting to note that within the NH$_2$-terminal region of e(P4) there is a highly cationic segment (amino acids 54–78) in which there are eight positively charged amino acid residues with only one negatively charged residue. Recently, Hoess et al. (50) have presented results suggesting that a highly cationic loop of the LPS-binding protein limulus antilipopolysaccharide factor (LALF) interacts with lipid A in much the same way as the cationic peptide antibiotic polymyxin B. Perhaps the cationic region of e(P4) noted above plays a similar role in binding lipid A and thus facilitates a localized disruption of the outer membrane in the vicinity of bound heme or PPIX.

Recently, Fleischmann et al. (39) reported the complete nucleotide sequence of $H$. influenzae strain Rd. It is interesting to note that the hel gene (HI0693) is apparently not located within an operon nor is it closely linked to another gene that encodes a product known to be involved in heme or iron acquisition. However, we note that a dyad repeat sequence TTTTCAATTGAAAA is located 62 bp upstream of the hel gene and hypothesize that this sequence may be involved in regulation of hel expression. This sequence is located in only one other place in the entire $H$. influenzae genome (position 1,558,736 bp). The second copy of the dyad repeat is near a gene cluster that includes a gene (HI1471) (39) that encodes a product that is highly homologous to HemU, an inner membrane heme permease of Yersinia enterocolitica (42). This putative operon also includes several other genes of which two (HI1470 and HI1472) apparently encode inner membrane transport and periplasmic binding proteins. Thus, these gene products may constitute part of a cytoplasmic membrane heme transport system that is coordinately regulated with hel.

Green et al. (17) originally reported the identification of the hel gene product as the outer membrane lipoprotein e(P4) and found that it was antigenically well conserved among $H$. influenzae type b, as well as among nontypable $H$. influenzae strains. These authors also demonstrated that anti-e(P4) polyclonal serum is bactericidal to clinical isolates of $H$. influenzae, but could not establish any other biologic function for e(P4). Given the essential role of e(P4) in heme uptake, it is not too surprising that antibodies directed against this protein might be deleterious for $H$. influenzae. Thus, our data support the use of e(P4) as an antigen for evaluation in future vaccines for nontypable $H$. influenzae and possibly other Haemophilus species.

We thank A. Wright for providing strain $H$. influenzae Rd, and also for his helpful discussions. We also thank G. Jander for providing the E. coli MC4100 hemA derivative and G. Siber for providing $H$. influenzae type b Eagan. For their careful reading of the manuscript, we thank M. Dziejman and K. Klose.

J. Reidl was supported by the German National Scholarship Foundation (Studienstiftung des deutschen Volkes, BASF-Sonderprogramm). This work was supported by National Institute of Health grant AI-26289 and a grant from the Shipley Institute of Medicine.

Address correspondence to Dr. John J. Mekalanos, Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115.

Received for publication 28 August 1995.
References


38. Reuter, K., R. Slany, F. Ullrich, and H. Kersten. 1991. Structure and organization of *E. coli* genes involved in bio-


