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Cloning and Characterization of the *Escherichia coli hemN* Gene Encoding the Oxygen-Independent Coproporphyrinogen III Oxidase

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Coproporphyrinogen III oxidase, an enzyme involved in heme biosynthesis, catalyzes the oxidative decarboxylation of coproporphyrinogen III to form protoporphyrinogen IX. Genetic and biochemical studies suggested the presence of two different coproporphyrinogen III oxidases, one for aerobic (HemF) and one for anaerobic (HemN) conditions. Here we report the cloning of the *hemN* gene encoding the oxygen-independent coproporphyrinogen III oxidase from *Escherichia coli* by complementation of a *Salmonella typhimurium hemF hemN* double mutant. An open reading frame of 1,371 bp encoding a protein of 457 amino acids with a calculated molecular mass of 52.8 kDa was identified. Sequence comparisons revealed 92% amino acid sequence identity to the recently cloned *S. typhimurium hemN* gene and 35% identity to the *Rhodobacter sphaeroides* gene. The *hemN* gene was mapped to 87.3 min of the *E. coli* chromosome and found identical to open reading frame o459 previously discovered during the genome sequencing project. Complementation of *S. typhimurium hemF hemN* double mutants with the *E. coli hemN* gene was detected under aerobic and anaerobic conditions, indicating an aerobic function for HemN. The previously cloned *E. coli hemF* gene encoding the oxygen-dependent enzyme complemented exclusively under aerobic conditions. Primer extension experiments revealed a strong transcription initiation site 102 bp upstream of the translational start site. DNA sequences with homology to a σ^{70}-dependent promoter were detected. Expression of the *hemN* gene in response to changing environmental conditions was evaluated by using *lacZ* reporter gene fusions. Under anaerobic conditions, *hemN* expression was threefold greater than under aerobic growth conditions. Removal of iron from the growth medium resulted in an approximately fourfold decrease of aerobic *hemN* expression. Subsequent addition of iron restored normal expression.

Tetrapyrroles, like hemes and chlorophylls, are integral parts of electron transport chains in members of the domains *Bacteria*, *Eucarya*, and *Archaea* (6, 9, 13). In *Escherichia coli*, the cellular levels of hemes vary drastically depending on whether the organism is growing aerobically, using oxygen as electron acceptor, or anaerobically, using a fermentative means of energy generation (6, 9, 11, 12, 17, 18, 21, 32). Genetic and biochemical investigations suggest the presence of one regulatory point at the formation of 5-aminolevulinic acid, an early precursor molecule for tetrapyrrole formation (10, 14–16, 19, 20, 30, 44). A second regulatory point was found at the formation of protoporphyrinogen IX from coproporphyrinogen III, a reaction in which the propionyl groups on the rings A and B are oxidatively decarboxylated to vinyl groups (6, 9, 21, 36). Since aerobic coproporphyrinogen III oxidase activity requires molecular oxygen as an electron acceptor for the oxidative decarboxylation of coproporphyrinogen III, it obviously could not function in the absence of molecular oxygen (34, 39, 40, 45). Consequently, anaerobic bacterial heme biosynthesis utilizes a different oxygen-independent enzyme, which was found to require Mg^{2+}, methionine, ATP, and NAD^+ or $NADP^+$ for its activity (25, 36, 39, 40). Various mechanisms were proposed for both enzymes (6, 9, 21, 25). However, the nature of aerobic and anaerobic protoporphyrinogen IX formation awaits final elucidation. Two loci for coproporphyrinogen III oxidase activity were mapped to min 50 (*hemF*) and min 85 (*hemN*) of the *Salmonella typhimurium* chromosome (45). The genes for the aerobic enzyme were cloned from yeasts (48), mammals (24, 27), and plants (26) and from the enterobacteria *S. typhimurium* (46) and *E. coli* (42). The genes for an oxygen-independent enzyme were cloned recently from *Rhodobacter sphaeroides* (7) and *S. typhimurium* (47). While the polypeptides for the aerobic enzymes share significant identity, no obvious homology was found to their anaerobic counterparts (7, 47). The expression of the yeast *HEM13* gene encoding an oxygen-dependent coproporphyrinogen III oxidase was found to be subject to transcriptional regulation in response to changing cellular levels of oxygen and heme (22, 43, 48). The presence of two bacterial genes encoding coproporphyrinogen III oxidase could provide the bacterial cell with a similar regulatory tool for the response to various changing environmental conditions.

Aerobic complementation of a heme-deficient *hemF hemN* double mutant of *S. typhimurium* with *E. coli* genomic DNA cloned in pBR322. We cloned the gene for the oxygen-independent coproporphyrinogen III oxidase (*hemN*) of *E. coli* by aerobic complementation of a heme-requiring *S. typhimurium* mutant defective for the anaerobic gene (*hemN*) and the aerobic gene (*hemF*). This strategy was chosen for several reasons. During our screen for *E. coli* genes encoding coproporphyrinogen III oxidase by using the complementation of heme-deficient yeast mutants (*HEM13*), the *E. coli hemF* gene but no *hemN* gene was cloned (42). Investigation of the *S. typhimurium* system revealed that a double mutant defective in both genes was needed for complementation experiments, since the

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TABLE 1. Bacterial strains, phages, and plasmids used

Strain, phage, or plasmid	Relevant genotype	Reference or source
E. coli		
DH5α	*hsdR recA1 lacZYA f80 lacZDM15 gyrA9*	35
BL21(DE3)	F^- *gal met* r^- m^- *hsdS* λ_{lys} *placUV5*::T7 gene 1 *placI*^{q}	38
MC4100	F^- *araD139* Δ(*argF-lac*)*U169 rpsL150 deoC1 relA1 ptsF25 flbB5301 rbsR*	4
BD25	F^- *supF supE hsdR galK trpR metB lacY tonA* Δ*lac*	29
BT18	MC4100 Φ(*hemN-lacZ*)	This study
S. typhimurium		
TE3006	*env-53 hemN704*::Mud-J(b) *hemF707*::Tn*10d*-Tet	45
TE2849	*env-53 hemN704*::Mud-J(b) *hemF705*	45
Bacteriophages		
$\lambda C17c_{190}$	Test phage for multiplicity of integration	1
λRZ5	*bla lacZ lacY*$^+$ *attP*$^+$	31
λBT18	λRZ5 [Φ(*hemN-lacZ*)] *bla*$^+$	This study
Plasmids		
pMLB1010	Vector for *lacZ* fusions	37
pBT18	704-bp 5′ region of *hemN* in pMLB1010	This study
pBluescript SK+	High-copy-number phagemid, Ap^r	Stratagene
pBlue13	6.5-kb *Hin*dIII fragment in pBluescript SK+ containing the *E. coli hemF* gene in the same orientation as the Φ10 promoter[a]	42
pBlueN7	2.6-kb *Hin*dIII-*Sal*I fragment from pBRN7 in pBluescript SK+ containing the *E. coli hemN* gene in the same orientation as the Φ10 promoter	This study
pBR322	Plasmid vector, Ap^r Tc^r	3
pBRN7	2.0-kb *Sau*3A fragment in pBR322 containing the *E. coli hemN* gene	This study

[a] Φ10 promoter = T7 RNA polymerase promoter.

anaerobic gene (*hemN*) sustained growth even under aerobic conditions (45). No complementing clones were obtained through complementation experiments with the *S. typhimurium* double mutant under anaerobic conditions (41, 47). However, the detected aerobic growth of an *S. typhimurium hemF* mutant in the presence of the intact genomic copy of the *hemN* gene made a screen for the oxygen-independent *hemN* gene of *E. coli* via complementation of the *S. typhimurium* double mutant under aerobic conditions possible (45). The outlined screen led to the parallel isolation of *hemF* and *hemN* genes. Complementing *E. coli hemF* genes were subsequently sorted out by the known restriction pattern of the *hemF*-containing plasmid DNA. *E. coli* K-12 genomic DNA was purified as outlined elsewhere (2) and digested with *Sau*3A. After size fractionation, 5- to 10-kb fragments were ligated into the *Bam*HI site of pBR322. The library was amplified in *E. coli* DH5α. The coproporphyrinogen III oxidase-deficient strain *S. typhimurium* TE3006 (Table 1) was transformed with the *E. coli* genomic library via electroporation. Transformants were screened aerobically for recovery of heme sufficiency on minimal medium containing glycerol as a nonfermentable carbon source. Several positive clones were obtained, and the complementing plasmid DNAs were extracted and subjected to *Pvu*II restriction analysis to identify and eliminate complementing *hemF* genes. Three identical clones without the typical *hemF* restriction pattern were found. Retransformation of one of the obtained clones termed pBRN7 into the *S. typhimurium* double mutant TE3006 and a second double mutant termed TE2849 confirmed the obtained phenotype (Table 2). The transformants with pBRN7 grew as well as both *S. typhimurium* double mutants transformed with pBlue13 containing the *E. coli hemF* gene (Table 2). Control transformants containing just the vector failed to grow (Table 2).

Nucleotide sequence and organization of the *E. coli hemN* gene. Plasmid pBRN7 contained an insert of approximately 2.0 kb (Fig. 1). For sequence determination and protein expression experiments, a 2.6-kb *Cla*I-*Sal*I fragment from pBRN7 was recloned into pBluescript SK+ to generate pBlueN7 (Table 1). Partial DNA sequence determination (5) identified the cloned DNA as part of a known *E. coli* DNA sequence determined during the *E. coli* genome sequencing project, containing one open reading frame of unknown function termed open reading frame o459 (33). Since several bases of the previously sequenced DNA remained undetermined, the complete insert of pBlueN7 was sequenced (Fig. 1). One open reading frame was found (Fig. 1). Open reading frame (*hemN*; see below) started with an ATG in position 300 and ended with an ochre codon in position 1673. The predicted protein of 457 amino acids had a calculated molecular mass of 52.8 Da. The deduced protein showed 92% identity to its previously cloned *S. typhimurium* counterpart and 35% identity to the *R. sphaeroides* oxygen-independent coproporphyrinogen III oxidase (after extension of the cloned open reading frame to an upstream ATG as described before [7, 47]). Moreover, protein sequences deduced from DNA sequences cloned from *Pseudomonas aeruginosa* and *Rhizobium phaseoli* revealed significant homology to

TABLE 2. Complementation of *S. typhimurium hemF hemN* double mutants with the *E. coli* genes encoding aerobic and anaerobic coproporphyrinogen III oxidases[a]

Plasmid	Complementation			
	S. typhimurium TE3006		*S. typhimurium* TE2849	
	$+O_2$	$-O_2$	$+O_2$	$-O_2$
pBlueN7 (*E. coli hemN*)	+	+	+	+
pBlue13 (*E. coli hemF*)	+	−	+	−
pBluescript	−	−	−	−

[a] *S. typhimurium* strains were transformed via electroporation with the plasmids indicated. Selection for heme sufficiency was performed as outlined in the text. + or − indicates whether the plasmid is able to restore growth on minimal plates with glycerol as a carbon source and without heme addition.

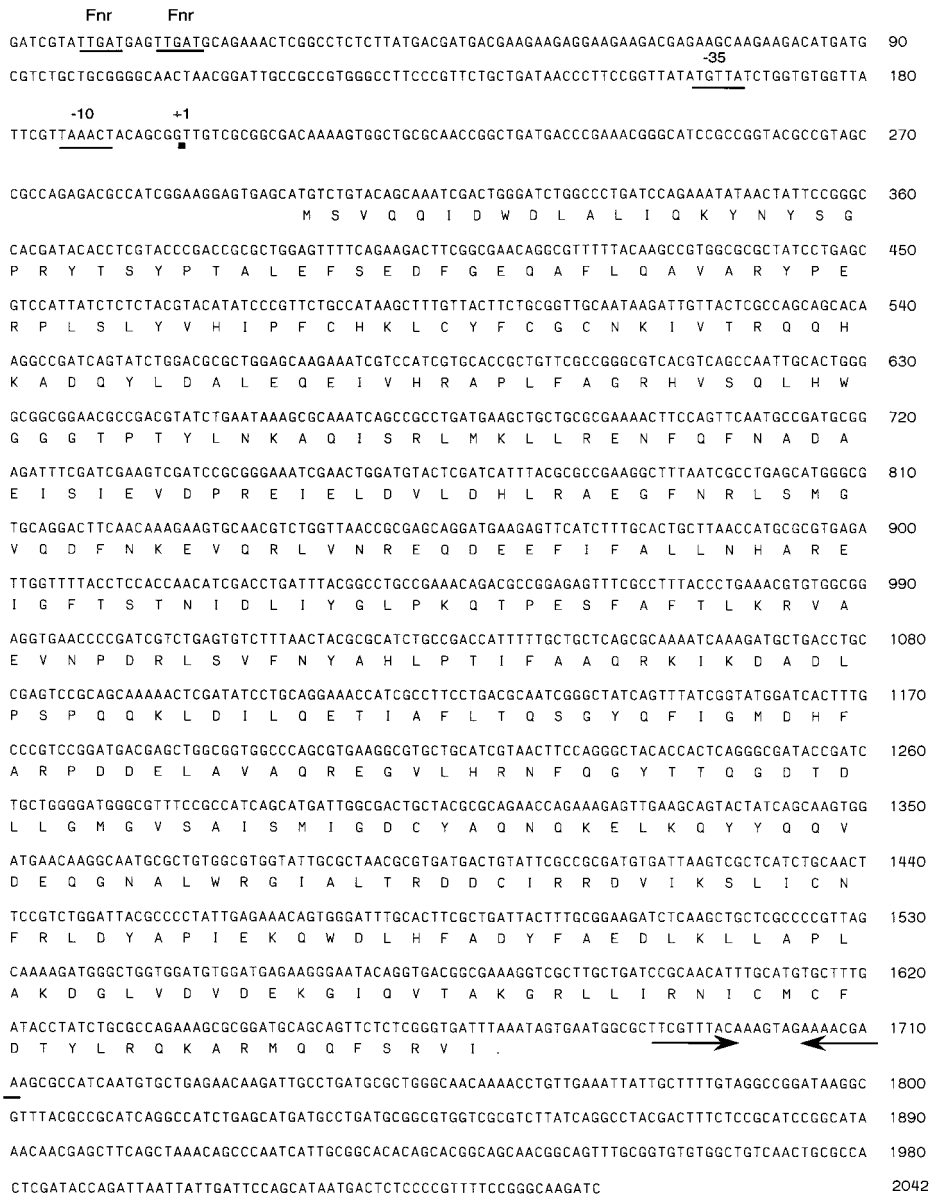

FIG. 1. Nucleotide and deduced amino acid sequences of the cloned *E. coli hemN* gene. The −10 and −35 regions of a potential σ^{70}-dependent promoter and potential Fnr half sites are underlined. The position of the 5′ end of the *hemN* mRNA indicated by primer extension analysis is marked with an arrow.

the *E. coli* HemN. However, sequence similarities were found distributed over more than one open reading frame in both unpublished sequences. Functional cloning of the *P. aeruginosa hemN* gene by our group identified one continuous open reading frame with strong homology to the *E. coli hemN* gene in a previously described region of the *P. aeruginosa* chromosome (13).

Detection of the protein encoded by the cloned DNA from *E. coli*. In vivo translational experiments were performed with pBlueN7, which contains the *hemN* gene cloned in the proper orientation to be expressed from the T7 RNA polymerase promoter of the plasmid. *E. coli* BL21(DE3), which carries T7 RNA polymerase under control of the *lacUV5* promoter, was transformed with plasmids pBlueN7 and, as a control, pBluescript SK+ (38). In vivo translation and analysis of the obtained products were performed as described earlier (42). A polypeptide of approximately 54 kDa, corresponding to the calculated molecular mass of HemN, was observed (Fig. 2, lane 2). Expression experiments with an empty vector and the cloned DNA in the opposite orientation yielded no additional labeled polypeptides (Fig. 2, lane 1, and data not shown).

Aerobic and anaerobic complementation of an *S. typhimurium hemF hemN* double mutant by the *E. coli hemN* gene indicates oxygen-independent coproporphyrinogen III oxidase activity. To further characterize the enzymatic nature of the *E. coli* HemN, complementation experiments using two *S. typhimurium hemF hemN* double mutants under aerobic and strictly anaerobic conditions were performed. The cloned *E. coli hemN* gene efficiently complemented both *S. typhimurium* mutants under aerobic and strictly anaerobic conditions (Table 2). The previously cloned *E. coli hemF* gene complemented the *S. typhimurium* double mutants only under aerobic conditions,

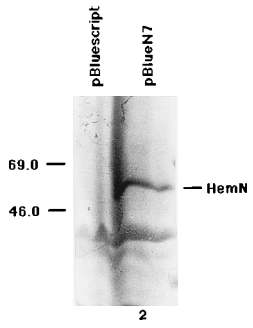

FIG. 2. In vivo expression of the *hemN* gene residing on the cloned *E. coli* DNA fragment. The plasmid-encoded gene product was labeled with [^{35}S]methionine as described in the text and detected by autoradiography after separation of proteins on sodium dodecyl sulfate-polyacrylamide gels. Plasmid pBlueN7 is described in Table 1. The positions and apparent molecular masses (in kilodaltons) of protein standards run on the same gel are indicated.

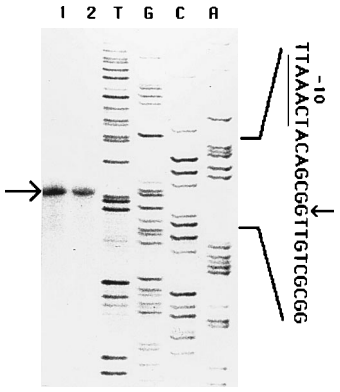

FIG. 3. Primer extension mapping of the 5′ ends of mRNAs encoded by the *hemN* gene. Labeled oligonucleotides were hybridized to total cellular RNA from *E. coli* grown under anaerobic conditions in the presence (lane 1) and absence (lane 2) of nitrate and were subsequently extended with avian myeloblastosis virus reverse transcriptase. Obtained products (lane 1 and 2) were analyzed parallel to DNA sequencing reactions (lanes T, G, C, and A) performed with the same primers on a denaturing polyacrylamide gel and visualized via autoradiography. The reverse transcripts and the positions of the 5′ ends of the mRNAs are indicated with arrows. For the exact positions of the sites found, see Fig. 1.

clearly indicating the oxygen dependence of the enzymatic reaction performed by the aerobic enzyme (Table 2). Transformations with an empty vector failed to grow without heme supplementation and served as a control. These results indicate aerobic expression of the *hemN* gene and aerobic function of the HemN protein. Interestingly, growth experiments with the *S. typhimurium* double mutants transformed either with the *E. coli hemF* gene or with the *E. coli hemN* gene showed similar aerobic growth (data not shown).

Chromosomal localization of the *E. coli hemN* locus. The previously sequenced region containing the *hemN* gene was aligned with sequences of the Kohara phages mapping at 87.3 min of the *E. coli* chromosome (23, 33). Hybridization of the insert of pBlueN7 to the Kohara phages (547)8B10(+) and (546)10P1(−) confirmed these results (data not shown). The restriction map of our clone was identical to the Kohara map published for this region, with the exception of an additional *Eco*RV site at position 1101 (23). Moreover, alignment of the *E. coli* and *S. typhimurium* chromosomal maps assigns min 87.3 of the *E. coli* chromosome to min 85 of the *S. typhimurium* chromosome (29). This is the exact position mapped by Xu and Elliott for the *S. typhimurium hemN* gene (47).

Mapping of the 5′ ends of the mRNAs and potential promoter elements for the *E. coli hemN* gene. The *E. coli hemN* gene was analyzed by primer extension for potential transcription start sites. Total cellular RNA was prepared from *E. coli* MC4100 as outlined elsewhere (2). The 5′ ends of mRNAs encoded by the *hemN* gene were mapped by using the primer extension method with an oligonucleotide complementary to positions 356 to 380 (CGGGCCACGATACACCTCGTACC CG) that has been labeled at its 5′ end by using T4 polynucleotide kinase and [γ-^{32}P]ATP. The exact protocol is given elsewhere (42). One potential transcription start site was located in the 5′ region of the gene in position 198, approximately 102 bp upstream from the translational start of *hemN* (Fig. 1 and 3). A potential σ^{70}-dependent promoter was found upstream of this initiation site in positions 160 to 166 (−35 region) and 186 to 191 (−10 region). Primer extension experiments using RNA prepared from *E. coli* grown anaerobically in the presence of nitrate (Fig. 3, lane 1) or utilizing a fermentative mode of energy generation (Fig. 3, lane 2) showed no significant difference in the intensity of the extension signals.

The *E. coli hemN* gene is regulated by oxygen and the iron contents of the growth medium. Coproporphyrinogen III is the major tetrapyrrole intermediate in a growing bacterial cell (11, 17, 18). The total cellular amount of the compound and its interconversion to heme are strictly dependent on the conditions of the growth environment and the metabolic capacities of the organism (11, 12, 17, 18). The existence of two bacterial enzymes for the oxygen-dependent (HemF) and oxygen-independent (HemN) conversion of coproporphyrinogen III into protoporphyrinogen IX suggests a strict regulation of their genes (*hemF* and *hemN*) by parameters of the growth environment, most likely the availability of oxygen. Moreover, the observed aerobic complementation of the *S. typhimurium hemF hemN* double mutant by the *E. coli hemN* gene indicated an additional aerobic function for HemN. To understand the influences of various environmental parameters on *hemN* expression, reporter gene fusions of the *hemN* 5′ region with the *lacZ* gene were constructed, inserted as a single copy into the chromosome of *E. coli*, and tested for their responses to changing growth conditions. The *hemN-lacZ* fusion was first constructed on a plasmid vector. To cover the complete region between the *hemN* gene and the potential open reading frame detected upstream during the genome sequencing project (33), a 704-bp DNA fragment starting at position 323, containing 323 bp of the cloned 5′ region and an additional 381 bp, was generated via PCR using phage (547)8B10(+) of the Kohara library as a template. For this purpose, primers from positions 311 to 335 (GCAAATCGACTGGGATCTGGCCCTG) and from outside the cloned fragment (ATCTTCGAATTCACGC AGCAAAGGTC) containing new *Eco*RI and *Bam*HI restriction sites were used. After purification and digestion with *Eco*RI and *Bam*HI, the fragment was cloned into pMLB1010 digested with the appropriate enzymes. The structure of the constructed plasmid (pBT18) was confirmed by DNA sequencing. The plasmid was transformed into *E. coli* BD25 missing *tonB* for the integration of λ phages into the chromosome. Transformed bacteria were subsequently infected with λRZ5 (31). Recombination events between the plasmid and the phage resulted in the integration of the *lacZ* fusion into the phage to generate λBT18. Single λBT18 plaques were isolated and amplified. The *hemN-lacZ* fusion was integrated into the chromosome of MC4100 (wild type) (4). The number of integrations into the chromosome was checked using the test phage λC17c_{190} as described previously (1). Strains containing

TABLE 3. Effects of various environmental conditions on *hemN-lacZ* expression[a]

Growth conditions		β-Galactosidase activity[b]
Addition(s) to M9 medium	Aerobic or anaerobic	
None	Aerobic	0.18
None	Anaerobic	0.56
Nitrate	Anaerobic	0.61
ALA	Aerobic	0.16
ALA	Anaerobic	0.52
$FeCl_3$	Aerobic	0.20
2,2-Dipyridyl	Aerobic	0.05
2,2-Dipyridyl + $FeCl_3$	Aerobic	0.16

[a] *E. coli* BT18 (*hemN-lacZ*) cells were grown in minimal medium with the indicated additions (10 mM KNO_3 [nitrate], 50 μM 5-aminolevulinic acid [ALA], 50 μM $FeCl_3$, 0.2 mM chelator 2,2′-dipyridyl, and excess $FeCl_3$ in the presence of the chelator) to an optical density at 600 nm of 0.35 to 0.5, harvested, and broken, and β-galactosidase activities were determined as described in the text.

[b] Expressed as micromoles of substrate (2-nitrophenyl-β-D-galactoside) cleaved per minute per milligram of protein.

single integrations were grown under the conditions indicated. Bacteria were grown on minimal media (M9) with glycerol or glucose as a carbon source and additions as indicated (35). Cells were harvested and broken, and β-galactosidase activity was measured as outlined by Miller (28). Table 3 shows weak expression of the *hemN* gene under aerobic conditions. However, a significant threefold increase of *hemN* mRNA was observed under anaerobic growth conditions. In agreement with the primer extension data, no significant difference between anaerobic growth in the presence and absence of nitrate was observed, indicating a general anaerobic induction of the gene (Table 3; Fig. 3). A similar induction by anaerobic conditions was observed for the *E. coli hemA* gene (10). Two half sites of the conserved DNA recognition site for the transcriptional regulator Fnr were detected approximately 300 bp upstream of the transcriptional start sites of *hemN* (underlined in Fig. 1). However, this would be an atypical location for an Fnr-regulated promoter. Therefore, the regulatory basis for the anaerobic induction process remains unknown. Future experiments using oxygen regulator (*fnr arcA/B*) mutants should provide further insights into the basis of the anaerobic induction process.

Addition of iron to the growth medium did not influence *hemN* transcription. Interestingly, growth of *E. coli* on minimal medium treated with the chelator 2,2′-dipyridyl led to an approximately fourfold decrease of *hemN* expression. Further addition of an excess of iron to the chelator-treated cells restored *hemN* expression. These findings were independent of the presence or absence of oxygen (data not shown). Similar effects were observed by Cotter et al. for the expression of various anaerobic respiratory genes, such as *narGHJI*, *dmsABC*, and *frdABCD* (8). Since all of these enzyme complexes contain iron, a direct response to iron limitation was postulated (8). Additionally, these enzyme systems possess iron-containing tetrapyrrole cofactors. The observed regulation of tetrapyrrole formation could be part of a coregulatory mechanism with previously observed regulation or merely the response of the cell to prevent accumulation of the highly light-sensitive and radical-forming iron-deficient tetrapyrroles. A variety of other tested parameters such as the heme contents of the cell increased by the addition of 5-aminolevulinic acid to the medium showed no significant influence on *hemN* expression (Table 3).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number X82073.

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