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Effect of Heme and Oxygen Availability on *hemA* Gene Expression in *Escherichia coli*: Role of the *fnr*, *arcA*, and *himA* Gene Products

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While many organisms synthesize δ-aminolevulinate, the precursor of heme, by condensing succinyl-coenzyme A and glycine, others use a glutamate-dependent pathway in which glutamyl-tRNA dehydrogenase catalyzes the rate-determining step. The *hemA* gene that encodes this latter enzyme in *Escherichia coli* has been cloned and sequenced. To examine how its expression is regulated, we constructed *hemA-lacZ* operon and gene fusions and inserted them into the chromosome in single copy. The effect of aerobic and anaerobic growth conditions and the availability of electron acceptors and various carbon substrates were documented. Use of different types of cell culture medium resulted in a fivefold variation in *hemA-lacZ* expression during aerobic cell growth. Anaerobic growth resulted in 2.5-fold-higher *hemA-lacZ* expression than aerobic growth. This control is mediated by the *fnr* and *arcA* gene products. Fnr functions as a repressor of *hemA* transcription during anaerobic cell growth only, whereas the *arcA* gene product activates *hemA* gene expression under both aerobic and anaerobic conditions. Integration host factor protein was also shown to be required for control of *hemA* gene regulation. To determine whether an intermediate or a product of the heme biosynthetic pathway is involved in *hemA* regulation, *hemA-lacZ* expression was analyzed in a *hemA* mutant. Expression was elevated by 20-fold compared with that in a wild-type strain, while the addition of the heme pathway intermediate δ-aminolevulinate to the culture medium restored expression to wild-type levels. These results suggest that the heme pathway is feedback regulated at the level of *hemA* gene expression, to supply heme as it is required during different modes of cell growth.

Heme, the prosthetic group of cytochromes, catalase, hemoglobin, and myoglobin, is an essential cofactor for cellular respiration in most procaryotic and eucaryotic organisms (21, 24). The first committed step in the biosynthesis of tetrapyrroles in many organisms involves the formation of δ-aminolevulinate (δ-ALA) (21, 24). In the animal mitochondrion, this occurs through the condensation of glycine and succinyl-coenzyme A in a reaction catalyzed by δ-ALA synthase, an enzyme whose regulation has been studied extensively for a variety of organisms, including humans. However, in plants and many bacteria, including *Escherichia coli* and *Bacillus subtilis*, δ-ALA is derived from a glutamate precursor in a C5 pathway through a series of alternative reactions (2, 22) (Fig. 1). The rate-limiting step in the C5 pathway of δ-ALA synthesis is catalyzed by glutamyl-tRNA dehydrogenase (15, 21, 22).

The *E. coli hemA* gene that encodes glutamyl-tRNA dehydrogenase has been cloned and sequenced (8, 19, 31). It is located at 26.7 min on the *E. coli* chromosome and specifies a 418-amino-acid polypeptide (8, 19, 31). Attempts to overexpress this gene in *E. coli* have been unsuccessful (32). However, a 45-kDa protein with glutamyl-tRNA dehydrogenase activity was isolated from a strain of *Saccharomyces cerevisiae* that was transformed with a plasmid containing the *E. coli hemA* gene. The yeast strain does not contain any glutamyl-tRNA dehydrogenase activity of its own (32). A second polypeptide of 85 kDa with glutamyl-tRNA dehydrogenase activity was also isolated from *E. coli* (15). Preliminary reports by Verkamp et al. indicate that this enzyme is active when the 45-kDa protein is defective (32). Nevertheless, *E. coli hemA* gene regulation in response to alternative cell growth conditions has not been analyzed (8, 19, 25, 31).

To examine how the *hemA* gene in this enteric bacterium is controlled, *hemA-lacZ* gene and operon fusions were constructed and introduced in single copy into the chromosome. Expression of these *hemA-lacZ* fusions was shown to be regulated by the *fnr* and the *arcA* gene products. A defect in heme synthesis caused by a *hemA* mutation resulted in abnormally elevated *hemA-lacZ* expression during aerobic and anaerobic culture. This control was δ-ALA dependent: when δ-ALA was added to the cell culture medium, *hemA-lacZ* expression was restored to wild-type levels, suggesting the existence of a heme pathway transcriptional regulator.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The sources of the bacterial strains, phages, and plasmids are described in Table 1. The SD1 (*himA*), SD6 (*fis*), and SD101 (*fur*) strains were constructed by P1 transduction of the respective mutations from K5185, MC4100 *fis*::*767*, and W3110 *fur* Kan^r into MC4100 (4, 7, 16, 26, 27). The *hemA-lacZ* strains containing deletions of *fnr*, *arcA*, or *fnr* and *arcA* were constructed by introduction of λSD1 as previously described (28). The *hemA*$^+$ gene was obtained from an *E. coli* genomic library by selection for vigorous growth of a *hemA* strain, PC40, which can only form pinpoint colonies on L agar medium without δ-ALA supplementation (8, 30, 31). Introduction of the *hemA*$^+$ gene into this strain restores the synthesis of NADPH-dependent glutamyl-tRNA dehydrogenase, and thus δ-ALA production, in the cell. Of 10 complementing plasmids obtained by this selection, 6 contained a 6-kb *Hin*dIII fragment corresponding

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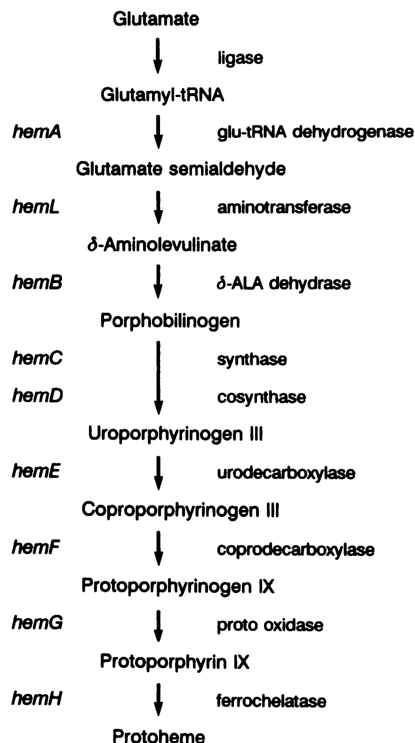

FIG. 1. Pathway of heme biosynthesis in *E. coli*. The gene designation is shown at the left of the arrow, and the corresponding gene product is shown to the right.

to the published restriction pattern of the *hemA* gene region (8, 19, 31). One plasmid, designated pHemA, was stocked and used as a source of DNA for construction of *hemA* gene and operon fusions.

Construction of *hemA-lacZ* operon and protein fusions. A 1.6-kb *Bam*HI-*Bgl*II fragment containing approximately 1.4 kb of DNA upstream of *hemA* plus the 246 bp 5′ of *hemA* was prepared from pHemA and inserted into the *Bam*HI site of the promoterless *lacZ* plasmid pRS415 (28). The *hemA-lacZ* operon fusion plasmid was designated pSD1. Introduction of the 1.6-kb fragment into the gene fusion vector pRS414 yielded pSD2, which contains an in-frame *hemA-lacZ* fusion between codon 82 of *hemA* and codon 9 of *lacZ*. The intended junction of each fusion was confirmed by DNA sequence analysis. Each fusion was then transferred onto λRS45 (28), and the resulting λ phages were used to produce single lysogens of *E. coli* MC4100 and its derivatives (Table 1).

Cell growth. Strains were maintained on Luria broth or solid LB medium (27). Ampicillin was added as needed at 80 mg/liter. Aerobic and anaerobic cell growth was performed as previously described (5). The effect of nitrate on *hemA-lacZ* expression was analyzed after cell growth in 40 mM glucose minimal medium (pH 7.0) supplemented with 40 mM $NaNO_3$ where indicated. To analyze how *hemA-lacZ* gene expression was affected by the availability of alternate carbon compounds, sorbitol, xylose, gluconate, succinate, or Casamino Acids (Difco) were used for cell growth in place of glucose. Each substrate was added at a final concentration of 40 mM except Casamino Acids, which were used at 1% (wt/vol). To limit heme, a *hemA* strain was grown in buffered Luria broth containing 30 mM pyruvate and supplemented with 0.25 mM δ-ALA where indicated. The effect of iron limitation on *hemA-lacZ* expression was examined in cells cultured in 40 mM glucose minimal medium supplemented with 200 μM 2,2′-dipyridyl, 40 μM $FeSO_4$, or both (4).

TABLE 1. Bacterial strains, phages, and plasmids

Strain, plasmid, or phage	Origin	Relevant genotype or phenotype	Source
Strains			
MC4100		F^- *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb-5301 deoC1 ptsF25 rbsR*	27
PC2	MC4100	Δ*fnr*	3
PC40	MC4100	*hemA41B* Kan^r	4
PC35	MC4100	Δ*arcA* Kan^r	6
PC36	PC2	Δ*arcA* Δ*fnr*	6
PC37	PC2	*hemA41B* Δ*fnr*	4
PC38	PC40	Δ*arcA hemA41B* Kan^r	4
SD1	MC4100	*himA*	27
SD6	MC4100	*fis*	27
SD14	MC4100	*recA56 srl*::Tn*10*	This study
SD15	PC2	Δ*fnr recA56 srl*::Tn*10*	This study
SD101	MC4100	*fur*::Tn*5* Kan^r	7
Plasmids			
pRS414		*lacZ* $lacY^+$ $lacA^+$	28
pRS415		$lacZ^+$ $lacY^+$ $lacA^+$	28
pHemA	pBR322	$hemA^+$	This study
pSD1	pRS415	Φ($hemA$-$lacZ^+$) $lacY^+$ $lacA^+$ (operon fusion)	This study
pSD2	pRS414	Φ(*hemA-lacZ*) $lacY^+$ $lacA^+$ (Hyb) (gene fusion)	This study
pfnr3	pACYC184	fnr^+	3
pRPG1	pBR322	$arcA^+$	13
Phages			
λRS45			28
λSD1	pSD1	Φ($hemA$-$lacZ^+$) $lacY^+$ $lacA^+$ (operon fusion)	This study
λSD2	pSD2	Φ(*hemA-lacZ*) $lacY^+$ $lacA^+$ (Hyb) (gene fusion)	This study

TABLE 2. Effect of alternative substrates on *hemA-lacZ* expression[a]

Addition	β-Galactosidase activity (nmol of ONPG hydrolyzed/ min/mg of protein)	
	$+O_2$	$-O_2$
Glucose	120	301
Glucose + nitrate	132	310
Sorbitol	163	565
Xylose	167	372
Gluconate	296	643
Succinate	211	NG[b]
Glycerol	171	NG
Acetate	239	NG
Casamino Acids	245	461
Buffered L broth	142	332
Buffered L broth + glucose	124	250
Buffered L broth + pyruvate	132	241

[a] MC4100(λSD1) was grown in minimal medium or buffered L broth with the indicated carbon additions at a final concentration of 40 mM.
[b] NG, no growth.

β-Galactosidase assay. β-Galactosidase activity was determined as previously described (5). The protein concentration was estimated by assuming that a cell density at an optical density at 600 nm of 1.4 corresponds to 150 μg of protein per ml (5). Values for β-galactosidase activity represent the averages for three or more experiments unless indicated otherwise, and the values varied no more than ±5% from the mean.

Materials. 2,2′-Dipyridyl, pyruvic acid, δ-ALA, ampicillin, and *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Sigma Chemical Co., St. Louis, Mo. Ferrous sulfate was obtained from Mallinckrodt Inc., Paris, Ky. All other reagents used were of reagent grade.

RESULTS

Effect of oxygen and nitrate on *hemA-lacZ* expression. To determine the effect of oxygen on *hemA-lacZ* expression, strains containing the operon (λSD1) or gene (λSD2) fusions were grown in a glucose minimal medium under aerobic and anaerobic conditions, and β-galactosidase activities were determined. Anaerobic cell growth generally resulted in a 2- to 2.5-fold-higher level of *hemA-lacZ* expression than aerobic growth in the λSD1 operon fusion strain (Table 2). Similar findings were observed for expression of the *hemA-lacZ* gene fusion λSD2 except that the level of activity was 10-fold lower under each condition tested (data not shown).

Expression of *hemA-lacZ* was also analyzed under anaerobic nitrate respiration conditions to determine whether nitrate stimulates *hemA* gene expression over that seen during fermentation (Table 2). Anaerobic cell growth with nitrate present results in synthesis of the four-heme-containing nitrate reductase enzyme complex encoded by *narGHJI*. Interestingly, nitrate addition did not affect *hemA-lacZ* expression significantly.

Effect of carbon substrates on *hemA-lacZ* expression. To determine whether the type of carbon compound used for cell growth affects *hemA-lacZ* expression, cells were grown aerobically and anaerobically in a minimal medium containing glucose, sorbitol, xylose, gluconate, succinate, or Casamino Acids (Table 2). Overall, *hemA-lacZ* expression varied over a 2.5-fold range during aerobic growth: β-galactosidase activity was lowest in glucose-grown cells and highest in a gluconate-containing medium. Interestingly, *hemA-lacZ* expression was generally about twofold higher during anaerobic cell growth than during aerobic culture for each medium tested (Table 2). When a rich medium (buffered L broth) was used, *hemA-lacZ* expression was similar to that observed in a minimal glucose-containing medium.

TABLE 3. Effect of *fnr*, *arcA*, *himA*, and *fis* gene products on *hemA-lacZ* expression[a]

Strain (genotype)	β-Galactosidase activity (nmol of ONPG hydrolyzed/min/mg of protein)	
	+ Oxygen	− Oxygen
MC4100 (wild type)	132	241
PC2 (*fnr*)	149	1,290
SD15(pfnr3) (*fnr*$^+$)	124	131
PC35 (*arcA*)	55	280
PC35(pRPG1) (*arcA*$^+$)	803	297
PC36 (*fnr arcA*)	49	141
PC36(pRPG1) (*fnr arcA*$^+$)	735	1,890
SD6 (*fis*)	158	268
SD1 (*himA*)	80	500

[a] The λSD1-containing strains were grown in buffered Luria broth supplemented with 20 mM pyruvate as described in Materials and Methods. Plasmids pfnr3 and pRPG1 carry *fnr*$^+$ and *arcA*$^+$, respectively.

Effect of *fnr* gene product on *hemA-lacZ* gene expression. The *fnr* gene product regulates the transcription of a number of genes involved in anaerobic respiration, including those for fumarate reductase (*frdABCD*), dimethyl sulfoxide reductase (*dmsABC*), and nitrate reductase (*narGHJI*), and aerobic respiration, i.e., the cytochrome *o* oxidase (*cyoABCDE*) and cytochrome *d* oxidase (*cydAB*) enzymes (12). During anaerobic growth, *hemA-lacZ* expression was elevated eight- to ninefold in a Δ*fnr* mutant compared with that in the wild-type strain, whereas expression during aerobic growth was unaffected (Table 3). This indicates that Fnr may function as an anaerobic repressor of *hemA* gene expression. The role of Fnr was confirmed when an *fnr*$^+$ gene on a multicopy plasmid was introduced into strain SD15, a recombination-deficient derivative of PC2(λSD1). Expression of *hemA-lacZ* was superrepressed when cells were grown anaerobically. Interestingly, the eightfold aerobic-anaerobic growth difference in *hemA-lacZ* expression seen in the Δ*fnr* strain indicates that an additional level of anaerobic regulation exists for *hemA* gene expression.

Effect of the *arcA* gene product on *hemA-lacZ* expression. To examine whether the ArcA/ArcB regulon of *E. coli* (14) is also involved in control of *hemA* gene expression, an *arcA* deletion was introduced into the MC4100(λSD1) *hemA-lacZ* lysogen. During aerobic growth, *hemA-lacZ* expression was reduced about 2.5-fold in the *arcA* mutant compared with the wild-type strain (Table 3). ArcA thus appears to function as an aerobic activator of *hemA* expression. In support of this conclusion, *hemA-lacZ* expression was elevated 15-fold when an *arcA*$^+$ plasmid was introduced into the *arcA* mutant. During anaerobic growth, *hemA-lacZ* expression was relatively unchanged in the wild-type strain relative to the *arcA* mutant (Table 3). *hemA-lacZ* expression was still regulated by fivefold in the Δ*arcA* strain in response to oxygen.

To determine how this aerobic-anaerobic control in the *arcA* strain is affected by Fnr, *hemA-lacZ* expression was examined in an *fnr arcA* double mutant (Table 3). The eightfold aerobic-anaerobic effect seen in the *fnr* strain was abolished in the *fnr arcA* double mutant. ArcA appears to function as an anaerobic activator of *hemA* gene expression, while Fnr repression is dependent on ArcA. A similar relationship was recently observed for ArcA and Fnr control of *cydAB* operon expression in *E. coli* (6, 10).

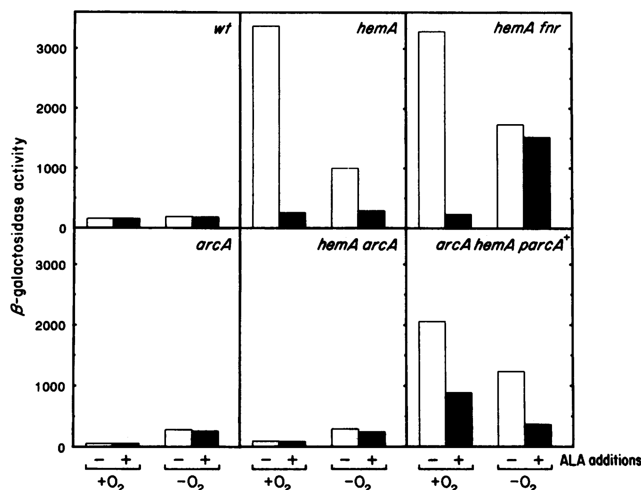

FIG. 2. Effect of a *hemA* mutation and δ-ALA supplementation on *hemA-lacZ* expression. The indicated strains that carried λSD1 were grown in buffered L broth with pyruvate (20 mM), pH 7.2. The medium was supplemented with 0.25 mM δ-ALA where indicated (solid bars). Units of β-galactosidase activity are given in nanomoles of ONPG hydrolyzed per minute per milligram of protein. wt, wild type.

During aerobic cell growth, expression in the *arcA fnr* double mutant was similar to that in the *arcA* strain. In contrast, the *fnr* mutant and the wild-type strain had about threefold-higher levels of *hemA-lacZ* expression. The aerobic regulation of *hemA* expression appears to be independent of Fnr. Overall, *hemA* expression varied by about 25-fold among the various *fnr* and *arcA* strains, which indicates considerable potential for mediating *hemA* transcription in response to oxygen.

Effect of a *hemA* mutation on *hemA-lacZ* expression. We also examined how *hemA-lacZ* expression varied in a *hemA* mutant (Fig. 2) which is defective for the first step in heme biosynthesis (Fig. 1). When the heme precursor δ-ALA was omitted from the growth medium, *hemA-lacZ* expression was dramatically elevated in the *hemA* mutant compared with the wild-type strain (ca. 20-fold aerobically and 5-fold anaerobically; Fig. 2). Addition of δ-ALA to the medium restored *hemA-lacZ* expression nearly to the level observed in the parent strain under either aerobic or anaerobic conditions (i.e., exogenous δ-ALA suppresses the HemA phenotype). These findings suggest that the first committed step of the heme biosynthetic pathway in *E. coli* is transcriptionally controlled directly or indirectly by an intermediate or a product of the pathway.

We also examined whether the effect of δ-ALA limitation on *hemA-lacZ* expression was dependent on either the Fnr or the ArcA regulatory protein (Fig. 2). The 20-fold aerobic elevation in *hemA-lacZ* expression seen in the *hemA* strain was still observed in the *fnr hemA* strain. The addition of δ-ALA to the *fnr hemA* double mutant restored *hemA-lacZ* expression to the level observed in the *fnr* strain during anaerobic growth (data not shown). The *fnr* gene product apparently functions independently of the δ-ALA-dependent control in the *hemA* strain. During aerobic growth, the pattern of *hemA-lacZ* expression in the *fnr hemA* strain was like that in the *hemA* strain, consistent with the notion that Fnr does not function under these conditions.

The ArcA and Fnr aerobic-anaerobic regulators act in quite distinct ways to modulate *hemA* gene expression. The 20-fold δ-ALA effect seen in the *hemA* mutant strain was totally abolished in an *arcA hemA* double mutant, and it was not further affected by δ-ALA addition. The *arcA* phenotype is epistatic to the *hemA* phenotype. Introduction of an *arcA*+-containing plasmid to the *arcA hemA* double mutant restored *hemA-lacZ* expression almost to the level seen in the *hemA* strain. When δ-ALA was also present, the introduction of an *arcA*+ plasmid also stimulated *hemA-lacZ* expression, in support of the proposal that ArcA is an activator of *hemA* gene expression. As the presence of a multicopy *arcA*+ plasmid causes poor cell growth, other cell functions are also apparently being perturbed.

Effect of iron limitation on *hemA-lacZ* expression. The final step in the heme biosynthesis pathway involves the insertion of iron into protoporphyrin IX by the ferrochelatase enzyme (Fig. 1) (24). To test whether iron limitation might also affect heme biosynthesis by altering *hemA-lacZ* expression, the iron chelator 2,2′-dipyridyl was added to the cell culture medium (4). During aerobic growth, *hemA-lacZ* expression was not significantly different in either the presence or absence of the chelator (data not shown). During anaerobic growth, iron limitation resulted in a modest 1.5-fold increase in *hemA-lacZ* expression (data not shown). Identical conditions were previously shown to affect the expression of several respiratory genes (4). Thus, limiting iron availability by this method did not greatly perturb *hemA-lacZ* gene expression, which suggests that the regulation of *hemA* expression does not depend on iron availability.

Effect of *fis* and *himA* mutations on *hemA-lacZ* expression. The *fis* and *himA* genes encode DNA-binding proteins involved in control of a variety of *E. coli* genes (9, 16). Whereas a *fis* mutation had little effect on *hemA-lacZ* expression, a *himA* mutation caused an altered pattern of expression under both aerobic and anaerobic conditions (Table 3). Expression of *hemA-lacZ* was reduced by twofold aerobically and elevated by almost twofold anaerobically compared with that in the wild-type strain, suggesting that integration host factor protein (IHF) is somehow important in controlling the DNA topology of the *hemA* regulatory region, since IHF is known to cause bending of DNA upon binding.

DISCUSSION

In this study, we show that *hemA* gene expression in *E. coli* grown under steady-state conditions varies over a fivefold range, depending on the oxygen level and the type of carbon source used for cell growth (Table 2). However, the overall potential for regulation of this gene is higher (ca. 35-fold), as revealed by *hemA-lacZ* expression in *hemA*, *fnr*, and *arcA* mutants (Table 3). Thus, δ-ALA synthesis, and presumably heme synthesis, can be adjusted over a considerable range.

Effect of oxygen on *hemA* expression. Expression of *hemA* is consistently higher during anaerobic than during aerobic cell culture (ca. 2- to 2.5-fold) regardless of the medium used for cell growth (Table 2). While this elevated expression of *hemA* under anaerobic conditions was unanticipated, the cellular need for heme under these conditions may be partially explained by the abundance of the heme-containing respiratory enzymes cytochrome *d* oxidase and nitrate reductase (11). Their synthesis requires insertion of 3 and 4 heme molecules per enzyme complex, respectively, which may create a higher demand for heme during anaerobic than during aerobic cell growth conditions (12). Rice and Hempfling demonstrated that the level of cytochrome *d* oxidase is more abundant under anaerobic conditions than it is during aerobic growth (23). This control is due in part to transcriptional regulation of the *cydAB* genes, as Cotter and coworkers reported a threefold-higher level of *cydA-lacZ* expression under anaerobic versus aerobic

conditions (3, 6). In contrast, cytochrome *o* oxidase, which contains 2 heme moieties per enzyme complex, is present optimally only under oxygen-rich growth conditions (3, 10, 23). The succinate dehydrogenase complex, which contains a single b-type heme, is also more abundant during aerobic growth (1). Although the absolute amount of heme in the cell is not clearly established for aerobic versus anaerobic cell growth conditions, a significant amount of heme is needed during either mode of growth. Even when the cell resorts primarily to a fermentation mode of metabolism to obtain energy (i.e., growth on glucose and in the absence of respiratory electron acceptors), the cell still continues to synthesize significant amounts of cytochrome *d* oxidase and nitrate reductase (3, 6). A shift from aerobic to anaerobic cell growth results in a 70-fold elevation in *narGHJI* expression (26), while the presence of nitrate can elevate expression a further 20-fold (26). Most interestingly, in spite of the increased synthesis of this anaerobic respiratory enzyme, the addition of nitrate to the culture medium had no significant effect on *hemA-lacZ* expression (Table 2). If heme demand in the cell is greatly increased under these conditions, it is not manifested at the level of *hemA* gene expression.

Effect of carbon source. Expression of the *hemA* gene varied fivefold, depending on the type of carbon substrate used for cell growth (Table 2). Growth on a glucose-containing medium resulted in the lowest level of *hemA-lacZ* expression, while growth on gluconate gave the highest *hemA* gene expression. These results are similar to the findings of Cotter et al. for *cydAB* and *cyoABCDE* expression with different carbon compounds (3). They noted that these respiratory enzymes, as monitored by expression of *cyd-lacZ* and *cyo-lacZ* fusions, are synthesized at higher levels when aerobic respiration is the only means of energy generation (i.e., growth on acetate versus glucose). Culture of cells in a rich medium such as buffered L broth or in a glucose-containing medium resulted in intermediate to low levels of *cyo-lacZ* and *cyd-lacZ* gene expression (3, 4). This regulatory pattern is like that seen for *hemA-lacZ* gene expression in *E. coli*. This is presumably also true for heme production and suggests that these related processes are coordinated within the cell.

Involvement of Fnr and ArcA. The *arcA* and *fnr* gene products are both required to regulate *hemA* gene expression in response to oxygen availability (Table 3). ArcA apparently functions as an activator of *hemA* transcription. When a wild-type *arcA* gene was introduced into the *arcA* deletion strain by using a multicopy plasmid, it resulted in a hyperactivation of *hemA-lacZ* expression aerobically but not anaerobically (Table 3). That ArcA can activate *hemA* gene expression anaerobically is suggested by the eightfold aerobic-anaerobic control seen in the *fnr* deletion strain, compared with the threefold aerobic-anaerobic control in the *fnr arcA* double mutant. These results are consistent with the idea that Fnr represses the ArcA-dependent expression of *hemA*. As indicated by the residual threefold aerobic-anaerobic control of *hemA* expression seen in the *arcA fnr* double mutant, some additional means of aerobic-anaerobic control of *hemA* gene expression appears to exist in *E. coli*. Similar residual control has been observed for the cytochrome *o* (*cyoABCDE*) and *d* (*cydAB*) oxidase operons (6) as well as the fumarate reductase (*frdABCD*) (17) and nitrate reductase (*narGHJI*) (26) operons of *E. coli*.

Fnr is a DNA-binding protein that regulates gene transcription by binding at highly conserved sequences located within the regulatory regions of the genes that it controls (29). Inspection of the *E. coli hemA* DNA sequence does not reveal the presence of an intact Fnr consensus binding sequence (TTGATnnnnATCAA, where n is any nucleotide), although a

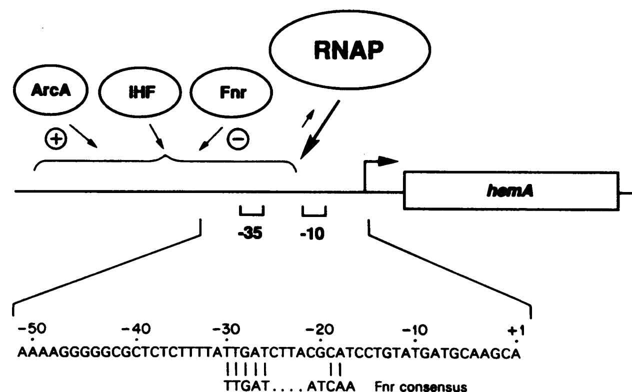

FIG. 3. Proposed regulatory scheme for control of *hemA* gene expression in response to oxygen availability. Positive control (i.e., transcriptional activation of gene expression) is provided by ArcA, while negative control (i.e., transcriptional repression of gene expression) is due to the Fnr protein. The contribution of δ-ALA via a hypothetical gene regulator is not indicated but is suggested from the data shown in Fig. 2. The DNA sequence shown in the lower portion of the figure is numbered relative to the 5′ terminus of the *hemA* mRNA (8). The consensus sequence of the RNA polymerase (RNAP) recognition sites in the −35 and −10 regions is indicated. Vertical dashes above a consensus Fnr-binding sequence (12, 29) indicate sequence similarity to a proposed *hemA* Fnr-binding site.

half-site (i.e., TTGAT that is located between the −35 and −10 regions for RNA polymerase recognition) is seen (Fig. 3). As Fnr has been shown to bind at this location (19a), Fnr may interfere either with the ability of RNA polymerase to bind at the *hemA* promoter or with productive initiation of transcription. It is not yet known what DNA sequences are recognized by ArcA for binding to DNA and where it binds within the *hemA* regulatory region. Given the requirement for a functional ArcA protein in the cell for Fnr to repress *hemA-lacZ* expression (Table 3), it will be of considerable interest to locate the binding site(s) of these proteins. From 5′ mRNA analysis, Verkamp and Chelm (31) proposed the location of a second *hemA* promoter 93 bp upstream of the promoter shown in Fig. 3. However, it is not clear if this upstream promoter contributes significantly to *hemA* expression, as evidenced by the relatively weak intensity of the corresponding S1-protected mRNA bands for the upstream promoter compared with that for the downstream promoter. If this putative second promoter is highly expressed under any conditions, control of *hemA* expression may be considerably more complex than suggested by the model shown in Fig. 3.

The dual pattern of *hemA* gene regulation by the Fnr and ArcA regulatory proteins is similar to that seen for the genes for cytochrome *d* oxidase in *E. coli* (Table 3), where Fnr functions to repress *cydAB* gene expression while ArcA somehow functions to activate their expression (6, 10). The model proposed for ArcA and Fnr control of *cydAB* expression may be proposed for *hemA* gene expression (6, 12). ArcA exists in a partially active DNA-binding form during aerobic cell growth and becomes fully activated by the ArcB protein when the cell perceives the microaerophilic condition. This proposal is consistent with the findings of our *hemA-lacZ* experiments, which indicate that ArcA is functioning to activate *hemA* expression during aerobic growth conditions (Table 3) while the major contribution of ArcA is seen anaerobically (e.g., best visualized in an *fnr* mutant, in which a 12-fold aerobic-anaerobic difference in *hemA* expression is seen). As the cells become fully

anaerobic, Fnr then acts to repress *hemA* gene expression to give the observed 2.5-fold control seen in the wild-type strain.

The dual regulation of *hemA* gene expression by the Fnr and ArcA proteins thus coordinates at least one step in the heme biosynthetic pathway with the synthesis of the various aerobic respiratory enzymes. This may ensure that sufficient heme is provided for its subsequent assembly into the respiratory apoenzyme complexes.

Effect of heme limitation. A mutation in the *hemA* gene resulted in a 20-fold elevation in *hemA-lacZ* expression during aerobic cell growth and a 5-fold elevation during anaerobic growth compared with that in the wild-type strain (Fig. 2). When the block in heme biosynthesis is overcome in the *hemA* mutant by supplementing cells nutritionally with exogenously added δ-ALA, *hemA-lacZ* expression is restored to nearly wild-type levels. It is unclear whether the effect is due to δ-ALA directly or to some subsequent metabolic product of the heme biosynthetic pathway following uptake of δ-ALA into the cell. Unfortunately, *E. coli* cannot take up heme or any of its precursors except δ-ALA from the environment; thus, it is difficult to assess what molecule is limiting. Interestingly, addition of excess δ-ALA to wild-type cells did not significantly affect *hemA-lacZ* expression (Fig. 2). Thus, δ-ALA or its metabolite(s) does not appear to be limiting in the wild-type cell during normal growth conditions. The identification of δ-ALA or some other molecule as a coregulator for control of *hemA* gene expression must await future investigations.

The effect of exogenous δ-ALA supplementation seen in the *hemA* strain is completely abolished in a *hemA arcA* double mutant (Fig. 2). The elevated level of *hemA* expression seen in the absence of added δ-ALA somehow requires the ArcA protein. There are several plausible models that could account for the observed δ-ALA-dependent regulation of *hemA* expression. In one model, a hypothetical regulatory protein is activated by the presence of δ-ALA or a δ-ALA-derived metabolite, and when it is in this activated state, the regulatory protein binds in the *hemA* regulatory region to control its expression. In this model, a functional ArcA protein is required for the hypothetical regulator to act properly. The hypothetical protein could conceivably act either as a repressor or as an activator of *hemA* transcription. Alternatively, ArcA could be a sensor (either directly or indirectly) of the δ-ALA concentration within the cell and be able to activate *hemA* transcription when the δ-ALA concentration drops below an acceptable threshold. By yet another scheme, ArcA could be required for the expression of some regulatory gene that encodes the δ-ALA sensor, which in turn regulates *hemA* expression when the δ-ALA level is low.

Role of iron in *hemA* regulation. Iron limitation had little effect on *hemA* gene expression, as determined by addition of the iron chelator 2,2′-dipyridyl to the cell growth medium (i.e., a less than twofold increase in *hemA-lacZ* expression; data not shown). In contrast, the addition of the metal chelator to anaerobically grown *E. coli* cultures caused a 12- to 14-fold reduction in *frdA-lacZ* (20), *narG-lacZ*, and *dmsA-lacZ* expression (4). The control of the anaerobic respiratory enzymes by iron extends to the synthesis of the respiratory apoenzymes but not to the biosynthesis of heme via synthesis of the glutamyl-tRNA dehydrogenase.

Estimate of glutamyl-tRNA dehydrogenase levels in the cell. Our in vivo studies with the *hemA-lacZ* operon (λSD1) and gene (λSD2) fusions support the notion that the *hemA* gene is expressed at a low level. An estimate of the number of HemA molecules per cell by the method of Grove and Gunsalus (11) gives a number for HemA monomers per cell in the neighborhood of 23 to 60 during aerobic and anaerobic growth, respectively. It is noteworthy that the *hemA-lacZ* operon fusion was expressed at 10-fold-higher levels than the corresponding gene fusion under all conditions examined. These findings suggest that *hemA* translation may be considerably less efficient than *lacZ* translation in *E. coli*.

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