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

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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 hemin through the outer membrane. The analysis of hybrids between *e*(P4) and β-lactamase demonstrated that a domain of *e*(P4) near its NH_2' terminus was required for its function in hemin use. Within this domain is a short amino acid sequence that displays similarity to *H. influenzae* hemin binding protein HbpA, hemin-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 hemin binding and/or transport.

Haemophilus influenzae type b is a Gram-negative coccobacillus, 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 hemin from certain host proteins that contain hemin. Some of the bacterial components required for utilization of various hemin-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 hemin or PPIX, there has been less progress in identifying the key components necessary for their utilization. Two hemin-binding proteins have been reported. A 39.5-kD outer membrane protein (15) was shown to bind to hemin-agarose and this interaction was inhibited by hemin or hemin-binding proteins such as hemoglobin, but not by PPIX. A 60.6-kD hemin-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

[1] *Abbreviations used in this paper:* ALA, 5-amino-levulinic acid; BHI, brain heart infusion; HAP-1, heme-activated protein; LB, Luria broth; NAD, nicotine 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$^-$ *araD* Δ*(argF-lac)*U69 *rpsL*150 *relA1 deoC1 ptsF25 flbB5301 rbsR*) (19) were grown on Luria broth (LB) medium supplemented with 50 μg/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 μg/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′-ATTGGATCCGAATTCTTAAAAGGAAT-3′; and AR16 (30′ mer): 5′-ATTAAATATTGGATCCAGTAAAAACTGAGC-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 Tn*10d-bla* (29) was used to construct hybrid fusion proteins between *e*(P4) and β-lactamase. Plasmid pJR207 was used to mutagenize plasmid pJRP4 as described earlier (29). The insertion sites of the Tn*10d-bla* element 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 Tn*10d-bla* insertions as described earlier (29).

Western Blot Analysis. Derivatives of *E. coli* strain MC4100 *hemA* carrying either plasmid pACYC177, pJRH1, or pJRP4 were grown in 30 ml LB at 37°C for 18 h. Cells were harvested by centrifugation, washed twice, and resuspended in $NaPO_4$ 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 Hantke (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 μg/ml), and kanamycin (20 μg/ml). After overnight incubation at 37°C, the colonies were replica plated onto LB agar plates containing hemin (10 μg/ml) and kanamycin (20 μg/ml). Several colonies that grew on the latter medium were purified and their plasmid DNA isolated. Retransformation into MC4100 *hemA* showed that plasmid pJRH1 was responsible for a hemin utilization phenotype.

Restriction and Southern blot analysis of plasmid pJRH1

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 NH_2-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, pJRH1 does allow strain MC4100 *hemA* to utilize hemin and PPIX, suggesting the *e*(P4) portion of the fusion protein encoded by pJRH1 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,

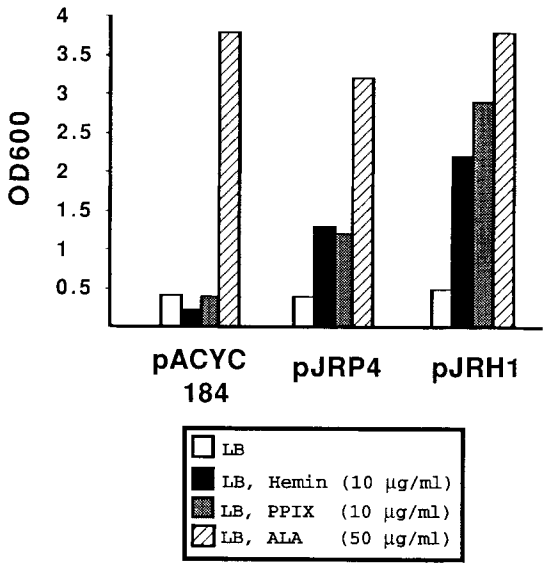


Figure 1. Growth analysis of MC4100 *hemA* transformants. Growth of different *E. coli* transformants were monitored after overnight growth. Strain MC4100 *hemA* was transformed with plasmids pJRP4, pJRH1, and control plasmid pACYC184. The strains were grown on LB containing chloramphenicol (15 µg/ml), and supplemented with hemin, PPIX, and ALA at the concentrations indicated below the graph. The cell density of overnight growth was measured optically at 600 nm.

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 heme utilization, we performed transposon mutagenesis of *hel* with the mini transposable element Tn*10d-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 Tn*10d-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 Tn*10d-bla* insertions on pJRP4 were determined by DNA sequence analyses. Seven Tn*10d-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 Tn*10d-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 Tn*10d-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

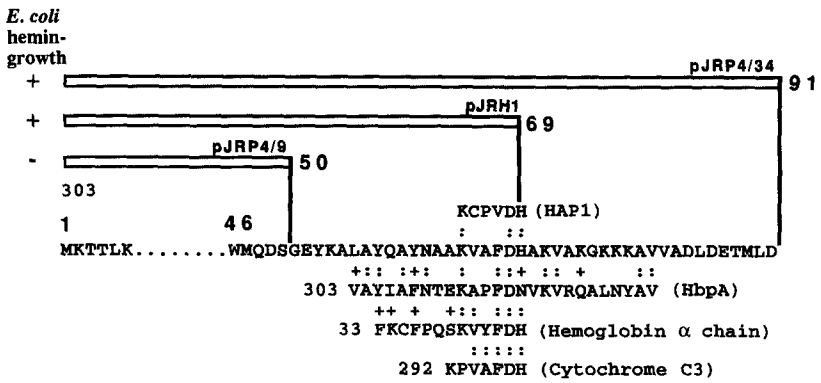


Figure 2. Similarity of *e*(P4) to hemin-binding proteins. (*Top*) The amino acid sequence in single letter code of a portion of the NH_2'-terminal region of *e*(P4). (*Bottom*) The residues of *e*(P4) remaining in fusion proteins encoded by plasmids pJRH1, pJRP4/9, and pJRP4/34. The sequence is aligned with short segments of homologous amino acid residues from hemin-binding proteins HbpA, HAP-1, hemoglobin α chain, and cytochrome C3. (:) Identity to *e*(P4); (+) conserved amino acids. (*Left*) The growth phenotype of *E. coli hemA* strains (carrying the indicated plasmids) on hemin (10 μg/ml)-supplemented media.

hemin uptake, we constructed insertion mutations in the *hel* gene of strain *H. influenzae* Rd. Plasmid pJRP4/9 and pJRP4/34 DNA were prepared and then linearized with restriction endonuclease BamHI to produce linear molecules in which the corresponding Tn*10d-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^r *H. influenzae* Rd colonies, REI1009 (*hel(9)*::Tn*10d-bla*) and REI1034 (*hel(34)*::Tn*10d-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^r 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 Tn*10d-bla* insertions (Tn*10d-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*::Tn*10d-bla* insertions 9 and 34 have been successfully recombined onto the chromosome of strains *H. influenzae* REI1009 and REI1034 .

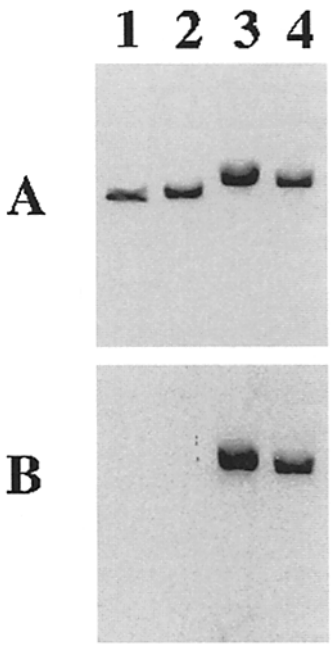
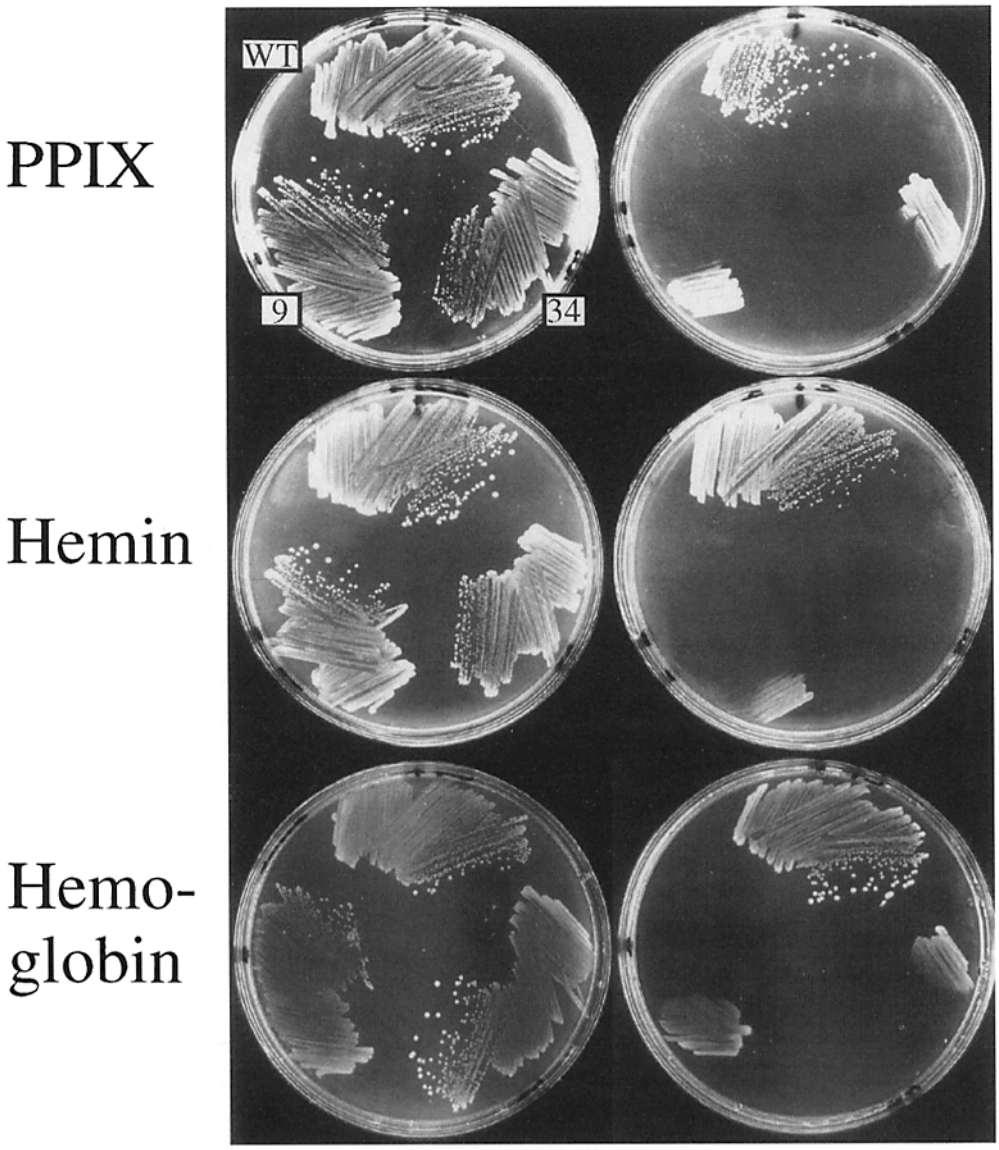


Figure 3. Southern blot analysis of *H. influenzae hel* mutant strains. Chromosomal DNA was digested with restriction endonuclease BamHI, separated on an agarose gel, and transferred to nylon membrane. DNA was prepared from *H. influenzae* type b Eagan (lane *1*), *H. influenzae* Rd (lane *2*), REI1009 (lane *3*), and REI1034 (lane *4*). (*A*) Membrane probed with *hel* gene–specific probe. (*B*) Membrane probed with *blaM* gene–specific probe.

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*::Tn*10d-bla* insertion mutant, we transformed REI1009 with plasmid pJRP4. 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 pJRP4 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



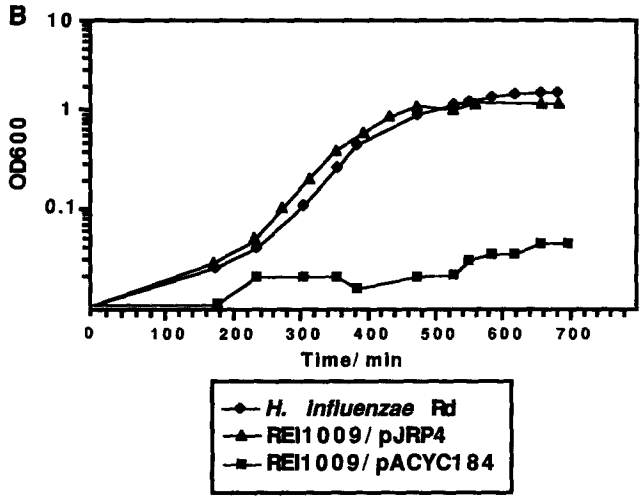


Figure 4. Growth phenotypes of *H. influenzae* Rd *hel* mutant strains. (*A*) Cells were grown on 3.8% BHI agar plates containing hemin (10 μg/ml), PPIX (10 μg/ml), or hemoglobin (200 μg/ml and previously dialyzed overnight in 10 mM $NaPO_4$ buffer, pH 7.4) at 37°C under anaerobic and aerobic conditions. 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.

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

[EDTA]	*H. influenzae* Rd*	REI1009*
mM	*OD600*	
0	1.6	0.08
0.001	1.7	0.35
0.01	1.7	1.9
0.1	1.3	1.4
0.5	0.09	0.06
1	0.06	0.06
3	0.05	0.05

*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.

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 in the presence of hemin. EDTA is known to disrupt the outer membrane of gram-negative bacteria, and thus enhance nonspecific diffusion across this membrane (40). The growth 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 NH_2-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 hemin 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" hemin 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, Tn*10d-bla* insertion analysis confirmed that as little as the first 90 amino acids of this protein could complement *E. coli* for hemin 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 NH_2'-terminal domain of *e*(P4), we compared the first 80 amino acids of *e*(P4) to entries in the GenBank da-

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 cytochrome C3 (48), also showed some homology to *e*(P4) in this region as did a eukaryotic transcription factor heme-activated protein ([HAP]-1) known to bind hemin (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 hemin. 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 hemin 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 hemin 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.

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