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Genetic Structure and Regulation of the *cysG* Gene in *Salmonella typhimurium*

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Siroheme, a cofactor of both sulfite and nitrite reductase in *Salmonella typhimurium*, requires the *cysG* gene for its synthesis. Three steps are required to synthesize siroheme from uroporphyrinogen III, the last common intermediate in the heme and siroheme pathways. All previously characterized *cysG* mutants were shown to be defective for the synthesis of cobalamin (B_{12}), which shares a common precursor with siroheme. Since few *cysG* auxotrophs had been previously analyzed and since there is no evidence of siroheme mutants outside of the *cysG* region, we sought to expand the analysis of the region by isolating more mutations and studying the transcriptional regulation of the *cysG* gene using *lacZ* fusions. We isolated and analyzed 66 *cysG* auxotrophs. All were defective for both siroheme and cobalamin synthesis. Five exceptional mutants were partially defective for the synthesis of both and appear to be leaky. Complementation tests with tandem duplications suggest that the mutations causing the Cys auxotrophy affect only one cistron. The *cysG* gene is transcribed in a clockwise direction; this was demonstrated by a method that permits determining the orientation of two genes of unknown orientation provided their relative map order is known. The *cysG* gene was not part of the cysteine regulon, but had a substantial basal level of expression which was induced fivefold when cells were grown anaerobically on nitrite. Finally, we used Mud-generated duplications to genetically determine the organization of the *cysG* and *nirB* genes.

The biosynthesis of cysteine is central to the assimilation of sulfur in the facultative anaerobic bacteria *Escherichia coli* and *Salmonella typhimurium* (22). Assimilation occurs by the reduction of sulfate (SO_4^{2-}) through sulfite (SO_3^{2-}) to sulfide (S^{2-}) (14, 15, 22); reduced sulfide reacts with *O*-acetyl-serine to form cysteine. All reduced sulfur in the cell is derived from cysteine (22). The aerobic reduction of sulfate to sulfide has been well characterized, and mutants defective in each step of the pathway have been isolated. Sulfate is taken into the cell by the sulfate permease which is encoded by the genes at the *cysA* locus (30). Internal sulfate is reduced to sulfite in three sequential steps and requires the products of the *cysH*, *cysD*, and *cysC* genes, which encode 3′-phosphoadenosine 5′-phosphosulfate reductase, ATP sulfurylase, and 5′-phosphosulfate kinase, respectively (14, 15). Sulfite is reduced in one step to sulfide; this step requires the *cysJ* and *cysI* genes, which encode sulfite reductase (14, 15). The *cysJ* and *cysI* genes, as part of the cysteine regulon, are coordinately regulated in response to cysteine levels (22). In addition to the *cysJ* and *cysI* genes, the *cysG* gene is also required for sulfite reduction; this gene encodes a protein involved in the synthesis of siroheme, an Fe^{2+}-containing cofactor of sulfite reductase which is also found in the NADH-dependent nitrite reductase of *E. coli* (10, 24, 25). Strains with a *cysG* mutation are cysteine auxotrophs and are unable to use nitrite (NO_2^-) as a nitrogen source during anaerobic growth; they are phenotypically Cys^- Nir^- (24). In *S. typhimurium*, a few *cysG* mutants have been tested for the ability to synthesize cobalamin (B_{12}), and all show a Cob^- phenotype (20). This is expected since cobalamin and siroheme are derived from a common precursor, dihydrosirohydrochlorin (DSC) (2). In *E. coli*, the *cysG* gene has been shown to encode an *S*-adenosylmethionine methyltransferase and to be in an operon with another gene(s) necessary for nitrite reductase activity (25, 31, 32, 39, 40).

The proposed biosynthetic pathways of heme, siroheme, and B_{12} in *S. typhimurium* are shown in Fig. 1. The biosynthetic pathway for siroheme branches from that of heme at uroporphyrinogen III (URO III); this heme precursor is converted to DSC. In the formation of DSC, URO III is methylated at two sites (2). In siroheme synthesis, DSC is oxidized to yield sirohydrochlorin (SC). The oxidation step occurs spontaneously in vitro under aerobic conditions, but may require catalysis in vivo (2). In the third and final step, the Fe^{2+} is chelated to form siroheme (28, 29, 35).

This scheme predicts two classes of mutants able to make heme but unable to make siroheme. These mutants should all be detected as cysteine auxotrophs since siroheme is essential for sulfite reduction. The first class would be blocked in the formation of DSC and unable to synthesize either siroheme or B_{12}. The other classes should be blocked after DSC; these should be defective in sulfite and nitrite reduction and retain the ability to synthesize B_{12}. The only heme-positive siroheme-negative mutants isolated so far were detected as cysteine auxotrophs of the first class; their mutations (*cysG*) map at min 72 of the chromosome. Of the few *cysG* mutants previously tested, all were defective for both B_{12} and cysteine synthesis.

Since *cysG* is the only locus for siroheme-defective mutants among the large number of *cys* auxotrophs studied in *S. typhimurium* (14, 15) and since only a few *cysG* mutants have previously been tested for B_{12} synthesis, it seemed possible that the whole pathway of siroheme synthesis might be encoded at the *cysG* locus. To check this possibility and to further investigate regulation of the *cysG* gene(s), we initiated a genetic investigation of this locus, which serves both B_{12} and siroheme synthesis.

We isolated a set of mutations blocked for the synthesis of siroheme (Cys^- Nir^-) and determined their ability to syn-

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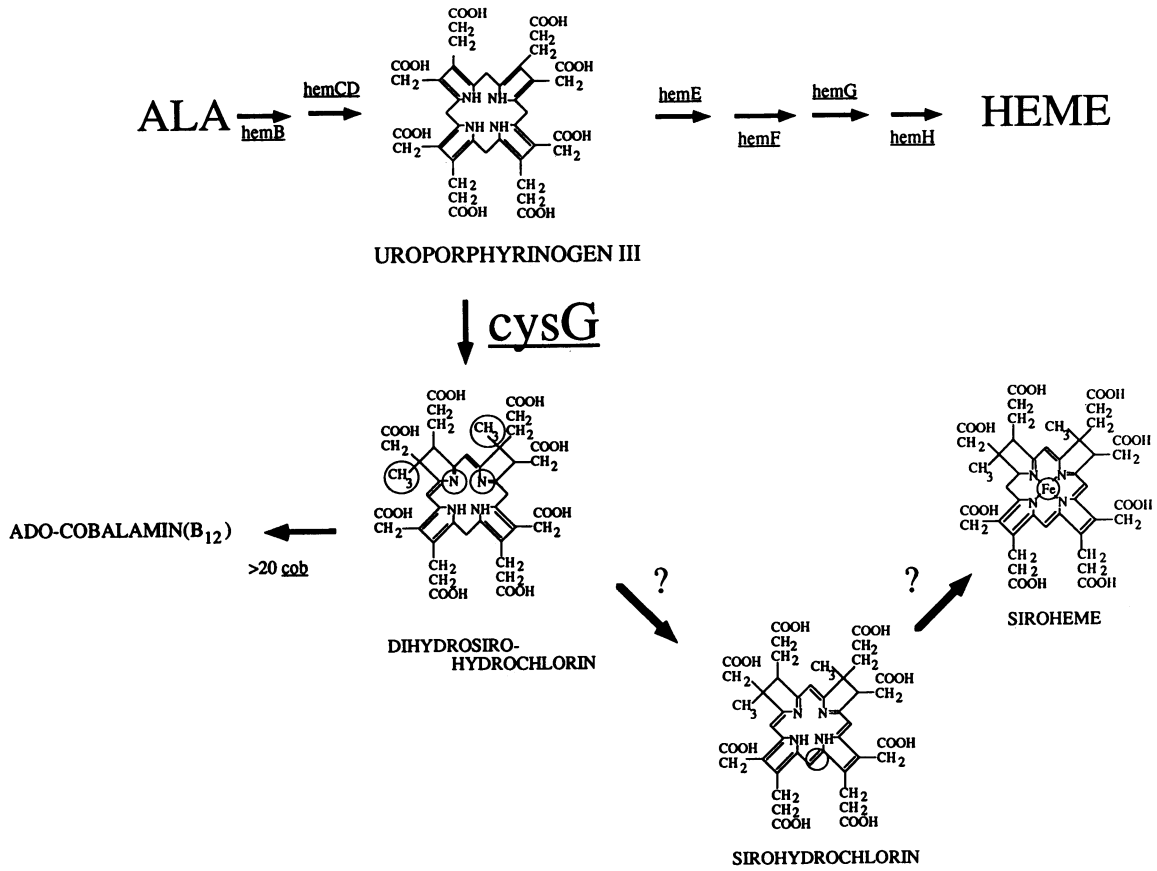


FIG. 1. Synthesis of siroheme from aminolevulinic acid in *E. coli* and *S. typhimurium*. Aminolevulinic acid (ALA) is converted in two steps to URO III, the last intermediate common to both heme and siroheme synthesis. URO III is methylated at the C-2 and C-7 carbons to form DSC. DSC, the last common intermediate in both siroheme and B_{12} synthesis, is oxidized to form SC. An Fe^{2+} is then chelated to SC to form siroheme, the cofactor of both sulfite and nitrite reductase.

thesize B_{12}. A deletion map of these mutations was constructed, and complementation tests were done. We found that all *cysG* mutants were defective for B_{12} synthesis and that only one complementation group necessary for cysteine synthesis exists at the *cysG* locus. We characterized the transcriptional regulation of this gene using Mud::*lac* fusions. We report here that the *cysG* gene was not regulated in response to cysteine levels and that its expression did not require the CysB activator protein. Therefore, the *cysG* gene is not part of the cysteine regulon. Transcription of the *cysG* gene was induced in response to nitrite when cells were grown under anaerobic conditions.

Molecular analysis of the *cysG* gene in *E. coli* suggests that the gene is in an operon with the *nirB* gene, which encodes the structural gene for the NADH-dependent nitrite reductase (25, 31, 32). Sequence analysis of the *cysG* gene suggests that this is also the case in *S. typhimurium* (41). We used Mud-generated duplications to show genetically that the *cysG* and *nirB* genes are in an operon and that the *cysG* gene is at least partially expressed from the *nirB* promoter.

MATERIALS AND METHODS

Bacterial strains and genetic methods. All strains used in this study are derived from *S. typhimurium* LT2 (Table 1). Two derivatives of bacteriophage Mu were used as transposons to form operon fusions; both are derived from the phage MudI (Amp^r *lac* cts) (6). The first derivative, MudI1-8, carries two mutations which make it conditionally defective for transposition (7). In this report, MudI1-8 (Amp^r *lac* cts) will be referred to as MudA. The second derivative, MudI1734, specifies kanamycin resistance (Kan^r) and lacks the Mu A and B genes necessary for transposition (18). In this report, MudI1734 (Kan^r *lac* cts) will be referred to as MudJ. The Tn*10* and Tn*10d*Tet insertion mutations were obtained from a pool of random insert mutants. Deletion mutations in *cysG* were isolated as tetracycline-sensitive derivatives of the Tn*10d*Tet strain TT15028 and of the Tn*10* strains TT1413 and TT1427; these were selected on Bochner-Maloy medium (5, 26). The selection and screening of Tet^s derivatives was done at 42°C. Transductional crosses were performed as described previously (13). Duplications were generated by the method of Hughes and Roth (18).

Media. Difco nutrient broth (NB, 0.8%) containing NaCl at a final concentration of 85 mM was used as the complex medium. The minimal medium was the E medium of Vogel and Bonner supplemented with 11 mM glucose, 22 mM glycerol, or 11 mM lactose (38). The sulfur-free minimal medium was NCE (38) supplemented with glucose or glycerol, 1.0 mM $MgCl_2$, 40 mM citrate (trisodium salt), and the various sulfur sources as indicated. The nitrogen-free medium was NCN (38) supplemented with glucose, $MgCl_2$, citrate, cystine, and either ethanolamine (0.2%; Aldrich) or 3 mM nitrite. Dimethylbenzimidazole (0.3 mM) was added to NCN medium when ethanolamine (EA) was used as the

TABLE 1. Strain list[a]

Strain	Genotype	Reference or source
LT2	Wild type	Laboratory collection
TN2063	*fnr2*::Tn*10 pepT7*::MudA *leuBCD485*	C. Miller (37)
TR6583	*metE205 ara-9*	Laboratory collection
TT1413	*cysG1518*::Tn*10*	Laboratory collection
TT1427	*cysG1526*::Tn*10*	Laboratory collection
TT9507	*pyrE2419*::MudA	Laboratory collection
TT9521	*pyrE2678*::MudA	Laboratory collection
TT9524	*pyrE2681*::MudA	Laboratory collection
TT9641	*cysJ1572*::MudA	Laboratory collection
TT9642	*cysG1573*::MudA	Laboratory collection
TT10607	*fnr2*::Tn*10*	Laboratory collection
TT10786	*aroE568*::MudA	Laboratory collection
TT13506	*cysG3170*::MudA	Laboratory collection
TT13507	*cysG3168*::MudA	Laboratory collection
TT14746	*cysG3169*::MudA	Laboratory collection
TT14747	*cysG3174*::MudA	Laboratory collection
TT15028	*zhc-3665*::Tn*10d*Tet	This study
TT15047	Δ*cysG3144*/DUP861(*aroE568*)*MudA*(*pyrE2681*)	This study
TT15048	DUP862(*aroE568*)*MudA*(*pyrE2681*)	This study
TT15049	Δ*cysG3172 lacZ*	This study
TT15052	*cysG3168*::MudA *cysB3305*::Tn*10d*Tet	This study
TT15053	*cysG3168*::MudA *metE205 ara-9*	This study
TT15126	*cysJ1572*::MudA *cysB3305*::Tn*10d*Tet	Laboratory collection
TT15815	*nirP1*::Tn*10 cysG*::MudA	This study
TT15816	*nirB2*::Tn*10 cysG*::MudA	This study
TT16899	DUP(*aroE-cysG*)*MudA*(*aroE-cysG*)	This study
TT16900	*fnr*::Tn*10 cysG*::MudA	This study

[a] All strains are derivatives of *S. typhimurium* LT2.

nitrogen source. Sulfur sources were added to the medium in the following concentrations: sulfate (1.0 mM), sulfite (0.3 mM), djenkolic acid (0.5 mM), and cystine (0.3 mM). Cyanocobalamin (Sigma Chemical Co.) was used as the exogenous B_{12} source (100 μg/liter). Auxotrophic supplements were added at final concentrations as described previously (13). Ampicillin was added to a final concentration of 30 μg/ml in NB and 15 μg/ml in NCE media. Tetracycline was added to a final concentration of 20 μg/ml in NB and 10 μg/ml in NCE media. Indicator plates containing X-Gal were made by adding 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside to a final concentration of 25 μg/ml. Solid rich medium contained agar (1.5%; Difco) or, when minimal medium was used, Noble agar (1.7%; Difco). Cells were grown at 37°C.

Scoring ability to reduce sulfite. The ability to reduce sulfite was scored as the ability to grow on minimal medium containing sulfite as the sole sulfur source. Alternatively, production of sulfide was scored directly by using bismuth sulfite plates (Difco). A black precipitate (Bi_2S_3) forms when bismuth interacts with the reduced sulfide. The latter is the more sensitive method for the detection of the ability to reduce sulfite.

Chemical mutagenesis. Phage P22 lysates for localized mutagenesis were concentrated and mutagenized with hydroxylamine by the method of Hong and Ames (17). The survival rate of the phage particles from this mutagenesis was 0.1%. This mutagenized lysate, grown on strain TT15028, was used to transduce LT2; among the Tet^r transductants, *cysG* mutants were identified.

Mapping of Tn*10* and Tn*10d*Tet mutations. The Tn*10* mutations were mapped by the method of Benson and Goldman (3). Briefly, Tet^r cells were streaked onto NB plates containing tetracycline. Single-colony isolates were inoculated into liquid NB medium containing tetracycline and grown to the mid-exponential phase. Cells were diluted 1/10, and 0.1 ml was plated onto Bochner-Maloy medium (5, 26). Portions (5 μl) of MudP22-transducing lysates were spotted onto the plates which were then incubated overnight at 42°C. The Tn*10* mutations were located by determining which lysate produced the greatest growth on Bochner-Maloy medium after 1 to 2 days of incubation. Once the general region of the chromosome was determined, transductional linkage to known markers was tested.

Deletion mapping. The endpoints of deletions were determined by selecting for recombinants in crosses between two *cysG* auxotrophs. Fifteen microliters of concentrated lysate was mixed with 150 μl of cells. This mixture was allowed to incubate for 30 min at room temperature and was then plated onto minimal plates containing low amounts of cystine (6 μM). The Cys$^+$ recombinants were scored after incubation for 48 h at 37°C.

β-Galactosidase assay. β-Galactosidase activity was assayed as described by Miller (27), using $CHCl_3$-sodium dodecyl sulfate to permeabilize whole cells. Enzyme activity is expressed as nanomoles of *o*-nitrophenyl-β-D-galactoside per minute per unit of optical density at 650 nm (1 unit = 1 ml of cell culture with an optical density at 650 nm of 1). All assays were performed in duplicate with early to mid-exponential-phase cultures.

Growth under anaerobic conditions. Cells were grown anaerobically on solid medium in an anaerobic chamber (model 1024; Forma Scientific) whose atmosphere contained nitrogen-hydrogen-carbon dioxide in a percentage of 90-5-5. Oxygen levels were determined with an oxygen detector (model 10 gas analyzer; Coy Laboratory Products). For anaerobic growth in liquid medium, oxygen was removed by the method of Balch and Wolfe (1). Carbon, nitrogen, and

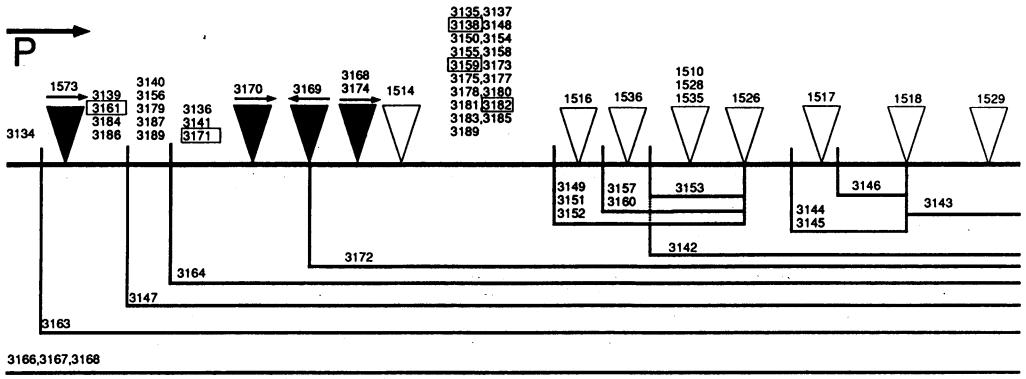


FIG. 2. Deletion map of the *cysG* gene. A fine-structure deletion map of the *cysG* gene was generated to assist in the analysis of the locus, as described in Materials and Methods. All numbers listed on the map are cysteine auxotrophic alleles. Open triangles represent Tn*10* insertion mutations, and closed triangles represent MudA insertion mutations. Arrows represent the orientation of the MudA insertion mutations. Open squares represent leaky alleles. The origin of replication is to the right on this map, and the gene is transcribed from left to right.

sulfur sources were added to sterile medium with a syringe. Such tubes were inoculated by injecting 0.1 ml of an overnight culture grown aerobically to saturation in NCE medium containing glucose, citrate, $MgCl_2$, and cystine.

RESULTS

Isolation and deletion mapping of *cysG* deletion and point mutations. The proposed biosynthetic pathway for cobalamin (B_{12}) and siroheme suggests that there should be two classes of siroheme auxotrophs (Fig. 1). The first class would be blocked in the formation of DSC, a precursor of B_{12}, and be defective for cysteine and cobalamin biosynthesis and nitrite reduction (Cys^- Nir^- Cob^-). The second class would be blocked after synthesis of DSC; they should be Cys^- Nir^- but retain the ability to synthesize B_{12}. Since all previously isolated Cys^- Cob^- mutants were detected as cysteine auxotrophs and map at min 72, it seemed possible that the whole pathway of siroheme synthesis might be encoded by the *cysG* locus. Only a few *cysG* mutants had previously been isolated and even fewer tested for B_{12} synthesis. We isolated a set of *cysG* point and deletion mutants as described in Materials and Methods and characterized their phenotypes with respect to their ability to make B_{12} (Cob) and use nitrite (Nir). A deletion map of the *cysG* locus was generated, as described in Materials and Methods, and the phenotype conferred by each deletion and point mutation was correlated with its map position (Fig. 2). We also analyzed 14 insertion mutations with respect to phenotype and map position.

Phenotypes of *cysG* mutants. The ability of *cysG* mutants to reduce sulfur was tested as described in Materials and Methods. Of the 66 *cysG* mutants tested, none of the 34 deletion and insertion mutants were able to reduce sulfite. Five point mutants were unable to grow on sulfite as a sole sulfur source but were positive in the bismuth assay for sulfite reduction. They appear to be only partially defective for sulfite reduction. The ability of *cysG* mutants to reduce nitrite was scored as the ability of cells to grow anaerobically on solid medium containing nitrite as the sole nitrogen source as described in Materials and Methods. Of the 66 *cysG* mutants tested, only those five mutants with a partial defect in sulfite reduction were able to use nitrite as a nitrogen source. The rest were unable to use nitrite but grew well when ammonia was added to the medium.

To determine the ability of *cysG* mutants to synthesize B_{12}, we introduced a *metE* mutation into each *cysG* auxotroph. The *metE* gene encodes homocysteine methyltransferase, the last enzyme in the methionine biosynthetic pathway (23). The auxotrophy of *metE* mutants is corrected in the presence of cyanocobalamin, because cobalamin permits the activity of an alternative B_{12}-dependent transferase, encoded by the *metH* gene (12, 23). Under anaerobic conditions, Cob^+ *Salmonella* strains produce B_{12}, activate the *metH* gene, and synthesize methionine despite the *metE* mutation (16, 20). The ability of *cysG metE* strains to synthesize B_{12} was scored as their ability to grow without methionine under anaerobic conditions.

The *metE* mutant grew anaerobically without methionine, while the *cysG metE* double mutants could not grow anaerobically unless the medium was supplemented with B_{12}. This showed that the *cysG* mutant was blocked for B_{12} synthesis. All *cysG* deletion and insertion mutants and most point mutants proved to be unable to synthesize B_{12}. The same five mutants (described above) that showed a leaky Cys^- phenotype appeared to synthesize B_{12} by virtue of residual CysG activity because they grew anaerobically without methionine.

A second, more stringent, test for the ability to make B_{12} was anaerobic growth on ethanolamine (EA), which can be used anaerobically as a nitrogen source if B_{12} is present to permit activity of EA ammonia lyase. All 66 *cysG* mutants were tested for the ability to use EA as a nitrogen source anaerobically. Most *cysG* mutants did not grow unless provided with B_{12}; only the five leaky *cys* auxotrophs described above were able to use EA as a nitrogen source. We did not recover *cysG* mutants defective in conversion of DSC to siroheme (Cys^- Nir^- Cob^+).

It seemed possible that the *cysG* region might include a set of genes involved in the last steps of siroheme synthesis, if such genes were located promoter-proximal to the methylase gene. If this were the case, it might be possible to isolate revertants that remove the polar block, generating strains with a Cob^+ $siroheme^-$ phenotype by relieving polarity. Several attempts to isolate such mutants were unsuccessful.

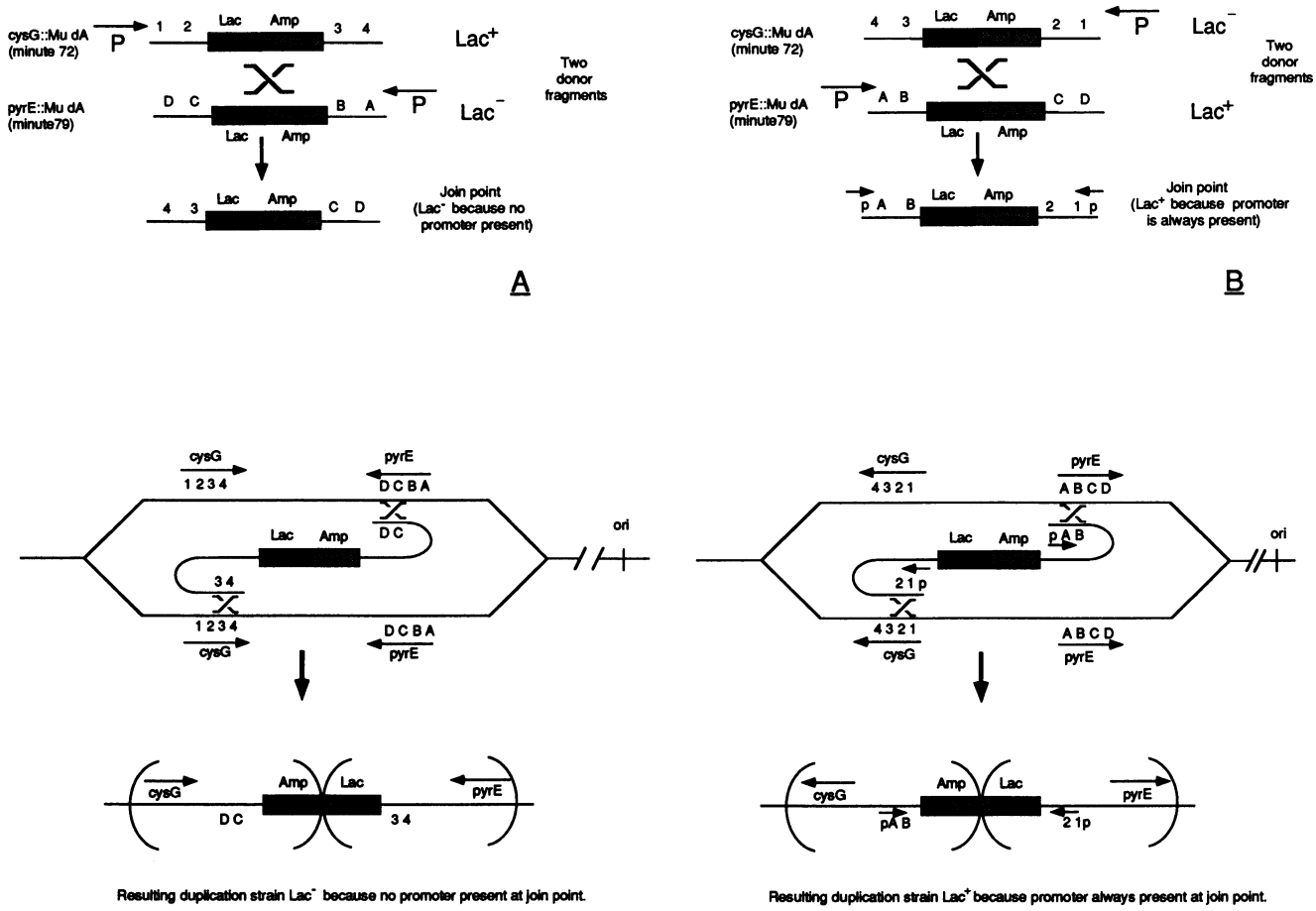
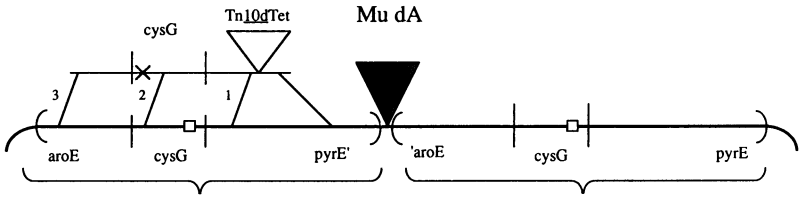


FIG. 3. Transcriptional orientation of the *cysG* and *pyrE* genes. The direction of transcription of both the *cysG* and *pyrE* genes is determined by using the mixed lysate assay to generate duplications with strain LT2 as the recipient. The Lac phenotype of the resulting duplication-containing recombinant is then scored. (A) The chromosome if the *cysG* and *pyrE* genes are transcribed convergently; (B) the chromosome if the genes are transcribed divergently. Arrows represent the proposed orientation of transcription. The numbers and letters by the genes are arbitrary designations to clarify gene orientation. The top half of the figure shows how the transducing fragment is made, and the bottom half of the figure shows how the transducing fragment recombines with the recipient chromosome at a replication fork. The duplications strains were all Lac$^-$, and therefore the genes are transcribed convergently.

Orientation of *cysG* gene transcription. The orientation of the *cysG* gene was determined by the method of Hughes and Roth (18), by generating duplications using mixed lysates of strains carrying MudA insertions, as described in Materials and Methods. Mud-generated duplications form by recombination between insertions in the same orientation.

We found that duplications formed when a lysate made on Lac$^+$ *cysG*::MudA strain TT13507 was mixed with a lysate made on the Lac$^-$ *pyrE*::MudA strain TT9507 and used to transduce LT2 to Amp^r. Duplications were also generated when Lac$^-$ *cysG*::MudA strain TT14746 and Lac$^+$ *pyrE*::MudA strain TT9521 or TT9524 were used as donors in the same cross. Mud-generated duplications never occurred when Lac$^+$ *cysG*::MudA strain TT13507 and Lac$^+$ *pyrE*::MudA strain TT9521 or TT9524 were used as donors or when Lac$^-$ *cysG*::MudA strain TT14746 and Lac$^-$ *pyrE*::MudA strain TT9507 were used as donors in this cross. Five *cysG*::MudA (four Lac$^+$ and one Lac$^-$) fusions and three *pyrE*::MudA (one Lac$^+$ and two Lac$^-$) fusions were tested in this manner. All four of the Lac$^+$ *cysG*::MudA fusions were oriented in the same direction; all are in opposite orientation to the Lac$^-$ *cysG*::MudA fusion (data not shown). The *cysG* and *pyrE* genes are thus oriented in opposite directions.

This test by itself gives no information whether that direction is convergent or divergent relative to the chromosome. Although the orientation of the *pyrE* gene is unknown, the direction of transcription of these genes relative to each other, and thus their orientation in the chromosome, can be determined from the Lac phenotype of the Mud::*lac* element at the join point of the duplications generated above. This determination depends on knowing the map positions of the two loci; the *cysG* gene maps at 72 min and the *pyrE* gene maps at 79 min (34). If the genes are transcribed toward each other, as diagrammed in Fig. 3A, the duplication strains will always be Lac$^-$ and have little or no β-galactosidase activity because no promoter is present at either side of the join point to express the *lac* genes. If the genes are transcribed away from each other (Fig. 3B), the final duplication strain will be Lac$^+$ because a promoter faces both ends of the Mud::*lac* element at the join point. Note in Fig. 3 that, although the fusions have been drawn in one direction, the orientation of the particular *lacZ* gene fusions used to generate the duplication is irrelevant to the Lac phenotype of the duplication



Recipient duplication recombinant
Aro+, Cys-, Pyr+, Amp^r.

FIG. 4. Generation of heteroallelic strain for complementation analysis. A strain containing a small deletion at the promoter-distal end of the *cysG* gene is used as a recipient to generate a strain containing a duplication of the *cysG* gene. The resulting strain (TT15047) is used as a recipient in a cross with a strain containing a Tn*10d*Tet insertion 90% linked to a *cysG* point mutation as described in Materials and Methods. Heteroallelic recombinants are scored for the ability to use sulfite as the sole sulfur source.

strain. In our experiments, the duplication strains formed were phenotypically Lac$^-$ and expressed less than 2 U of β-galactosidase activity regardless of which donors were used (data not shown). The genes were thus transcribed convergently. That is, *cysG* transcription is clockwise and *pyrE* transcription is counterclockwise.

In previous studies, the direction of transcription of a gene or operon using mixed lysates has been determined by comparing the orientation of an unknown gene with that of a known gene (18). Analyzing the lactose phenotype of the duplication strain allows one to determine the direction of transcription of two unknown genes if the relative map positions of the two genes are known.

Orienting the *cysG* genetic map vis-à-vis the direction of transcription. The direction of transcription relative to the *cysG* genetic map was determined in two ways. In the first, three *cysG*::Tn*10* *cysG*::MudA double mutants were constructed to test whether these Tn*10* insertions were polar on *lacZ* expression from the *cysG*::MudA insertion. Mutants containing the *cysG3170*::MudA (TT13506) allele alone and all the *cysG*::Tn*10* *cysG*::MudA double mutants were phenotypically Lac$^+$, suggesting that the Mud elements are upstream of the Tn*10* insertions. Furthermore, the β-galactosidase activity of the double mutants was the same as that of the MudA insertion alone (data not shown). Apparently all the Tn*10* insertions tested are promoter distal from the MudA insertion. This suggests that the gene is transcribed as the map is drawn in Fig. 2.

In the second test, strain TT14746, a Lac$^-$ *cysG*::MudA insertion, was used to select for Lac$^+$ revertants. It was presumed that a Lac$^-$ insertion was oriented such that transcription from the *cysG* promoter would cross the *lac* operon in the wrong orientation. Some Lac$^+$ revertants could arise by means of deletions, which fuse a foreign promoter to the *lacZ* gene. Lac$^+$ revertants of TT14746 were found at a frequency of $1/10^8$. Eleven independent Lac$^+$ revertants were isolated in this manner. All were tested, by recombinational analysis, for loss of *cysG* material. Two of the Lac$^+$ revertants carried deletions of all the material to the right of the *cysG*::MudA insertion. One of these is listed in the deletion map as *cysG3172*. None of the Lac$^+$ revertants carried detectable deletions to the left of the *cysG*::MudA insertion, as determined by recombinational analysis. Thus, the direction in which the deletions were generated and the lack of polarity of Tn*10* insertions on the *cysG*::MudA insertion strongly suggest that the gene is transcribed as depicted in Fig. 2.

Complementation tests of *cysG* mutants. The above results suggest that all *cysG* mutants are blocked for the synthesis of DSC; none appeared to have a defect limited to either of the last two steps of siroheme synthesis. It could be argued that siroheme genes are all located at the *cysG* locus but are essential. We believe this is unlikely since deletions that remove the entire region could be generated. It was also possible that the *cysG* locus encodes all siroheme genes but that all mutations in the later steps also lack the first activity. Alternatively, siroheme might be an essential cofactor required for later steps in the synthesis of cobalamin. To explore the possibility of multiple genes at the *cysG* locus, we did complementation tests.

Complementation tests were done by using a tandem duplication of the chromosomal segment between the *pyrE* and *aroE* loci by the method of Hughes and Roth (18). Heteroallelic *cysG* mutants were generated by introducing the *pyrE-aroE* duplication into deletion mutant *cysG3144*. The *cysG* deletion affects the promoter-distal end of the genetic map of the *cysG* locus (Fig. 2) and should therefore not show a polar effect on the rest of the *cysG* region. The duplication formed carries the *cysG* deletion in both copies of the duplicated region.

The diploid Cys$^-$ Amp^r strain was transduced to Tet^r with phage grown on strains carrying a Tn*10d*Tet insertion (*zhc-3665*) 90% linked to the *cysG* gene and containing one of the point mutations listed on the *cysG* map (Fig. 2). Figure 4 shows the three possible outcomes of the cross. Class one transductants inherited only the Tn*10* element without the *cysG* point mutation and remained Cys$^-$. Class two transductants have a wild-type *cysG* gene. The third class inherited the donor point mutation in place of one copy of the deletion and became heterozygous for the *cysG* locus; 90% of the Tet^r transductants were of the third class and became heterozygous for the *cysG* mutations. Of 27 tight point mutations tested, none showed complementation with the small *cysG* deletion used in these tests. We conclude that the mutations causing *cys* auxotrophy affect only one gene.

The *cysG* gene is not in the cysteine regulon. Since siroheme is required for cysteine synthesis, one might expect that the *cysG* gene would be coregulated with the cysteine biosynthetic genes. The *cysJ* and *cysI* genes, which encode sulfite reductase, are part of the cysteine regulon. Expression of these genes requires a positive regulatory protein, encoded by the *cysB* gene, which is activated by sulfur limitation (21, 22). Growth of a cysteine auxotroph on djenkolic acid, a poor source of reduced sulfur, causes derepression of the genes of the cysteine regulon (22).

A Φ(*cysG-lacZ*) fusion strain (TT13506) was used to study the transcriptional regulation of the *cysG* gene (Table 2). Expression of the *cysG* gene was not derepressed when a

TABLE 2. β-Galactosidase levels of Φ(*cysG-lacZ*) and Φ(*cysJ-lacZ*) fusions under indicated growth conditions[a]

Fusion	Genotype	β-Galactosidase level[b]			
		Aerobic		Anaerobic	
		Djenkolic acid	Cystine	Djenkolic acid	Cystine
Φ(*cysG-lacZ*)	*cysB*$^+$	80	100	60	120
	cysB::Tn*10d*Tet	40	90	60	100
Φ(*cysJ-lacZ*)	*cysB*$^+$	1,360	5	1,860	15
	cysB::Tn*10d*Tet	<1	<1	<1	<1

[a] Cells were grown in NCE medium with either cystine or djenkolic acid as the sole sulfur source.
[b] Specific activity is measured in Miller units.

cysG mutant was grown on djenkolic acid and it was not repressed by growth on cystine. Under our conditions, growth on djenkolic acid seemed to cause a slight decrease in the expression of the *cysG* gene compared with that seen during growth on cystine. This difference may be due to differences in growth rate. The Φ(*cysJ-lacZ*) fusion (TT9641), on the other hand, was derepressed over 100-fold during growth on djenkolic acid. This regulation was independent of oxygen levels.

We tested the role of the CysB protein on *cysG* expression by transducing a *cysB*::Tn*10d*Tet insertion into a strain containing a Φ(*cysG-lacZ*) fusion. The *cysB*::Tn*10d*Tet insertion had little effect on the expression of a *cysG*::*lac* operon fusion when cells were grown in djenkolic acid and no difference when cells were grown on cystine (Table 2). In comparison, the *cysJ* gene required the *cysB* protein for its expression. Since the *cysG* gene was not regulated in response to cystine levels and was not affected by a *cysB* mutation, we conclude that the *cysG* gene is not part of the cysteine regulon.

***cysG* gene expression is induced by nitrite.** Since siroheme is also required for the reduction of nitrite to ammonia, we reasoned that nitrite might stimulate transcription of the *cysG* gene. Under anaerobic conditions, the addition of NO_2^- increased the transcription rate of four different Φ(*cysG-lacZ*) operon fusions (Table 3). This increase in activity was not seen when cells were grown anaerobically without nitrite or when cells were grown under aerobic conditions in the presence of nitrite. The induction by NO_2^- was not affected by the presence of ammonia. All four Φ(*cysG-lacZ*) operon fusions were tested and exhibited this pattern of expression.

Isolation of mutations which reduce *cysG* gene expression. The *cysG* gene of *S. typhimurium* was expressed constitutively under most conditions but was induced fivefold during anaerobic growth in the presence of NO_2^- (see above). To determine the mechanism(s) of this induction, we isolated mutations which reduced *cysG* gene expression. On aerobic MacConkey lactose plates without NO_2^-, cells containing a Φ(*cysG-lacZ*) transcriptional fusion form white colonies. When NO_2^- is added to this medium, colonies form which have a red center (fish-eyed phenotype), indicating that NO_2^- induces *cysG* gene expression in anaerobic cells within the colony. To isolate mutations which were no longer induced by nitrite, we subjected a Φ(*cysG-lacZ*)-containing strain (TT13506) to Tn*10* mutagenesis. Tetracycline-resistant (Tet^r) transductants were screened on MacConkey lactose nitrite plates to look for colonies which had lost the fish-eye phenotype (i.e., formed white colonies). From 20,000 tetracycline-resistant colonies, six mutants were identified. For all six mutations, the white colony phenotype was 100% linked to the Tet^r phenotype. The six Tet^r strains were compared with the parent for β-galactosidase production by the Φ(*cysG-lacZ*) fusion. All six double mutants expressed about 100 U of activity when grown anaerobically on nitrite, compared with 500 U of activity for the Φ(*cysG-lacZ*) fusion alone.

TABLE 3. Nitrite induction of Φ(*cysG-lacZ*) operon fusions[a]

Strain	β-Galactosidase sp act[b]			
	Aerobic		Anaerobic	
	$-NO_2^-$	$+NO_2^-$	$-NO_2^-$	$+NO_2^-$
TT9642	50	40	70	240
TT13506	100	100	140	480
TT13507	90	90	120	450
TT14747	110	100	130	500

[a] Cells were grown aerobically or anaerobically in E medium with glucose.
[b] Specific activity is measured in Miller units.

TABLE 4. Expression of Φ(*cysG-lacZ*) fusion strain in various backgrounds[a]

Genotype	β-Galactosidase sp act[b]			
	Aerobic		Anaerobic	
	$-NO_2^-$	$+NO_2^-$	$-NO_2^-$	$+NO_2^-$
Wild type	100	95	150	500
fnr::Tn*10 cysG*::*lacZ*	110	100	100	100
nirB::Tn*10 cysG*::*lacZ*	100	100	90	105
nirP::Tn*10 cysG*::*lacZ*	100	100	100	95
DUP *nirB*$^+$ (*cysG*::*lacZ*)	90	100	130	460
DUP *nirB*::Tn*10 cis cysG*::*lacZ*	90	110	145	90
DUP *nirB*::Tn*10 trans cysG*::*lacZ*	105	100	90	450

[a] Cells were grown aerobically or anaerobically in E medium with or without nitrite.
[b] Specific activity is measured in Miller units. DUP refers to strains containing tandem duplications of the *nirB-cysG* region.

Mapping the Tn*10* insertions. The Tn*10* mutations which affect the activity of the Φ(*cysG-lacZ*) fusion were mapped by the method of Benson and Goldman (3) and fell into three classes. The first class (*nirP*) mapped between min 7 and min 12, the second class (*fnr*) mapped between min 29 and min 33, and the third class (*nirB*) mapped between min 71 and min 72 of the *Salmonella* linkage map (34).

The *nirP* mutations were 50% linked, by P22-mediated transduction, to the *proAB* operon, but were unlinked to the *proC* gene. The *fnr* mutations were not found to be cotransduced to any known markers. The third class of mutations was 80% linked to the *cysG* gene and 50% linked to the *crp* gene. Of the six mutations isolated, two were of the first class, three were of the second class, and one was of the third class. The effect of the mutations on *cysG* expression is shown in Table 4.

Phenotypes of the Tn*10* mutants. The *nirB* gene, the structural gene for an NADH-dependent nitrite reductase, maps very close to the *cysG* gene (25) in *E. coli*. Since wild-type *S. typhimurium* can use nitrite as a nitrogen source, the *nirB* phenotype could be checked by scoring growth on NCN medium containing nitrite. All three classes of mutations, when moved into an otherwise wild-type

background lacking the parental Φ(*cysG-lacZ*) fusion, were unable to use nitrite as a nitrogen source. All mutants were able to use ammonia as a nitrogen source, and all strains were cysteine prototrophs. The *nirP* mutants could use nitrate, but not nitrite (2 mM), as a nitrogen source. These mutants (*nirP*) could also grow on nitrite supplied at higher concentration (15 mM). The name *nirP* reflects our suggestion that these mutations affect nitrite transport.

The mutations tentatively classified as *fnr* mapped in a region known to include the *fnr* gene. Therefore, the *fnr* phenotype of the mutants was tested. The *fnr* gene encodes a positive regulatory protein required for the induction of several anaerobic respiratory systems, including nitrate, fumarate, and nitrite reductases in *E. coli* (36). These mutants were found to be defective for the ability to grow on nitrate as an electron acceptor. A known *fnr*::Tn*10* mutation (37) was moved into a Φ(*cysG-lacZ*) strain; the double mutant was no longer inducible by nitrite.

The mutants inferred to affect the *nirB* gene were unable to grow on either nitrite or nitrate as a nitrogen source and mapped near the *cysG* gene. The *nirB* gene in *E. coli* also maps near the *cysG* gene (25). It seemed likely that the class three mutants isolated here were also defective for the structural gene for nitrite reductase and reduced *cysG* gene expression by a polar effect. Strains containing defects in the *nirB* gene have not previously been isolated in *S. typhimurium*.

A *cis/trans* test of the *nirB* gene. It has been suggested that the *nirB* and *cysG* genes of *E. coli* are in an operon (31, 32). To genetically test the possibility that *nirB*::Tn*10* insertions may cause a polar effect on *cysG* expression, we constructed a duplication strain (DUP) by the method of Hughes and Roth (18). This duplication strain contains two copies of the *nirB-cysG* region with a Mud::*lacZ* fusion at the join point expressed by the *cysG* promoter and, presumably, any other upstream promoter. Transcriptional expression of the *cysG* gene in the duplication strain was the same as expression in the haploid strain (Table 4).

The *nirB*::Tn*10* insertion was introduced into one copy of the duplication. Strains with the Tn*10* close to the MudA fusion (*cis*), as determined by P22-mediated transductional linkage, formed white colonies on MacConkey-lactose medium; those with the Tn*10* far away from the Mud fusion (*trans*) formed the fish-eye colony typical of a normally regulated Φ(*cysG-lacZ*) fusion strain. The *cis* strain was not induced when grown anaerobically in the presence of nitrite; the *trans* strain was induced when grown anaerobically on nitrite (Table 4). The *nirB* mutation was, therefore, *cis* dominant in its effects on the expression of the *cysG* gene. This supports an operon structure.

DISCUSSION

Our results suggest that only one gene involved in cysteine synthesis is present at the *cysG* locus; this gene encodes a protein required for both vitamin B_{12} and siroheme synthesis. All *cysG* mutants which are defective for cysteine synthesis and for the ability to use nitrite as a nitrogen source are also defective for B_{12} synthesis. Mutants of the *cysG* gene that are leaky for cysteine synthesis and nitrite reduction are also leaky for B_{12} synthesis.

Complementation data, using a chromosomal duplication, suggest that all *cys* mutations which map to min 72 affect one gene. These data suggest that all *cysG* mutations are blocked in the synthesis of DSC and that no other enzyme of the siroheme synthetic pathway is encoded at the *cysG* locus. Work in *E. coli* has shown that the *cysG* locus encodes the URO III methylase (39, 40).

Work on B_{12} and siroheme synthesis suggests that there should be three steps involved in converting URO III to siroheme (2, 28, 29, 35). The first step requires a methyltransfer activity and converts URO III to DSC. In the second step, DSC is oxidized to form SC. In the third step, Fe^{2+} is chelated to SC to form siroheme. It is unclear why the last two steps of siroheme synthesis have not been identified genetically. It is known that the formation of SC from DSC occurs spontaneously in the presence of air (2). In cells grown aerobically, this reaction may proceed spontaneously. Catalysis is probably required, however, in cells grown in an anaerobic reduced environment. If the conversion of DSC to SC occurs spontaneously in cells grown aerobically, mutations affecting DSC oxidation may only be observable anaerobically. It may be possible to isolate mutants that are cysteine auxotrophs only under anaerobic growth conditions. Repeated searches on our part have failed to reveal such mutants.

An SC ferrochelatase should perform the last step in siroheme synthesis; however, mutants lacking this function have never been isolated. Two reasons might explain this: there may be multiple enzymes which perform the same reaction or the ferrochelatase may have a secondary function essential for cell growth. Cysteine auxotrophs could not be isolated in either of these cases. A candidate for an alternative enzyme could be the *hemH* protein. This gene encodes a ferrochelatase that catalyzes the last step in heme biosynthesis (33). However, *hemH* mutants have been isolated in *S. typhimurium*, and none require cysteine for growth (42). Thus, the protein encoded by *hemH* is not required for siroheme synthesis. If the *hemH* gene encodes a redundant ferrochelatase activity, it may be possible to isolate SC ferrochelatase mutants by mutagenizing *hemH* mutants and screening for cysteine auxotrophs. Some of these auxotrophs could be SC ferrochelatase mutants. Attempts to isolate SC ferrochelatase mutants in a *hemH* parent strain have been unsuccessful. Of 47 Cys^- Nir^- mutants isolated in a *hemH* background, 13 carried *cysG* mutations and the rest carried either an *alu hemB*, *hemC*, or *hemD* mutation (data not shown).

These results do not eliminate the possibility that a single *cysG* protein catalyzes DSC synthesis and the formation of siroheme. The *cysG* protein of *Pseudomonas denitrificans* is a 30-kDa protein which methylates URO III to DSC, while the *cysG* protein of *E. coli* is a 56-kDa protein and catalyzes the same reaction (4, 39). Sequence analysis suggests that the *cysG* protein of *S. typhimurium* is almost identical to the *E. coli* protein (32, 41). It is possible that the "extra" 26 kDa of the *E. coli* and *Salmonella* protein may constitute a second domain which catalyzes the final steps of siroheme synthesis.

The orientation of transcription of the *cysG* gene was determined by using mixed lysates of phage grown on strains containing MudA insertions in the *cysG* and *pyrE* genes. Our data suggest that *cysG* transcription proceeds clockwise and that of the *pyrE* gene runs counterclockwise. The technique used in this study has previously been used for determining the orientation of transcription of only an unknown gene by comparison to a known reference gene (18). The results here demonstrate that one can determine the orientation of transcription of two genes whose map positions are known by analyzing the lactose phenotype of the MudA fusion generated at the duplication join point.

The *cysG* gene is not in the cysteine regulon. Strains

containing *cysG*::MudA mutations grown on cystine have slightly higher levels of β-galactosidase activity. This could be due to better growth conditions in general and not related to cysteine levels. The positive regulator of the cysteine regulon, encoded by the *cysB* protein, has no effect on the regulation of the *cysG* gene. This is surprising in that the other two genes required for aerobic sulfite reduction, the *cysJ* and *cysI* genes, are tightly regulated by both cysteine and the *cysB* protein. Vitamin B_{12}, oxygen, carbon source, and cyclic AMP also have no effect on the rate of transcription of the *cysG* gene (data not shown).

Nitrite reduction requires the siroheme cofactor, and the presence of nitrite increases expression of the *cysG* gene when cells are grown anaerobically. This induction, which is fivefold, is independent of the presence of ammonia and is only observed when cells are grown without oxygen. We isolated three classes of mutations which affect the ability of the *cysG* gene to be induced under anaerobic conditions in the presence of nitrite. These mutations prevent induction and leave the *cysG* gene with the basal levels provided by an internal unregulated promoter. All these mutations are defective in the ability to grow on nitrite as a nitrogen source.

Two of the affected functions, *nirP* and *fnr*, act in *trans* on the *cysG* gene. The *nirB* mutation is *cis* dominant in its effect on the *cysG* gene. The *nirP* and *nirB* mutations have not previously been described in *Salmonella* species and map at min 7 and 72, respectively, on the *Salmonella* chromosome. The third mutation, *fnr*, maps to min 30 on the *Salmonella* chromosome and causes a defect in the ability to reduce both nitrate and nitrite. Due to the fact that mutations in the *nirP* gene are phenotypically suppressed by nitrate or by high amounts of nitrite, the *nirP* gene is tentatively regarded as encoding a nitrite permease. Due, however, to the cellular toxicity of nitrite, it seems unlikely that nitrite uptake is the major physiological role of the *nirP* gene.

Previous work on *E. coli* and *S. typhimurium* has suggested that the *cysG* gene is in an operon with and downstream of the structural gene for nitrite reductase, *nirB* (31, 32, 41). Our data agree with that conclusion. An operon structure of this design allows the cell to increase expression of siroheme synthesis when cells synthesize nitrite reductase. A promoter immediately upstream of the *cysG* gene is constitutively expressed and provides enough *cysG* transcript to prevent cysteine starvation in the absence of nitrite or in the presence of oxygen. The open reading frames, found by molecular analysis, in the operon suggested the possibility that other genes required for siroheme synthesis might also be in this operon; however, our data suggest that this is not the case.

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