Alternative Pathways for Siroheme Synthesis in *Klebsiella aerogenes*

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Siroheme, the cofactor for sulfite and nitrite reductases, is formed by methylation, oxidation, and iron insertion into the tetrapyrrole uroporphyrinogen III (Uro-III). The CysG protein performs all three steps of siroheme biosynthesis in the enteric bacteria *Escherichia coli* and *Salmonella enterica*. In either taxon, *cysG* mutants cannot reduce sulfite to sulfide and require a source of sulfide or cysteine for growth. In addition, CysG-mediated methylation of Uro-III is required for de novo synthesis of cobalamin (coenzyme B₁₂) in *S. enterica*. We have determined that *cysG* mutants of the related enteric bacterium *Klebsiella aerogenes* have no defect in the reduction of sulfite to sulfide. These data suggest that an alternative enzyme allows for siroheme biosynthesis in CysG-deficient strains of *Klebsiella*. However, *Klebsiella cysG* mutants fail to synthesize coenzyme B₁₂, suggesting that the alternative siroheme biosynthetic pathway proceeds by a different route. Gene *cysF*, encoding an alternative siroheme synthase homologous to CysG, has been identified by genetic analysis and lies within the *cysFDNC* operon; the *cysF* gene is absent from the *E. coli* and *S. enterica* genomes. While CysG is coregulated with the siroheme-dependent nitrite reductase, the *cysF* gene is regulated by sulfur starvation. Models for alternative regulation of the CysF and CysG siroheme synthases in *Klebsiella* and for the loss of the *cysF* gene from the ancestor of *E. coli* and *S. enterica* are presented.

Sulfur is a constituent of numerous critical biomolecules in the cell, including cysteine, methionine, glutathione, thiamine, biotin, lipoic acid, and coenzyme A. In these organic molecules, sulfur is typically found in its fully reduced state of sulfide (S²⁻), while the dominant form of sulfur in the environment is the fully oxidized form, sulfate (SO₄²⁻). Bacterial assimilation of sulfur typically entails the acquisition and reduction of sulfate to sulfide before its incorporation into activated serine to form cysteine (11). Alternatively, fungi and some bacteria assimilate sulfide into activated homoserine to form homocysteine (25). Cysteine then serves, either directly or indirectly, as the source of reduced sulfur in all other molecules in the cell.

A key enzyme in the sulfate reduction process is sulfite reductase, which performs a stepwise, six-electron reduction of sulfite to sulfide (22). Sulfite reductase employs an unusual heme cofactor, siroheme, in the electron transfer pathway from NADPH to the bound sulfur compound. Siroheme is synthesized by methylation, oxidation, and insertion of iron (24, 27, 28) from uroporphyrinogen III (Uro-III), an intermediate in the heme biosynthetic pathway (Fig. 1). In the enteric bacteria *Escherichia coli* and *Salmonella enterica*, this process is catalyzed by the product of the *cysG* gene; mutants lacking this gene fail to reduce sulfite and are cysteine auxotrophs. The methylation functions of CysG are catalyzed by the C-terminal portion of the enzyme; proteins homologous to this domain perform methylation of Uro-III and other tetrapyrroles (20). Metal insertion appears to be catalyzed by the N-terminal portion of CysG (4).

Salmonella also synthesizes cobalamin (coenzyme B₁₂) from

Uro-III (7), a process that requires at least the methylation activity of the CysG enzyme (Fig. 1). Since cobalamin contains a central cobalt atom, it is likely that an iron-free intermediate in siroheme biosynthesis is released from the CysG enzyme, making it available to cobalamin biosynthetic enzymes (encoded by the *cbi* and *cob* genes). While it has been proposed that CysG also performs cobalt insertion (4), this activity has also been ascribed to the CbiK protein, encoded within the *cbi/cob* operon (19). Unlike *Salmonella*, *E. coli* cannot synthesize B₁₂ de novo (14) and uses CysG activity solely for siroheme biosynthesis.

Despite their central role in the synthesis of siroheme and coenzyme B₁₂, the *cysG* genes of *E. coli* and *S. enterica* are not regulated by the need for B₁₂ production or sulfite reduction. Rather, the *cysG* gene is cotranscribed with the *nirBCD* operon, which encodes a siroheme-dependent nitrite reductase (16, 18). The *nirBCDcysG* transcription unit is induced anaerobically in the presence of nitrate or nitrite, allowing for nitrite reduction during anaerobic nitrate respiration (6). Neither the CysB protein (positive activator of the *cys* regulon) nor the PocR protein (positive activator of the *cbi/cob* operon) appears to regulate *cysG* expression. Although induced only anaerobically in the presence of nitrate or nitrite, baseline production of CysG appears to be sufficient to supply siroheme and cobalamin under other growth conditions.

Here we describe siroheme biosynthesis in the related enteric bacterium *Klebsiella aerogenes*. We show that two pathways mediate siroheme biosynthesis in this organism; however, only one pathway allows for B₁₂ biosynthesis. The gene encoding the second siroheme biosynthetic pathway (*cysF*) was likely lost from the ancestor of *E. coli* and *Salmonella* after divergence from the *Klebsiella* lineage. We present models for the role of CysF in *Klebsiella* and for its loss from the ancestor of *E. coli* and *S. enterica*.

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FIG. 1. Structure and assembly of siroheme. Biochemical reactions noted along the center are catalyzed by the CysG protein in enteric bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this study are listed in Table 1. The parental strain, LD561 (*hsdR hutC515*(Con) *dadA lac Δbla-2*), is a derivative of *K. aerogenes* W70 and was kindly provided by R. Bender; this strain had been cured of plasmids bearing antibiotic resistance genes and carries a spontaneous mutation conferring sensitivity to bacteriophage P1 (5). To confer sensitivity to bacteriophage λ, plasmid pTAS1 was introduced in *Klebsiella* strains by electroporation. Plasmid pTAS1 was derived from pTROY11 (3) by deletion of DNA between the *Hind*III and *Bam*HI sites, removing part of the *tet* gene (21). Plasmid pMMK1 was constructed by inserting an *Eco*RI fragment of pNK2881 (10)—encoding the *Tn10* altered-target specificity (ATS) transposase, which mediates transposition with relaxed target site specificity—into the corresponding site of pTAS1.

Media and growth conditions. The rich medium used was Luria-Bertani medium (LB); chloramphenicol was added to 20 μg/ml, tetracycline was added to 10

μg/ml, and ampicillin was added to 200 μg/ml. MacConkey plates were supplemented with 20 μM CoCl₂ and 1% propanediol. The defined medium was E (26); NCE medium comprises carbon-free E medium lacking the citrate chelator. Glucose was added at 0.2%, glycerol was added at 0.4%, cysteine was added at 0.005%, KNO₃ was added at 20 mM, fumarate was added at 20 mM, and cyanocobinamide or cyanocobalamin was added at 100 nM. Solid media contained 1.2% agar. Cultures were incubated at 37°C; anaerobic growth was performed in a Forma Scientific anaerobic chamber at 30°C. A 1/1,000 dilution of liquid LB was added to defined medium to provide micronutrients under anaerobic growth conditions.

Genetic techniques. Insertion mutations were created by delivery of transposition-defective derivatives of *Tn10* via bacteriophage λ vectors (10) into pTAS1- or pMMK1-bearing strains; λNK1323 (delivering *Tn10dCm*), λNK1316 (delivering *Tn10dTc*), and λ1205 (delivering *Tn10LK*) were provided by N. Kleckner. The bacteriophage vectors were amplified in *E. coli* C600 cells. To create inser-

TABLE 1. Bacterial strains

Strain ^a	Relevant genotype	Source or reference
LD561	Wild type	KC2668, R. Bender
LD806	pTAS1 (LamB ⁺)	21
LD807	<i>metE4021::Tn10dKn</i>	21
LD821	pMMK1 (LamB ⁺ <i>Tn10</i> ATS ^b transposase)	This study
LD808	<i>cysG4066::Tn10dCm</i>	This study
LD809	<i>cysG4066::Tn10dCm metE4021::Tn10dKn</i>	This study
LD810	<i>cysG4067::Tn10dTc</i>	This study
LD811	<i>cysG4067::Tn10dTc metE4021::Tn10dKn</i>	This study
LD812	<i>cbiG4035::Tn10dCm</i>	This study
LD813	<i>cbiG4035::Tn10dCm metE4021::Tn10dKn</i>	This study
LD814	<i>cysF4068::Tn10dCm</i>	This study
LD815	<i>cysF4069::Tn10dCm</i>	This study
LD844	<i>cysF4073::Tn10LK</i>	This study
LD816	<i>cysF4068::Tn10dCm cysG4067::Tn10dTc</i>	This study
LD817	<i>cysF4069::Tn10dCm metE4021::Tn10dKn</i>	This study
LD818	<i>cysF4069::Tn10dCm cysG4067::Tn10dTc metE4021::Tn10dKn</i>	This study
JM107	<i>E. coli endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac proAB) F' traD36 proAB lacI^a lacZΔM15</i>	Laboratory collection
LD819	JM107 with pMMK23	This study
LD820	JM107 with pMMK12	This study
LD845	<i>E. coli</i> XL2-Gold-Kn (Stratagene) with pLAK1	This study
LD846	<i>E. coli</i> XL2-Gold-Kn (Stratagene) with pLAK2	This study
LD839	<i>cysD4072::Tn10dCm</i>	21
LD855	<i>E. coli</i> XL2-Gold-Kn (Stratagene) with pTAS6	21

^a All strains are derivatives of *K. aerogenes* W70 unless otherwise noted.

^b ATS, altered-target specificity.

tion mutants, cells were mixed with bacteriophage lysates and incubated for 60 min at 37°C before being plated on selective media. Since λ 1205 does not encode a *Tn10* transposase, pMMK1-bearing cells were used for mutagenesis with *Tn10*LK; after being screened on selective media, candidate mutations were immediately transduced into a transposase-free background.

Transduction employed bacteriophage P1 *vir*. To prepare transducing lysates, cells were diluted 1:100 into LB–5 mM CaCl₂ and grown for 1 h prior to the addition of bacteriophage P1 to a final titer of 10⁷ PFU · ml⁻¹. Lysates that cleared within 6 h were sterilized by chloroform addition. For transduction, recipient cells were grown to mid-log phase, concentrated by centrifugation, and resuspended in 1/4 volume of 5 mM CaCl₂–10 mM MgSO₄. After incubation on ice for 30 min, 100 μ l of cells was added to 100 μ l of bacteriophage lysate and the mixture was incubated at 30°C for 60 min and added to 150 μ l of 1 M sodium citrate. The resulting mixture was plated on the appropriate medium.

Cloning and sequencing. Bacterial chromosomal DNA was partially digested with endonuclease *Sau3AI* and size-fractionated on an agarose gel. Large-molecular-weight fragments were isolated and ligated to pUC18 or pNEB193 DNA cleaved with *Bam*HI and treated with alkaline phosphatase. Ligations were introduced in JM107 cells by electroporation (for *cysG*) or into XL2-Gold-Kn cells (Stratagene) by chemical transformation according to the manufacturer's instructions; transformants were isolated on LB-ampicillin and screened for chloramphenicol-resistant colonies by replica printing to LB-chloramphenicol. For both the *cysG* and *cysF* loci, the nucleotide sequences of both strands of several clones were determined using an ABI 310 sequencer; sequencing reactions were performed according to the manufacturer's instructions.

Data analysis. Sequencher (GeneCodes) was used for sequence assembly. Sequence analysis employed the DNA Master, BLAST (1), and GCG program packages (2).

Enzyme assays. Assays for β -galactosidase activity were performed as described previously (17). Assays for nitrite reductase activity were performed using a modification of the procedure of Snell and Snell (23), whereby residual nitrite, following consumption by cellular nitrite reductase, is assayed colorimetrically. Cells were pregrown anaerobically in E medium-glucose before dilution into E-glucose assay medium; assay medium optionally contained 20 mM KNO₃ and/or 0.005% cysteine. After growth to an optical density at 600 nm (OD₆₀₀) of ~0.5, cells were rinsed in E salts and resuspended in E-glucose–1 mM KNO₂. Periodically, a 100- μ l aliquot was removed and diluted into 4.4 ml of 1% sulfanilamide (in 1 M HCl)–0.5 ml of 0.02% *N*-1-naphthylethylenediamine dihydrochloride. After incubation at room temperature for 20 min, absorption was measured at 540 nm. Nitrite concentrations were estimated by comparison to a standard curve; the rate of nitrite consumption was estimated by linear regression of nitrite concentration versus time. Protein concentration was estimated from the absorbance of the final cell suspension at 550 nm. Nitrite reductase activity was calculated as nanomoles of nitrite consumed per minute per milligram of protein.

Nucleotide sequence accession numbers. DNA sequences determined in this study have been deposited in GenBank under accession no. AF308467 and AF308468.

RESULTS

Isolation of B₁₂-deficient mutants. To isolate *K. aerogenes* insertion mutants, two strategies were adopted. First, λ NK1324 was used to deliver *Tn10dCm* into a wild-type strain bearing plasmid pTAS1 (LD806). A library of ~50,000 independent mutants was collected. To identify mutants defective in the biosynthesis of cobalamin (coenzyme B₁₂), colonies were screened on MacConkey-propanediol agar; mutants incapable of synthesizing coenzyme B₁₂ were unable to degrade propanediol and appeared as white colonies, whereas normal propanediol degradation produces prodigious amounts of propionic acid, turning the colonies red. Mutants defective in cobalamin biosynthesis were corrected by the addition of cobalamin to the MacConkey agar, which allowed for acid production. A total of 52 *Tn10dCm* insertion mutants defective in B₁₂ biosynthesis were isolated. In the second strategy, λ NK1323 was used to deliver *Tn10dTc* into a *metE* mutant bearing plasmid pTAS1; *metE* mutants must use the cobalamin-dependent MetH protein to synthesize methionine. Tet-

racycline-resistant mutants were isolated and screened under anaerobic conditions for methionine auxotrophs that were corrected by the addition of cobalamin to the growth medium. A total of 11 *Tn10dTc* insertion mutants defective in B₁₂ biosynthesis were isolated.

In both screens, mutations were transduced back to the parental background to verify 100% linkage between a single transposon insertion and the mutant phenotype. Mutations were sorted into linkage groups by analysis of frequencies of cotransduction between the chloramphenicol-resistant and tetracycline-resistant insertions. One linkage group comprised two insertions, one *Tn10dCm* (strain LD808) and one *Tn10dTc* (strain LD810), that were 100% linked to each other and unlinked to the remaining mutations. Mutations defining additional linkage groups of B₁₂-biosynthetic genes will be discussed elsewhere (M. M. Kolko, N. M. Scott, and J. G. Lawrence, unpublished data).

Physical characterization of the *cysG* region. A plasmid library was constructed from strain LD808 as described above. Plasmids were introduced into JM107, and transformants were screened for chloramphenicol resistance conferred by a cloned *Tn10dCm* by replica plating. Plasmids were isolated from 24 chloramphenicol-resistant transformants, and the sequences of their inserts adjoining the vector were determined. From these data, two overlapping clones (pMMK12 and pMMK23) were identified and their sequences were determined completely; in total, a 7,343-bp contiguous sequence was assembled. A good fit (GGTTAAGCA) to the canonical *Tn10* 9-bp target duplication (NRYYNRRYN) was observed adjacent to the *Tn10dCm* sequence; excision of these regions yielded a 5,854-kb sequence. The sequence across the *Tn10dCm* insertion site was determined from a PCR-amplified fragment of the corresponding region in wild-type cells. The location of the *Tn10dTc* insertion site in LD810 was determined by PCR and by sequencing the appropriate fragment; *Tn10dTc* is found in the same position as *Tn10dCm* in LD808 (Fig. 2).

The 5,854-bp region contained five open reading frames (Fig. 2). Genes were identified by the homology of their products to *E. coli* proteins as revealed by BLAST; sequences were 75 to 85% identical to *E. coli* genes, and encoded proteins were between 83 and 96% identical to their *E. coli* homologues. A single amino acid insertion at codon 85 of the *Klebsiella* *gdh* gene was the only length polymorphism observed. Only the 3' portion of the *nirB* gene, encoding the C-terminal 715 of 848 amino acids, was cloned.

Three of the genes may form an operon and contribute to a single function, reduction of nitrite to ammonia. The *nirB* and *nirD* genes encode subunits of nitrite reductase, while the product of the *cysG* gene, siroheme synthase, provides the necessary cofactor for the NirBD enzyme. A homologue of the *E. coli* *nirC* gene was not observed; the contribution of the *nirC* gene product to nitrite reduction in *E. coli* is not clear. The two *Tn10* insertions were located in the 5' end of the *Klebsiella* *cysG* gene, encoding siroheme synthase (Fig. 2). Homologues of the *E. coli* *trpS* and *gph* genes are located downstream of the *cysG* gene. Since the *cysG* gene is the last gene in its transcription unit, the phenotype of *cysG* mutants is not likely to be the result of polar effects. A likely terminator (5' CCCCCCGGC CACGGGGGG) is located downstream of the *nirD* gene, and a strong, likely bidirectional terminator (5' AAAAAAGGGC

FIG. 2. (A) The *cysG* regions of *K. aerogenes* and *E. coli*. Open reading frames (ORFs) in *Klebsiella* are named after their *E. coli* homologues; percentages of nucleotide/protein identities to homologous *Klebsiella* ORFs and proteins are noted above each *E. coli* gene. Triangle, common site of Tn10 insertions in strains LD808 and LD810; gray bars, extent of the inserts in plasmids pMMK12 and pMMK23. (B) The *cysFDNC* operon region of *K. aerogenes* and the corresponding region of *E. coli*. Triangles, sites of Tn10 insertions in strains LD839, LD814, and LD844.

GGGATATCATCCCGCCCTTTT [underlined portions are inverted repeats]) is located between the *cysG* and *trpS* genes.

Phenotype of the *cysG* mutants. Both *cysG* insertion mutants were tested for defects in B₁₂ biosynthesis using three independent assays. First, B₁₂-dependent degradation of propanediol was examined on MacConkey indicator medium, which turns red upon the production of propionate from propanediol; the lack of propanediol degradation was corrected by the addition of exogenous B₁₂ (note that, unlike *S. enterica*, *Klebsiella* synthesizes prodigious amount of B₁₂ under aerobic conditions 15). Second, methionine synthesis by MetH was examined in a *metE* mutant background; both mutants were tight methionine auxotrophs under aerobic and anaerobic conditions that were correctable by the addition of B₁₂. Last, it was found that the function of the B₁₂-dependent glycerol dehydratase was also compromised in *cysG* mutants, preventing glycerol fermentation but not glycerol respiration to fumarate (Table 2). In all assays, *cysG* mutants showed defects that were correctable by the addition of exogenous cobinamide or cobalamin (Table 2), suggesting that *cysG* mutants in *Klebsiella*, like those of *Salmonella*, are unable to synthesize coenzyme B₁₂. However, unlike those of *Salmonella*, *Klebsiella cysG* mutants have no defect in cysteine biosynthesis under either aerobic or anaerobic conditions, indicating that an alternative mechanism for siroheme synthesis must be employed.

Isolation of *cysF* mutants. To isolate mutants that failed to synthesize any siroheme, strain LD810 (*cysG*::Tn10dCm) was mutagenized with transposon Tn10dCm and screened for mutants that required cysteine for growth. Among 59 cysteine auxotrophs, 21 mutants were defective in sulfite reduction and were corrected only by the addition of sulfide or cysteine to the medium. Nineteen of the associated mutations were likely to affect the *cysII* genes (the structural genes for siroheme synthase), since they conferred cysteine auxotrophy after transduction into a wild-type background. The other two insertion mutations conferred cysteine auxotrophy only in combination with a *cysG* mutation; in an otherwise wild-type background they resulted in no phenotype (Table 2). P1 transduction showed that the two mutations, termed *cysF*, were linked to the *cysDNC* operon, being 98% linked to the *cysDN* locus and ~75% linked to the *cysII* locus.

Physical characterization of the *cysF* region. A plasmid library was constructed from strain LD814 as described above. Plasmids were introduced into *E. coli* XL2-Gold-Kn (Stratagene), and transformants were screened for chloramphenicol resistance by replica plating. Plasmids were isolated from two chloramphenicol-resistant transformants (LD845 and LD846), and the sequences of their inserts adjoining the vector were determined. A contiguous sequence was constructed by primer walking from these clones. This sequence overlapped the se-

TABLE 2. Phenotypes of *K. aerogenes* *cys* mutants

Strain (genotype)	Aerobic growth on Mac-PD supplemented with ^a :			Anaerobic growth in ^b :								
				NCE-Glu supplemented with:				NCE-Gly supplemented with:				
	0	B ₁₂	0	Met	B ₁₂	Cys	Cys+B ₁₂	0	B ₁₂	Fum	Cys	Cys+B ₁₂
LD561 (wild type)	R	R	+	+	+	+	+	+	+	+	+	+
LD807 (<i>metE</i>)	R	R	+	+	+	+	+	+	+	+	+	+
LD808 (<i>cysG</i>)	W	R	+	+	+	+	+	+	+	+	+	+
LD809 (<i>cysG metE</i>)	W	R	+	+	+	+	+	+	+	+	+	+
LD812 (<i>cbiG</i>)	W	R	+	+	+	+	+	+	+	+	+	+
LD813 (<i>cbiG metE</i>)	W	R	+	+	+	+	+	+	+	+	+	+
LD814 (<i>cysF</i>)	R	R	+	+	+	+	+	+	+	+	+	+
LD817 (<i>cysF metE</i>)	R	R	+	+	+	+	+	+	+	+	+	+
LD816 (<i>cysF cysG</i>)	W	R	+	+	+	+	+	+	+	+	+	+
LD818 (<i>cysF cysG metE</i>)	W	R	+	+	+	+	+	+	+	+	+	+

^a Growth was indicated by color (R, red; W, white) on MacConkey agar with 1% propanediol and 20 μ M CoCl₂ (Mac-PD) supplemented with 100 nM coenzyme B₁₂ (B₁₂) or with no supplement (0).

^b NCE-Glu, NCE minimal medium with 0.2% glucose; NCE-Gly, NCE medium with 0.4% glycerol. Supplements: 0, no supplement; Met, 0.0045% methionine; B₁₂, 100 nM coenzyme B₁₂; Cys, 0.0022% cysteine; Fum, 20 mM Na-fumarate.

quence of pTAS6, which was isolated upon cloning a Tn10dCm in the *cysD* gene (21). The 1,416-bp *cysF* gene was located upstream of the *cysD* gene in the *cysFDNC* operon (Fig. 2B); the Tn10dCm in strain LD814 was located in the downstream portion of the *cysF* gene, after bp 1082. The corresponding *cysDNC* operon in *E. coli* does not contain a *cysF* gene (Fig. 2B). The region between the *cysF* and *cysD* genes, five bases, likely does not contain a promoter. Rather, the two *cysF* insertions we isolated appear to be nonpolar, the downstream *cysDNC* genes being expressed from the promoter driving the Tn10dCm-encoded *cat* gene. Other *cysF* insertions, including the Tn10LK insertion discussed below, are polar on the *cysDNC* genes and show the appropriate phenotypes (21).

The CysF protein is 51% identical (60% similar) to the *Klebsiella* CysG protein over its entire length and has similarity

to siroheme synthases from a variety of taxa; the sequence most similar to CysF (58% identical, 67% similar) is the siroheme synthase from *Neisseria meningitidis* (Fig. 3). Therefore, we predict that it encodes a bona fide siroheme synthase. Similarity to siroheme synthases extended over both domains of CysF, including the methylation domain (residues 217 to 475 in Fig. 3) and the iron chelation domain (residues 1 to 152 in Fig. 3).

The roles of CysG- and CysF-produced siroheme. Unlike *cysG* mutants, *cysF* mutants have no defect in B₁₂ biosynthesis either aerobically or anaerobically (Table 2). Indeed *cysF* mutants have no discernible phenotype in an otherwise wild-type background. Although neither *cysG* nor *cysF* mutants conferred cysteine auxotrophy alone, the double mutant failed to reduce sulfite to sulfide (Table 2). These results suggest that

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FIG. 3. Alignment of siroheme synthases. Periods, residues identical to those of the *E. coli* CysG protein; dashes, deleted residues. EcoCysG, *E. coli* CysG; StyCysG, *S. enterica* serovar Typhimurium CysG; KaeCysG, *K. aerogenes* CysG; KaeCysF, *K. aerogenes* CysF; NmeCysG, *N. meningitidis* siroheme synthase.

TABLE 3. Nitrite reductase activity of *Klebsiella* *cys* mutants

Strain (genotype)	Nitrite reductase activity for cells grown in ^a :			
	NSE-glucose-Met		NSE-glucose-Met-20 mM KNO ₃	
	- Cysteine	+ Cysteine	- Cysteine	+ Cysteine
LD561	7.8 ± 0.7	6.4 ± 0.6	20.8 ± 0.5	42.1 ± 2.8
LD814 (<i>cysF</i>)	8.4 ± 0.5	5.6 ± 0.9	32.0 ± 1.4	55.4 ± 1.6
LD810 (<i>cysG</i>)	1.4 ± 0.9*	-1.5 ± 0.9*	4.1 ± 0.8	-0.1 ± 1.0*
LD816 (<i>cysF cysG</i>)	0.6 ± 0.7*	-0.3 ± 0.9*	1.0 ± 1.9*	0.3 ± 1.4*

^a Nitrite reductase activity is reported as nanomoles of nitrite consumed per minute per milligram of protein. Values are means ± standard deviations. *, not significantly greater than zero. Cells were grown anaerobically in NSE-glucose-0.0045% methionine; cysteine was added to 0.005% (+ cysteine). Cells were pregrown anaerobically in NSE-glucose-methionine.

cysF-encoded functions allow for siroheme biosynthesis via different mechanisms. If so, then NirBD should be able to use CysF-produced siroheme.

To test this hypothesis, we assayed the nitrite reductase activity of *cysF* and *cysG* mutants grown anaerobically with or without additional cysteine (Table 3). As expected, no aerobic nitrite reductase activity was observed (data not shown), and maximal anaerobic activity was observed when the *nirBD* operon was induced with nitrate (Table 3). *cysF* strains showed no consistent defect in anaerobic nitrite reductase activity, indicating that CysF-produced siroheme was not necessary for full NirBD activity. However, *cysG* mutants showed a significant reduction in nitrite reductase activity in media lacking cysteine and no appreciable nitrite reductase activity in the presence of cysteine. Since the addition of cysteine is expected to repress the *cysF* gene and deplete siroheme pools (see below), CysF-produced siroheme must be available to the NirBD nitrite reductase and must serve as an appropriate cofactor. No nitrite reductase activity was seen in *cysF cysG* double mutants, suggesting that the siroheme allowing NirBD activity in *cysG* mutants was provided by CysF.

NirBD binding of CysF-produced siroheme may deplete siroheme pools in *cysG* mutants, thereby impeding the function of the CysIJ sulfite reductase. To examine this possibility, we assayed the growth of *cysF* and *cysG* mutants in minimal media (Fig. 4). Neither *cysG* nor *cysF* mutants had a significant growth defect when using sulfate as a sole sulfur source, demonstrating that an appropriate cofactor for CysIJ could be synthesized by either CysF or CysG. However, *cysG* mutants showed a growth defect when grown anaerobically in the presence of nitrate; this defect is almost entirely corrected by the addition of cysteine (Fig. 4). These results support the hypothesis that the NirBD enzyme will bind CysF-produced siroheme, thereby inducing a modest cysteine auxotrophy. We interpret the remaining growth defect of *cysG* mutants grown in cysteine-nitrate (the presence of nitrate results in a consistent, modestly slower growth rate rather than the reproducibly slightly faster growth rate conferred by nitrate respiration in wild-type and *cysF* strains) as inhibition caused by the accumulation of nitrite.

Regulation of the *cysF* gene. The location of the *cysG* gene downstream of the *nirBD* operon (Fig. 2A) suggests that it may be induced by nitrite under anaerobic growth conditions, as was demonstrated for the *nirBD* genes above; the presence of

cysteine did not affect the levels of nitrite reductase in the cell (Table 3; Fig. 4). On the other hand, the location of the *cysF* gene within the *cysFDNC* operon suggests that it will be regulated by sulfur starvation by the CysB protein, analogous to the regulation of the *cysDNC* operon in *E. coli*. To test this hypothesis, we isolated Tn10LK insertions in *cys* genes; Tn10LK forms translational fusions to the *lacZ* reporter gene at the site of insertion (10). The Tn10LK insertion in strain LD844 creates a fusion after codon 132 of the CysF gene (Fig. 2B). Unlike the Tn10dCm insertions in the *cysF* gene described above, the Tn10LK insertion is polar on *cysDNC* and confers cysteine auxotrophy.

We examined the expression of the *cysF::lacZ* fusion when the cell was grown on cysteine (noninducing conditions) or on sulfite or methionine (inducing conditions) as a sole sulfur source. As expected, the *cysFDNC* operon was induced 8.6-fold when grown on sulfite as a sole sulfur source and 10.6-fold when grown on methionine as a poor sulfur source. These data demonstrate that sulfur starvation controls the expression of the *cysFDNC* genes, likely via a homologue of the CysB protein (8, 9, 21). Therefore, the *cysF* and *cysG* genes are regulated in a different fashion.

DISCUSSION

Alternative pathways for siroheme biosynthesis. The cysteine auxotrophy of *cysF cysG* double mutants and the prototrophy of each single mutant suggest that either protein can synthesize siroheme. The nitrate-induced CysG enzyme likely synthesizes siroheme for use in the NirBD nitrite reductase, and the cysteine-repressed CysF protein synthesizes siroheme for the CysIJ sulfite reductase. Upon induction with nitrate in a *cysG* mutant, the NirBD nitrite reductase depletes the pool of siroheme produced by the CysF protein, thereby inducing cysteine auxotrophy (Fig. 4). Consistent with this result, high-level expression of the CysIJ sulfite reductase in *E. coli* also requires increased siroheme production via a plasmid-borne *cysG* gene (29). In addition, NirBD nitrite reductase activity in a *cysG* mutant is dependent on CysF-produced siroheme (Table 3). These data support the hypothesis that both the CysF and CysG proteins synthesize siroheme and that CysIJ and NirBD both utilize this same cofactor. The sequence of the CysF protein aligns well with those of siroheme synthases, providing no evidence that it performs any additional biochemical function. Yet despite the similarities between CysF and CysG and each's ability to provide siroheme to NirBD or to CysIJ, the proteins appear to function in a different manner. That is, only the CysG protein can provide a methylated intermediate competent to enter the cobalamin biosynthetic pathway.

One explanation for this observation is that the CysF enzyme may bind its intermediates more tightly, reducing the flux of methylated intermediates into the cobalamin biosynthetic pathway. To estimate the amount of B₁₂ synthesized in a *cysG* mutant, we examined the growth rate of a *metE cbiG* mutant when provided with limiting amounts of B₁₂ (Fig. 5). The *metE* mutation makes the cell reliant on the B₁₂-dependent MetH enzyme for methionine synthesis, and the *cbiG* insertion eliminates de novo B₁₂ biosynthetic capability. In minimal medium, the growth rate is correlated to the amount of available B₁₂,

FIG. 4. Growth curves of *cysG* and *cysF* mutants grown anaerobically in minimal media with glucose as a carbon source; sources of sulfur were added as indicated. (A) LD561. (B) LD814 (*cysF*). (C) LD810 (*cysG*). Doubling times were calculated from the slope of a line estimated from the linear range of log-transformed data; at least four data points were used, and the correlation coefficients ranged from 0.989 to 0.999.

and the relationship between extracellular B₁₂ concentration and growth rate is robust (Fig. 5; $r^2 = 0.998$). If we assume that the relationship between extracellular and intracellular B₁₂ concentrations is at best linear, we can use this relationship to predict the maximal amount of B₁₂ produced by a *cysG* mutant (corresponding to 0.011 pM extracellular) relative to that produced by an otherwise wild-type cell (corresponding to 20 pM extracellular). These results suggest that *cysG* mutants could allow for the biosynthesis of 1,000-fold less B₁₂ than a *cysG*⁺ cell. More specifically, the fully induced amount of CysF protein would release 1,000-fold-less methylated intermediate competent for B₁₂ biosynthesis than the uninduced amount of CysG protein. Yet the growth yield of *metE cysG* mutants (maximal OD of 0.250 after 5 days) is comparable to that of a *metE cbiG* mutant and far lower than that of *metE cbiG* mutants (maximal OD of 0.850 after 4 days) that were provided with the amount of B₁₂ (0.01 pM) that supports a similar growth rate. These data imply that very little, if any, B₁₂ is synthesized in *cysG* mutants, and the scant growth we initially observe may be attributed to the depletion of internal methionine stores.

Although the CysF enzyme may merely bind its intermediates more tightly, the very tight growth phenotype of *cysG* mutants belies this scenario. Alternatively, it is possible that the CysF- and CysG-catalyzed reactions use different routes to synthesize siroheme from Uro-III. Siroheme synthesis requires four modifications of Uro-III: two methylations, oxidation, and iron insertion (Fig. 1). The CysG protein performs the two methylation reactions first, making the intermediate tetrapyrrole (dihydrosirohydrochlorin) available as a substrate for B₁₂ biosynthesis (4, 24, 27, 28). We postulate that the CysF protein may first reduce, or insert iron into, Uro-III thereby preventing its flux into the B₁₂ biosynthetic pathway; alternatively, the CysF protein may perform only a single methylation, which may yield a product capable of functioning in NirBD but not competent for B₁₂ biosynthesis. The modular nature of siroheme synthetases allows for either alternative.

Why alternative routes? Methylation of Uro-III serves to divert tetrapyrroles from heme synthesis (hemes *a*, *b*, *c*, *d*, and *o* in enteric bacteria) into pathways for making either siroheme or coenzyme B₁₂ (Fig. 1). Hence, the combined action of the CysF and CysG enzymes regulates the relative pools of three separate cofactors. Yet siroheme is used for two entirely different purposes in the cell, the assimilatory reduction of sulfite and the dissimilatory reduction of nitrite. Therefore, alternative enzymes for siroheme biosynthesis would contribute to more-efficient regulation of tetrapyrrole biosynthesis in *Klebsiella*. In addition, the synthesis of coenzyme B₁₂ represents yet a third destination for methylated Uro-III, whose biosynthesis would decrease potential pools of siroheme. Since the CysF-

FIG. 5. Growth rate of LD813(*metE cbiG*) grown anaerobically in E medium plus glucose and the indicated amounts of cyanocobalamin (B₁₂). The anaerobic growth rates for LD807 (*metE*) and LD811 (*metE cysG*) were 127 and 816 min, respectively, in E medium plus glucose.

catalyzed pathway does not provide large amounts of intermediates competent for B₁₂ biosynthesis, the production of siroheme via the CysF protein would be unaffected by B₁₂ biosynthesis and vice versa. On the other hand, the induction of B₁₂ biosynthesis would affect CysG-catalyzed siroheme pools and vice versa, as CysG-generated intermediates can be shunted into the B₁₂ biosynthetic pathway. The CysF protein may allow for more-consistent production of siroheme for the CysIJ protein.

Why was the *cysF* gene lost from the ancestor of *E. coli* and *Salmonella*? It is likely that the presence of the *cysF* gene in *Klebsiella* and its absence from *E. coli* and *Salmonella* represent a loss from the ancestor of the latter two bacteria rather than a gain into the *Klebsiella* lineage. Neither the character of the *cysF* gene nor its position within the *cysFDNC* operon is consistent with sequence features indicative of recent horizontal genetic transfer (12, 13). If the CysF protein served such an important role in siroheme biosynthesis, why was it lost from the ancestor of *E. coli* and *Salmonella*? Although both *Klebsiella* and *Salmonella* synthesize coenzyme B₁₂, this capability was lost from the ancestor of *E. coli* and *Salmonella* and was recently regained in the *Salmonella* lineage (14, 15). Without B₁₂ biosynthesis, the constitutive expression of the *cysG* gene may have been sufficient to provide siroheme for CysIJ in the ancestor of *E. coli* and *Salmonella*. In this scenario, the *cysF* gene would have provided an insufficiently important function to be maintained and was lost. In this way, the loss of B₁₂ biosynthesis could have resulted in the indirect loss of the *cysF* gene in the ancestor of *E. coli* and *Salmonella*. Testing this model awaits further examination of *cysF* genes in enteric bacteria.

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