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Characterization of Ferrochelatase (*hemH*) Mutations in *Haemophilus influenzae*

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Haemophilus influenzae **lacks most of the biosynthetic enzymes for hemin synthesis. However, the organism has retained ferrochelatase activity, which we identified to be encoded by a *hemH*-homologous gene. In this report we characterize the growth physiology conferred by *hemH* mutations under infection and laboratory conditions.**

Haemophilus influenzae is a gram-negative bacterium that is responsible for significant morbidity and mortality in young children (4, 20). Under normal growth conditions *H. influenzae* needs two essential growth factors: NAD and hemin (2). Hemin can serve as a source of both iron and porphyrin, and protoporphyrin IX (PPIX) can substitute for hemin if exogenous iron is available (5, 21). The activity of a putative ferrochelatase was found in whole-cell extracts of *H. influenzae* (11), indicating that Fe^{2+} and PPIX are chelated into heme. Iron-citrate or ferric ions can be utilized by an uptake system, which was found to be encoded by the genes *hitABC* (18). Since free iron is limited under in vivo conditions and no siderophores are synthesized, *H. influenzae* has evolved strategies to scavenge host iron-binding proteins as sources of iron (19). Human transferrin has been shown, for example, to be a suitable substrate and is specifically recognized by two outer-membrane-located receptor proteins encoded by *tbpAB* (6). Free hemin or PPIX may also be utilized by *H. influenzae* if it is present in growth media, and they are also scavenged from hemin-containing host proteins by specific hemopexin- and hemoglobin- or haptoglobin-binding protein complexes (1, 7–9, 12, 13, 16, 22).

H. influenzae has only a rudimentary hemin biosynthetic pathway, in which no other enzymes except that encoded by the *hemH* homologue are known to exist. In this study, we establish that the *hemH* gene identified by Fleischmann et al. (3) and designated HI1160 encodes a ferrochelatase and that defined mutations in the gene region corresponding to *hemH* confer a profound growth phenotype.

Construction of *hemH* mutants. Utilizing the genome sequence provided by Fleischmann et al. (3) (http://www.tigr.org), *hemH* (HI1160) was PCR amplified from *H. influenzae* strain Rd chromosomal DNA with oligonucleotides containing flanking *Eco*RV restriction sites, i.e., hemH1 (AAGATATCAGTGGATCATCGTACTATGC) and hemH2 (AAGATATCGCTGATTTTAGCAAAGTGCG) (synthesized by MWG-Biotech, Ebersberg, Germany). The resulting 1,315-bp product of HI1160 (Fig. 1A) was cleaved with *Eco*RV and subcloned into *Hin*cII- and *Fsp*I-linearized pACYC177 (17), resulting in plasmid pMR1 (Fig. 1A). A PCR-generated chloramphenicol acetyltransferase-encoding gene (*cat*) with flanking *Pst*I restriction sites (10) was inserted into a unique *Pst*I site, resulting in pMR2 (Fig. 1B). An 837-bp segment of the 5′ *hemH* sequence was deleted by PCR amplification of pMR2 with oligonucleotides del1*Bgl*II (GAAGATCTCAGGCGTTTAAGGGCACC) and del2*Bgl*II (GAAGATCTTTTGCCAAACTTGGATATT), containing flanking *Bgl*II restriction sites. Subsequent ligation of the *Bgl*II sites resulted in plasmid pMR3 (Fig. 1C). The *hemH*::*cat* gene from pMR2 and the *′hemH*(Δ*837*)::*cat* gene from pMR3 were amplified again by PCR, using oligonucleotides hemH1 and hemH2, and the DNA fragments were retransformed into *H. influenzae* strain Rd and *H. influenzae* type b strain Eagan (Hib), respectively. Chloramphenicol-resistant (Cm^r) colonies were obtained on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) supplemented with hemin (20 μg/ml) (Sigma), NAD (10 μg/ml) (Sigma), and chloramphenicol (2 μg/ml) (Sigma). Transformants were designated SCH01 (Rd *hemH*::*cat*) and SCH02 [Hib; *hemH*(Δ*837*)::*cat*]. Correct gene replacement in both strains was verified by PCR (Fig. 1D) and Southern blot analysis (data not shown).

Characterization of *hemH* growth phenotypes. Strains SCH01 and SCH02 were tested for their abilities to grow on PPIX- or hemin-supplemented BHI medium. Both *hemH* mutants failed to grow on medium supplemented with PPIX (20 μg/ml) (Sigma) but grew well on medium containing hemin (20 μg/ml) (Table 1). Complementation of both strains with plasmid pMR1 resulted in growth of both strains on PPIX-containing medium (Table 1), indicating that *hemH* was responsible for utilization of PPIX. We further tested the ability of strain SCH01 to grow on hemin as an intracellular iron source. To establish iron-limiting conditions, BHI medium was supplemented with the iron chelator deferoxamine mesylate (DFX) (Sigma), which preferentially chelates extracellular iron. We observed no growth of the wild-type (wt) Rd strain on BHI medium with PPIX (20 μg/ml) supplemented with DFX (0.08 mM), indicating that under these conditions no iron source was available for hemin biosynthesis (Table 1). With hemin instead of PPIX, both the wt Rd and the *hemH* mutant SCH01 could grow in the presence of DFX. This finding demonstrates that both strains can utilize hemin as an iron source and that if HemH could release iron from hemin, as has previously been suggested (11), then an additional cytoplasmic hemin-utilizing and iron liberation system must coexist with *hemH*.

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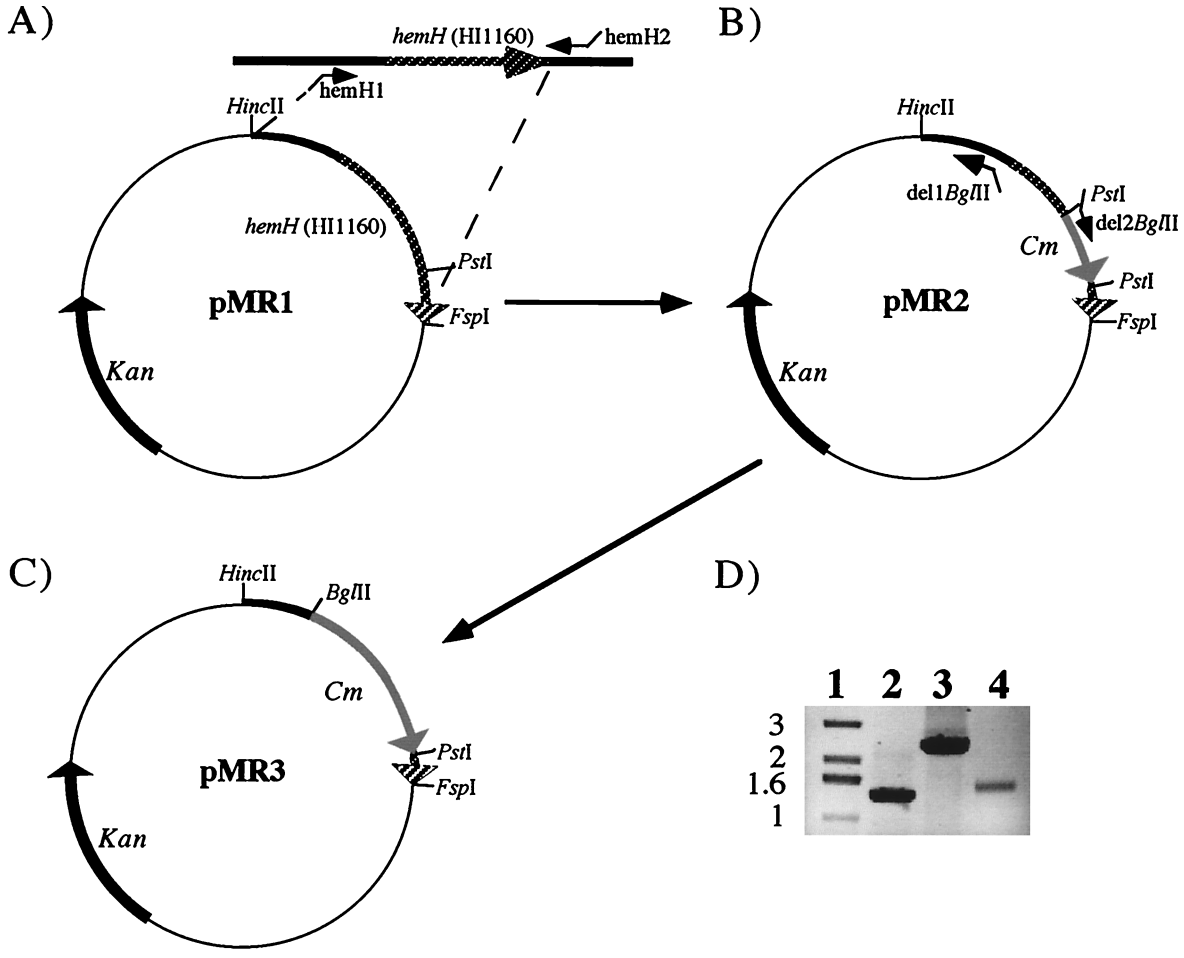

FIG. 1. Cloning of *hemH* and construction of *hemH* mutants. (A) Cloning of *hemH* PCR product, containing flanking *Eco*RV restriction sites, into a *Hinc*II- and *Fsp*I-linearized pACYC177 plasmid. (B) pMR1 was digested with *Pst*I, and *cat* was ligated into *hemH*. (C) Plasmid pMR3 was derived from pMR2 by deletion of the 5′ end of *hemH* (see text). Antibiotic markers and relevant restriction sites are indicated. The *hemH* gene (hatched arrows), chloramphenicol resistance gene (*cat*) (light arrows), and kanamycin resistance marker (Kan) (black arrows) are shown. (D) A 0.7% agarose gel with PCR-generated fragments obtained by using oligonucleotides hemH1 and hemH2 and purified chromosomal DNAs of *H. influenzae* strains Hib (lane 2), SCH01 (lane 3), and SCH02 (lane 4). Lane 1, 1-kbp standard (Gibco Life Technologies).

The in vivo relevance of *hemH* was assessed by intraperitoneal and intranasal inoculation of 5-day-old Sprague-Dawley infant rats (14, 15). Infant rats were inoculated intraperitoneally with 100 μl of 0.1% gelatin in phosphate-buffered saline containing 10^2 CFU of wt Hib ($n = 5$) or the *hemH* mutant Hib strain SCH02 ($n = 5$). At 48 h, there was no difference in bacteremia ($2.78 \times 10^6 \pm 1.9 \times 10^6$ CFU/ml for wt Hib [mean ± standard deviation] versus $2.98 \times 10^6 \pm 2.1 \times 10^6$ CFU/ml for SCH02), and all animals died by 72 h, suggesting that the two strains had similar virulence. Infant rats were also inoculated intranasally with 10 μl of 0.1% gelatin–phosphate-buffered saline containing 10^7 CFU of wt Hib ($n = 3$) or SCH02 ($n = 3$). There was no difference in recovery (around 10^3 to 10^4 CFU) of either strain from 40-μl nasal washings at 48 h. These experiments indicate that HemH is not essential for bloodstream survival or nasopharyngeal colonization and suggest that PPIX is not a major in vivo source of factor X.

TABLE 1. Growth abilities of *H. influenzae hemH* mutant and complemented strains on hemin and PPIX

Supplement(s)[a]	Growth phenotype				
	wt	SCH01	SCH01(pMR1) (*hemH*$^+$)	SCH02	SCH02(pMR1) (*hemH*$^+$)
Hemin	+	+	+	+	+
Hemin, DFX	+	+	ND[b]	ND	ND
PPIX	+	−	+	−	+
PPIX, DFX	−	−	ND	ND	ND

[a] BHI medium containing NAD (10 μg/ml) was supplemented with hemin (20 μg/ml) or PPIX (20 μg/ml), with or without DFX (0.08 mM).
[b] ND, not done.

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