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Evidence that the CysG Protein Catalyzes the First Reaction Specific to B_{12} Synthesis in *Salmonella typhimurium*, Insertion of Cobalt

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The *cysG* gene of *Salmonella typhimurium* is involved in synthesis of both cobalamin (B_{12}) and siroheme (a cofactor required for SO_3^{2-} and NO_2^{2-} reductases). The failure to reduce SO_3^{2-} leads to cysteine auxotrophy, for which the enzyme is named. Although *Escherichia coli* does not synthesize B_{12} de novo, it possesses a very similar CysG enzyme which has been shown to catalyze two methylations (uroporphyrinogen III to precorrin-2), ring oxidation (precorrin-2 to factor II), and iron insertion (factor II to siroheme). In *S. typhimurium*, precorrin-2 is a precursor of both siroheme and B_{12}. All previously known *Salmonella cysG* mutants are defective in the synthesis of both siroheme and cobalamin. We describe two new classes of *cysG* mutants that cannot synthesize B_{12} but still make siroheme. For class I mutants, exogenous cobalt corrects the B_{12} defect but inhibits ability to make siroheme; B_{12} synthesis is inhibited by added iron. Class II mutants are unaffected by exogenous cobalt, but their B_{12} defect is corrected by derepression of the B_{12} biosynthetic genes (*cob*). We propose that all mutants are defective in insertion of cobalt into factor II and that the *Salmonella* CysG enzyme normally catalyzes this insertion—the first reaction dedicated to cobalamin synthesis. Although *E. coli* does not make B_{12}, its CysG enzyme has been shown in vitro to insert cobalt into factor II and may have evolved to support B_{12} synthesis in some ancestor common to *Salmonella* species and *E. coli*.

In both *Escherichia coli* and *Salmonella typhimurium*, *cysG* mutants were initially identified as cysteine-requiring (Cys^-) auxotrophs (10, 26). These mutants, as well as *cysIJ* mutants were shown to be defective in reduction of sulfite to sulfide (14), which is required for cysteine biosynthesis. The auxotrophy of *cysG* mutants is due to their failure to produce siroheme, the cofactor of the CysIJ enzyme (11, 24, 27, 28). Since siroheme is also required for nitrite reductase (NirB), *cysG* mutants are also defective in reduction of nitrite (Nir^-) (11). After the discovery of B_{12} synthesis in *S. typhimurium* (18), it was found that *cysG* mutants fail to make cobalamin (i.e., are B_{12}^-) (16). Thus, *cysG* mutant phenotypes could be accounted for if the CysG enzyme were required to make some precursor common to both siroheme and cobalamin, possibly precorrin-2; this will prove to be an oversimplification.

The common precursor, precorrin-2, is formed by methylation of the heme precursor uroporphyrinogen III (UroIII) (38); these reactions are diagrammed in Fig. 1. Synthesis of siroheme from precorrin-2 requires ring oxidation followed by iron insertion (40). Mutants defective for either of these individual reactions would be expected to show a Cys^- Nir^- phenotype. If such mutants retained the ability to make precorrin-2 (and if precorrin-2 is the direct precursor of B_{12}), they should be phenotypically B_{12}^+. No mutants of this type have been discovered, despite extensive attempts to find them (16). This suggested that all activities are encoded by the *cysG* locus.

To dissect the *cysG* locus, a large number of *cysG* mutants were screened for siroheme-defective (Sir^-; equivalent to Cys^- Nir^-) mutants that retained the ability to form precorrin-2 (and maintained a B_{12}^+ phenotype). All Sir^- *cysG* point mutants tested had an additional defect in B_{12} synthesis and fell into a single complementation group, suggesting that a single multifunctional protein is encoded at the *cysG* locus (16). If a single CysG protein provides all three activities needed to make precorrin-2 and to convert it to siroheme, none of the mutants tested caused loss of a single activity.

Enzymological studies by Spencer et al. showed directly that purified *E. coli* CysG protein could promote the methyl transfer reactions leading to precorrin-2, the NAD-mediated ring oxidation, and the iron insertion (into factor II) leading to siroheme production (40). In a parenthetical note, the authors mentioned that when cobalt was provided in place of iron, the enzyme could insert cobalt into factor II. Such an activity might be relevant to synthesis of B_{12}, but *E. coli* does not synthesize this cofactor de novo (22).

Most closely related enteric bacteria (*Salmonella*, *Citrobacter*, and *Klebsiella* species) do make cobalamin. Therefore, it is very possible that cobalt insertion by the *E. coli* CysG enzyme reflects an ancient role in B_{12} synthesis. The CysG enzyme of *E. coli* is homologous to that of *S. typhimurium* (which synthesizes B_{12} de novo); the two enzymes are 90% identical and 95% similar over the entire lengths of both proteins (45). While the *Salmonella* enzyme has not been tested for the ability to insert cobalt, it is known that salmonellae synthesize B_{12} by a pathway in which cobalt insertion occurs at a very early step (36). Thus, the idea of biologically relevant cobalt insertion by the *Salmonella* CysG protein is an attractive possibility. While this possibility has been suggested previously (29), there is no evidence to show that the *Salmonella* CysG enzyme inserts cobalt or that this inferred activity is biologically relevant.

Previously known genetic phenotypes of *cysG* mutants demonstrate that at least the methylation reactions are significant in vivo since these reactions lead to precorrin-2, the known precursor of both cofactors. There is no genetic evidence to support the biological role of the in vitro oxidation and metal insertion activities, since no mutants that lack only these activities have been isolated. Similarly, it is not clear whether the observed cobalt insertion ability of the *E. coli* CysG enzyme is biologically important.

Here we describe the isolation of several *Salmonella cysG*

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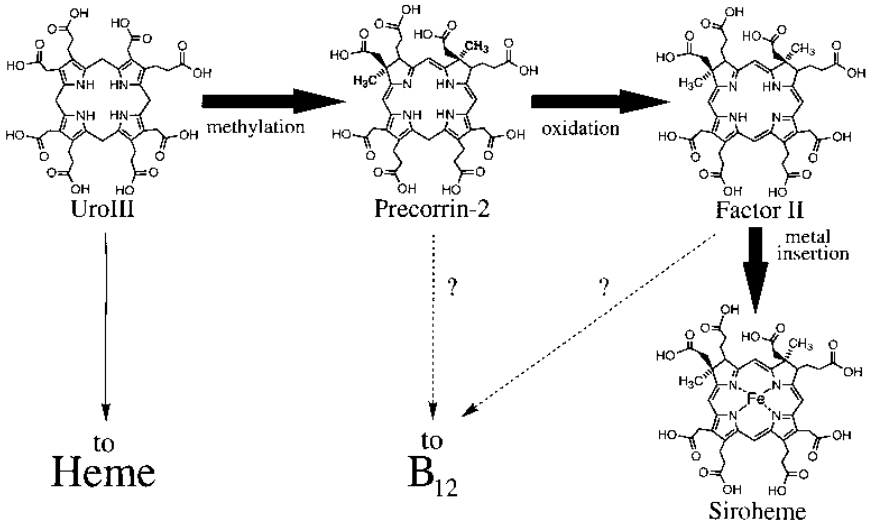

FIG. 1. Structures of compounds involved in CysG-catalyzed reactions. UroIII is the last precursor common to heme, siroheme, and B_{12}. UroIII is methylated at C-2 and C-7 to form precorrin-2. Precorrin-2 is oxidized at C-14 and C-15 to form factor II, into which an Fe^{2+} ion is inserted to form siroheme. Wide arrows represent reactions shown to be catalyzed by CysG (40). Specific alterations in porphyrin molecules resulting from CysG-catalyzed reactions are shaded. The thin, solid arrow marks the branch point of the heme biosynthetic pathway. The dotted arrows marked by question marks denote possible branch points of the B_{12} pathway.

mutants defective only in cobalamin synthesis. These mutants provide evidence that the *Salmonella* CysG enzyme catalyzes a biologically relevant reaction unique to B_{12} synthesis, presumably insertion of cobalt into factor II. Secondary phenotypes of these mutants also support a biological role of its ring reduction and iron insertion activities. Thus, it appears that a single remarkable enzyme performs five biologically important reactions: two methyl transfers, a ring oxidation, insertion of iron to form siroheme, and insertion of cobalt (as an alternative to iron) to generate an intermediate in B_{12} synthesis (Fig. 2). This places the CysG enzyme in a unique position to regulate distribution of UroIII into the pathways for heme, siroheme, and B_{12} synthesis. Despite the strategic position of the CysG enzyme in these pathways, no evidence that the relative levels of its several activities vary in response to cellular conditions has been presented.

MATERIALS AND METHODS

Phage and bacterial strains. All strains used in this study are derivatives of *S. typhimurium* LT2 (Table 1). Transductional crosses used the high-frequency generalized transducing mutant of bacteriophage P22 (HT105/1 *int-201*) as described previously (12). The Tn*10d*Tc element is a transposition-defective derivative of transposon Tn*10* which confers tetracycline resistance (44). The Tn*5* transposon confers kanamycin resistance (7).

Media and auxotrophic supplements. Difco nutrient broth (NB; 0.8%) supplemented with 85 mM NaCl was used as the complex medium. For most experiments, E medium supplemented with glucose (0.2%) was used as the minimal medium. For maximal *cob* expression, we used NCE (no-citrate E) medium supplemented with sodium pyruvate (0.44%), disodium fumarate (0.32%), and DL-1,2-propanediol (0.2%). Other supplements were added at the following concentrations: cyanocobalamin, 0.1 μg/ml; cysteine, 0.3 mM; methionine, 0.3 mM; homocysteine (HC), 0.3 mM; $CoCl_2$, 2.0 μg/ml; and $FeSO_4$, 2.5 μg/ml. Solid medium contained 1.5% agar. For transductional crosses in which a Cys^+ phenotype was selected for mapping of *cysG* point mutations (see below), a small amount of NB (0.01%) was added to E medium as a micronutrient source.

Scoring *cys* and *cob* phenotypes. Ability to reduce sulfite was determined by growing strains on solid minimal media with and without cystine or Na_2S. Since cobalamin is not essential for growth of wild-type *S. typhimurium*, B_{12} production was scored by including a *metE* mutation in each strain, forcing use of the B_{12}-dependent homocysteine methyltransferase (MetH) (18). In *metE* mutants that fail to make B_{12}, growth depends on addition of either B_{12} or methionine to the medium. Since salmonellae synthesize cobalamin only anaerobically, most experiments described here were performed in an anaerobic chamber (Forma Scientific model 1024) containing an (89:5:6) atmosphere N_2-CO_2-H_2.

Scoring sulfite reductase phenotypes. The presence of sulfite reductase (CysIJ) activity was determined qualitatively under aerobic conditions, using solid Difco bismuth sulfite medium, as described previously (16). Relative levels of sulfide production by the various strains were inferred from the color intensity of bismuth sulfide produced from sulfite by bacterial colonies growing on this medium.

Localized mutagenesis of the *cysG* locus. Hydroxylamine mutagenesis was performed by the method of Hong and Ames (17). Bacteriophage P22 was grown on a strain (TT15028) carrying a Tn*10d*Tc insertion near the *cysG* locus; this lysate was mutagenized to 0.1% survival and used to transduce a *metE* recipient

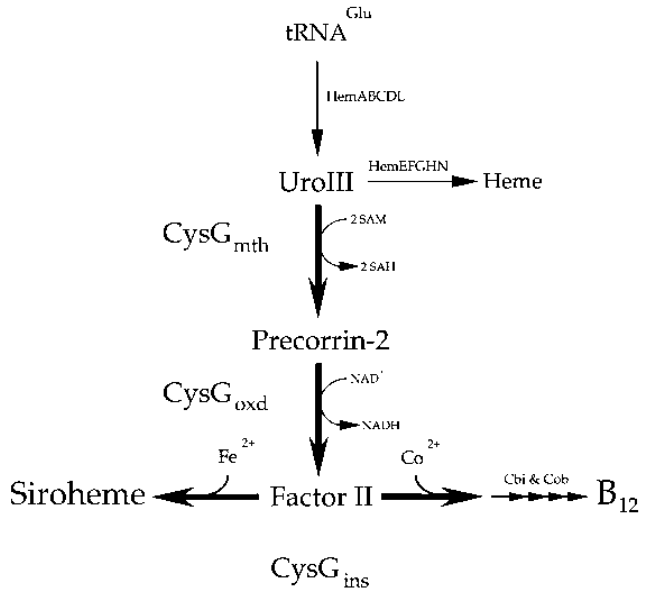

FIG. 2. Proposed synthetic pathway for siroheme and B_{12}. Large arrows represent CysG-catalyzed reactions. Gray bars represent the different catalytic activities of the CysG enzyme, with their respective functions labeled below or on the side. $CysG_{mth}$, methylation; $CysG_{oxd}$, oxidation; $CysG_{ins}$, metal insertion. The siroheme/B_{12} pathway diverges from the heme pathway when UroIII is methylated by CysG to form precorrin-2. Precorrin-2 is then oxidized by CysG to form factor II. The metal insertion domain of CysG inserts iron to form siroheme or (postulated here) cobalt to form first specific B_{12} precursor (factor II-chelated cobalt). SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomoserine.

TABLE 1. Bacterial strains used

Strain	Genotype
TT2095	*metB869*::Tn*10 his-9533*
TT2742	*aroB542*::Tn*5*
TT15028	*zhc-3665*::Tn*10d*Tet (90% linked to *cysG*)
TT15696	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet)
TT18449	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *zhc-3665*::Tn*10d*Tc (90% linked to *cysG*)
TT18450	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *zhc-3665*::Tn*10d*Tet *cysG3323*
TT18451	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *zhc-3665*::Tn*10d*Tet *cysG3324*
TT18452	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *zhc-3665*::Tn*10d*Tet *cysG3325*
TT18453	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *zhc-3665*::Tn*10d*Tet *cysG3326*
TT18454	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *zhc-3665*::Tn*10d*Tet *cysG3327*
TT18455	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *zhc-3665*::Tn*10d*Tet *cysG3328*
TT18577	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *cysG3170*::MudA
TT18578	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *zhc-3665*::Tn*10d*Tet *cysG3326 aroB542*::Tn*5*
TT18579	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *zhc-3665*::Tn*10d*Tet *cysG3327 aroB542*::Tn*5*
TT18586	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *metB869*::Tn*10*
TT18587	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *metB869*::Tn*10 cysG3324*
TT18588	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *metB869*::Tn*10 cysG3325*
TT18589	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *metB869*::Tn*10 cysG3326*
TT18590	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *metB869*::Tn*10 cysG3327*
TT18591	*ara-9* DEL*1077* (*metE zid-3680*::Tn*10d*Tet) *metB869*::Tn*10 cysG3328*
TT19215	*cysG3324 eut-38*::MudJ
TT19216	*cysG3325 eut-38*::MudJ
TT19217	*cysG3326 eut-38*::MudJ
TT19218	*cysG3327 eut-38*::MudJ
TT19219	*cysG3328 eut-38*::MudJ
TT19220	Δ*cysG3166 eut-38*::MudJ

strain (TT15696) to tetracycline resistance. Transductant (tetracycline-resistant) colonies were scored for the ability to produce siroheme or cobalamin. Mutants defective for siroheme or B_{12} synthesis were identified as potential *cysG* mutants.

Mapping of *cysG* point mutations. Point mutations used in this study were mapped to sites within the *cysG* gene by transductional crosses using previously mapped *cysG* deletion mutants (16). A P22 lysate (100 μl) prepared on each point mutant was mixed individually with 150 μl of a fresh overnight NB culture of each *cysG* deletion mutant. The mixture was incubated for 1 h and then plated on minimal medium containing a small amount of NB (0.01% [wt/vol]). Point mutations lying outside of the region removed by a recipient deletion mutation recombined to form Cys^+ transductants. A lysate prepared on strain LT2 gave approximately 1,000 Cys^+ transductants per plate for each deletion. Lysates grown on a point mutant gave an average of 300 to 500 Cys^+ transductants when crossed with a nonoverlapping deletion; point mutations that gave no Cys^+ recombinants when crossed with a particular deletion were assumed to map within the region removed by the deletion.

Introduction of a *metB*::Tn*10* insertion. As isolated, the original *cysG* mutants carried the *zhc-3665*::Tn*10d*Tc insertion near the *cysG* locus and were therefore tetracycline resistant. This insertion was removed to permit selective introduction of a *metB*::Tn*10* mutation.

Two different procedures were used to separate the Tn*10d*Tc insertion mutation from the five *cysG* mutations. In the first method, the *cysG* mutation was moved into a new recipient strain, making use of the fact that the five *cysG* point mutants are Cys^+ when grown aerobically with added methionine. To construct strains TT18581, 18582, and 18585 (tetracycline-sensitive derivatives carrying mutation *cysG3324*, *-3325*, or *-3326*), a P22 lysate was grown on each of the *cysG* point mutants and used to transduce recipient strain TT18577 (*metE cysG3170*::MudA). Cys^+ transductants that acquired the donor *cysG* mutation without the nearby Tn*10d*Tc insertion were saved.

A second method was used to construct tetracycline-sensitive derivatives of mutants bearing *cysG3326* or *cysG3327*. Insertion *aroB542*::Tn*5* (TT2742), closely linked to the *cysG* locus, was transduced into recipient strains TT18453 and 18454 (*metE cysG zhc-3665*::Tn*10d*Tc). Each of the resulting Aro^- strains was transduced to Aro^+, and transductants (TT18583 and TT18584) that carried the *cysG* mutation but not the nearby Tn*10d*Tc insertion were saved. Into the tetracycline-sensitive *cysG* mutants generated by either of the two methods outlined above, the *metB869*::Tn*10* insertion was transduced by selecting tetracycline resistance; this yielded strains TT18587 through 18591 (*metE cysG metB*).

Assay of β-galactosidase. β-Galactosidase activity was assayed as described by Miller (25). Conditions for pregrowth of cells were a modification of those described earlier (8). Cells were grown to saturation in NB, brought into an anaerobic chamber, and diluted 1:50 into NCE pyruvate-fumarate medium containing ethanolamine and a trace amount of $CoCl_2$ (2 ng/ml) in a crimp-sealable culture tube. The medium had been made anaerobic by prior incubation in an anaerobic chamber for at least 7 h. Tubes were capped with sterile rubber stoppers, sealed, and removed from the chamber. Gas within the tubes was replaced with nitrogen by at least three cycles of evacuation and pressurization (3). Cultures were shaken at 37°C until cells had reached log phase, at which point they were assayed for β-galactosidase activity.

RESULTS

Isolation of *cysG* mutants with a Cys^+ B_{12}^- phenotype. All previous *cysG* mutants were isolated as cysteine auxotrophs and were thus defective in synthesis of siroheme (required for sulfite and nitrite reductases) (10, 11, 16, 24, 26). Previously, a set of 30 such *Salmonella cysG* mutants were found to be defective for cobalamin synthesis (B_{12}^-) (16). We initiated a new search for *cysG* mutants singly defective for either B_{12} or siroheme synthesis but not both. The parent strain used carried a *metE* mutation which renders methionine synthesis dependent on the B_{12}-dependent MetH enzyme. Thus, a deficiency in B_{12} synthesis can be scored as a B_{12}-correctable methionine requirement. Since wild-type salmonellae make B_{12} only anaerobically, these tests were made in the absence of oxygen. Siroheme deficiency (Sir^-) was detected as cysteine auxotrophy, also under anaerobic growth conditions. Following local mutagenesis of the *cysG* locus, we isolated 35 new point mutants. Thirty of these were of the standard Sir^- B_{12}^- (Cys^- Met^-) type seen previously. The remaining five appeared to be defective only for B_{12} synthesis (Sir^+ B_{12}^-).

Growth phenotypes of the five new mutants are shown in Table 2. The parent (*metE cysG*$^+$) strain (TT18449) can synthesize B_{12} and therefore grew anaerobically on minimal media. A standard (Sir^- B_{12}^-) *cysG* null mutant (TT18450) grew anaerobically only when supplemented with both cysteine (or sulfide) and B_{12} (or methionine). Each of the five new *cysG* mutants (*cysG3324* to *cysG3328*) grew anaerobically on minimal medium when provided with only B_{12} or methionine, indicating a defect in only cobalamin synthesis. These mutants did not require cysteine or sulfide, indicating proficiency in siroheme synthesis.

The ability of the new mutants to reduce sulfite was confirmed by growing cells on bismuth sulfite indicator plates. Strains capable of reducing sulfite to sulfite form a black precipitate of bismuth sulfide; this precipitate is not formed by strains lacking sulfite reductase activity. Null mutants of the *cysG*, *cysI*, or *cysJ* gene characteristically form white patches on bismuth sulfite agar, since the *cysI* and *cysJ* mutations eliminate subunits of the siroheme-dependent sulfite reductase, and the *cysG* mutation eliminates the required siroheme cofactor. When tested in this manner, the five new CysG mutants each gave an intermediate result; *cysG3324*, *cysG3325*, and *cysG3328* mutants formed dark brown patches, while *cysG3326* and *cysG3327* mutants appeared light brown. Thus, the B_{12} auxotrophy observed under anaerobic conditions appears to result from a *cysG* defect which strongly reduces the produc-

TABLE 2. Growth phenotypes of *cysG* mutants

Strain[a]	Relevant genotype	Mutant class	Anaerobic growth on minimal medium[b] with:									
			No addition	B_{12}	Cys	Cys, B_{12}	Co^{++}	Co^{++}, Cys	Co^{++}, B_{12}	Co^{++}, Cys, B_{12}	Fe^{++}, Co^{++}, Cys	Fe^{++}, Co^{++}, Cys, B_{12}
TT18449	*cysG*$^+$	Wild type	+	+	+	+	+	+	+	+	+	+
TT18450	*cysG3323*	Null	−	−	−	+	−	−	−	+	−	+
TT18451	*cysG3324*	I	−	+	−	+	−	+	+	+	−	+
TT18452	*cysG3325*	I	−	+	−	+	−	+	+	+	−	+
TT18455	*cysG3328*	I	−	+	−	+	−	+	+	+	−	+
TT18453	*cysG3326*	II	−	+	−	+	−	−	+	+	−	+
TT18454	*cysG3327*	II	−	+	−	+	−	−	+	+	−	+

[a] All strains carry a *metE* mutation and are *cob*$^+$.
[b] Strains were grown anaerobically on solid E medium with glucose and indicated supplements: cobalamin (CN-B_{12}), 100 ng/ml; cysteine, 36.5 μg/ml (0.3 mM); $CoCl_2 \cdot 6H_2O$, 2 μg/ml; and $FeSO_4 \cdot 7H_2O$, 5 μg/ml.

tion of B_{12} but allows continued production of siroheme (albeit at a reduced rate).

Mapping of *cysG* point mutations. The five Sir^+ B_{12}^- mutations were mapped to the *cysG* locus by transductional crosses as described in Materials and Methods. The five new Sir^+ B_{12}^- mutations mapped to three contiguous deletion intervals near the promoter-proximal end of the gene (Fig. 3). This general region of the gene is thought to encode the portion of the enzyme responsible for ring oxidation and metal insertion activities (43); the C-terminal region is known to perform the two methylations (40, 43). We will propose that the wild-type CysG enzyme is able to insert cobalt into factor II to form the first intermediate in B_{12} production and that the five new mutants described are defective in this activity.

Methionine requirement is not due to cysteine limitation. Since the five *cysG* mutants were phenotypically Cys^+ Met^- under anaerobic growth conditions (Table 2), they appear to make siroheme but not cobalamin (Sir^+ B_{12}^-). However, interpretation of this phenotype is complicated by the relationship of the cysteine and methionine biosynthetic pathways (Fig. 4). Cysteine is used as a substrate of the methionine biosynthetic pathway; it serves as a sulfide donor in the production of HC, which is then (by a B_{12}-dependent reaction) converted to methionine. Consequently, limitation of cysteine production might secondarily limit methionine production. The partial defect of the five new *cysG* mutants for siroheme synthesis might create a cysteine shortage which is seen phenotypically as a methionine requirement, accounting for the phenotype (Cys^+ Met^-) observed for the five new mutants.

To test this possibility, we separated the Cys and Met synthetic pathways from one another by introducing a *metB* mutation into each of the *cysG* mutants. The *metB* gene encodes cystathionine-γ-synthase, which joins a molecule of cysteine to *O*-succinylhomoserine and eliminates succinate to form cystathionine (21) (Fig. 4). To grow, a *metB* mutant requires either methionine or one of its sulfur-bearing precursors; it uses no cysteine for the synthesis of methionine. Triple mutants (*metE cysG metB*) were tested for the ability to make B_{12} when provided with HC. While none of the five *metE cysG metB* triple mutants grew on HC alone, all grew on HC plus B_{12}. This finding indicates that the five *cysG* mutants are defective in methionine synthesis due only to a defect in B_{12} synthesis; their Met^- phenotype is not due to limited production of Met precursors.

A second assay for the B_{12} defect of cysG mutants. To provide independent evidence that the *cysG* mutants described here are deficient in B_{12} synthesis, we used induction of the ethanolamine (*eut*) operon as a test for B_{12} production. This operon requires both ethanolamine and B_{12} for induction and is subject to catabolite repression (30–32, 39). During growth on a poor carbon source (to stimulate cyclic AMP production) under anaerobic conditions (to allow B_{12} synthesis), the *Sal-*

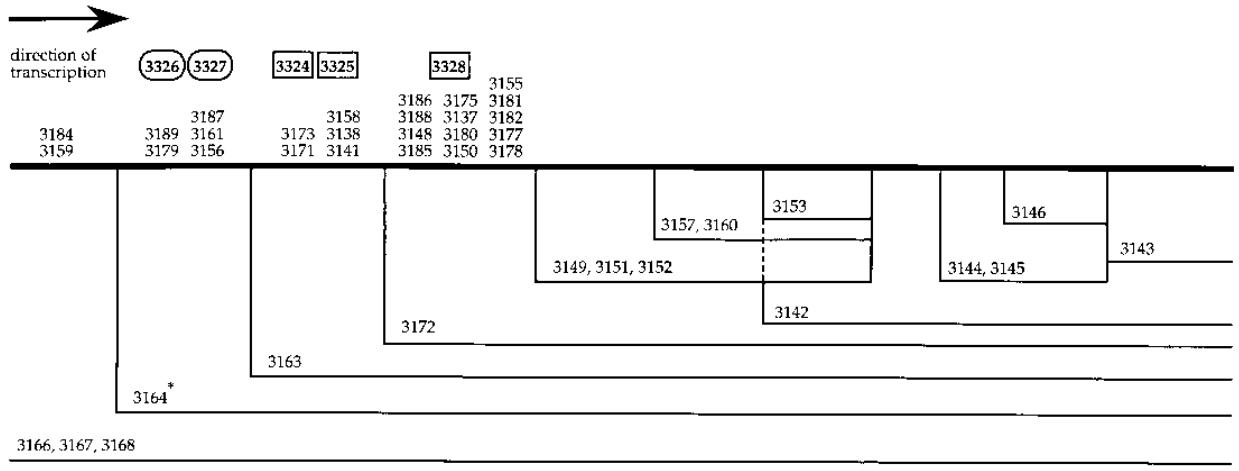

FIG. 3. Deletion map of the *cysG* gene. Deletion mutations shown were characterized previously (16). Map positions of point mutations in relation to deletion endpoints are given. Class I mutations are enclosed by boxes; class II mutations are enclosed by ovals. *The map is revised slightly from that presented earlier (16); specifically, the endpoints of deletions *cysG3163* and *cysG3164* have been reversed.

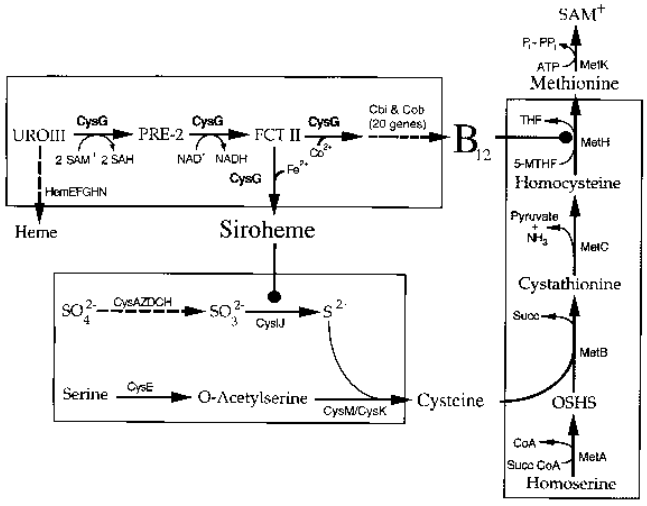

FIG. 4. Relationship of the cysteine and methionine pathways to B_{12} and siroheme. Boxes enclose individual pathways; enzymes catalyzing various reactions are indicated by their genetic designations. In the methionine pathway (shaded box), HC and methionine levels regulate the *metA*, *-B*, *-C*, *-H*, and *-K* genes and thus reduce the drain on cysteine caused by methionine synthesis. Pre-2, precorrin-2; Fct II, factor II; OSHS, *O*-succinylhomoserine; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomoserine; THF, tetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; Succ, succinate; CoA, coenzyme A.

monella eut operon is induced by addition of ethanolamine. This induction depends on endogenous B_{12} synthesis. To test this induction, we used a MudJ insertion within the transcribed region of the *eut* operon but outside of all coding regions. This insertion mutant (*eut-38*::MudJ) is completely wild type for ethanolamine utilization and for operon control. Strains carrying this insertion and various *cysG* mutations were grown anaerobically on minimal pyruvate-fumarate medium containing ethanolamine and assayed for β-galactosidase. All strains are *metE*$^+$; therefore, their ability to synthesize methionine is independent of B_{12} production.

Assay results in Table 3 show that the *eut* operon of a *cysG*$^+$ strain is fully induced during growth on ethanolamine with or without exogenous B_{12}. A *cysG* null mutant (deletion *cysG3166*) cannot induce the *eut* operon unless B_{12} is provided. The five *cysG* point mutations described here are all defective for induction of the operon without added B_{12}. Since their defect is corrected by B_{12}, we infer that the mutants are unable to produce B_{12}. It should be noted that the defect in B_{12} production assayed in this way is as great as that seen for a *cysG* deletion mutant. All of the strains assayed are able to grow on pyruvate-fumarate medium in the absence of cysteine, demonstrating their continued ability to synthesize siroheme.

Effects of added cobalt on mutant phenotypes. The five new *cysG* mutations block anaerobic B_{12} production while maintaining some sulfite reductase activity. Their phenotypes might be explained if the CysG enzyme were responsible for cobalt insertion in addition to its demonstrated role in iron insertion (diagrammed in Fig. 2). Thus, the mutants might be generally impaired in metal insertion, maintaining enough iron insertion activity to be phenotypically Cys$^+$ (Sir$^+$) but lacking sufficient cobalt insertion activity (B_{12}^-). Such a defect might be overcome by the addition of exogenous cobalt.

When grown in the presence of excess cobalt (2 μg/ml), the five mutants fell into two classes, shown in Table 2. Cobalt, by itself, did not restore growth on minimal medium to any of the five new *cysG* mutants. However, cobalt plus cysteine allowed growth of three of the five mutants (*cysG3324*, *-3325*, and *-3328*; referred to as class I). Thus, for class I mutants, addition of cobalt to the medium had two phenotypic consequences: it corrected the B_{12} defect and induced an apparent Sir$^-$ defect (cysteine auxotrophy). This result suggested that in class I mutants, cobalt and iron might compete for a reduced ability of the mutant enzyme to insert metal ions; this balance could be altered by increasing cobalt levels. Class II mutants (*cysG3326* and *-3327*) appear to have a more stringent defect for cobalt insertion that is not affected by higher cobalt levels; they remained Sir$^+$ B_{12}^- in the presence of high cobalt.

Effect of added iron on *cysG* mutants. If iron and cobalt compete for the same active site in the CysG enzyme, addition of iron to the medium might affect growth phenotypes in the five *cysG* mutant strains. To test this possibility, cells were grown on minimal media containing cysteine, cobalt, and iron, with and without B_{12}, and were incubated anaerobically. Results are shown in Table 2. The *cysG*$^+$ *metE* deletion mutant (TT18449) grew anaerobically with and without added iron and B_{12}. The ability of the three class I *cysG* mutants (*cysG3324*, *-3325*, and *-3328*) to make B_{12} when provided with cystine plus cobalt was eliminated by the addition of iron. The B_{12} deficiency caused by iron was corrected by added B_{12}; this result demonstrates that iron inhibits B_{12} production, consistent with a competition between the metals for insertion into the porphyrin ring. The effect of iron on the cobalt-induced Sir$^-$ phenotype could not be tested since the addition of B_{12} alone restored a Sir$^+$ phenotype (see below). Class II mutants could not be tested for iron sensitivity, since these strains remain Sir$^+$ B_{12}^- with cobalt.

Effect of B_{12} on siroheme synthesis by *cysG* mutants. The three class I (B_{12}^- Sir$^+$) mutants that were made B_{12}^+ Sir$^-$ by added cobalt regained a Sir$^+$ phenotype on medium containing B_{12} in addition to cobalt (Table 2). This observation could be attributed to allosteric effects of B_{12} on the CysG enzyme. Alternatively, it could be due to repression of *cob* operon expression by B_{12} (2, 15, 20) or inhibition of some step of the B_{12} synthetic pathway; either of these B_{12} effects would reduce metabolic flux through the cobalamin biosynthetic pathway and might increase siroheme synthesis.

We tested the repression hypothesis by providing B_{12} at a level (1 nM) below that required for repression of the *cob* operon (2), with and without added cobalt (data not shown). Low levels of B_{12} had the same growth effect as high levels; that is, all three class I mutants were Cys$^-$ on cobalt alone and became Cys$^+$ with a low level of added B_{12}. Thus, B_{12}-mediated repression of the *cob* genes does not account for the Cys$^+$ phenotype observed in the presence of cobalt and B_{12}.

TABLE 3. Estimation of B_{12} production by measuring induction of the *eut* operon[a]

Strain	Relevant genotype[b]	Mutant class	Expression of *eut-lac* fusion (Miller units)	
			$-B_{12}$	$+B_{12}$
TT18827	*cysG*$^+$	Wild type	226	195
TT19220	*cysG3166*	Null	3.2	182
TT19215	*cysG3324*	I	3.2	119
TT19216	*cysG3325*	I	1.6	103
TT19219	*cysG3328*	I	2.0	121
TT19217	*cysG3326*	II	3.4	142
TT19218	*cysG3327*	II	3.5	149

[a] Strains were grown on NCE pyruvate-fumarate medium containing ethanolamine and indicated supplements. Strain TT19220 was supplemented with cysteine (0.3 mM).

[b] All strains were *metE*$^+$ and contained a *eut-lac* fusion (*eut-38*::MudJ).

TABLE 4. Phenotypes of *cysG* mutants under high levels of *cob* operon expression

Strain[a]	Genotype	Mutant class	Anaerobic growth on minimal pyruvate-fumarate propanediol medium[b] with:				
			No addition	B_{12}	Cys	HC	Met
TT18449	*cysG*$^+$	Wild type	+	+	+	+	+
TT18450	*cysG3323*	Null	−	−	−	−	−
TT18451	*cysG3324*	I	−	−	+	+	+
TT18452	*cysG3325*	I	−	−	+	+	+
TT18455	*cysG3328*	I	−	−	+	+	+
TT18453	*cysG3326*	II	−	+	+	+	+
TT18454	*cysG3327*	II	−	+	+	+	+

[a] All strains carry a *metE* mutation and are *cob*$^+$.
[b] Strains were grown anaerobically on solid NCE medium with pyruvate as the carbon and energy source, fumarate as the electron acceptor, DL-1,2-propanediol as an inducer of the *cob* operon, and indicated supplements: cobalamin (CN-B_{12}), 100 ng/ml; cysteine, 36.5 μg/ml (0.3 mM); HC, 45 μg/ml (0.3 mM); and methionine, 45 μg/ml (0.3 mM).

Inducing the *cob* operon reduces cysteine production. The initial characterization of the Sir$^+$ B_{12}^- phenotypes of the new *cysG* mutations (described above) was performed on anaerobic minimal medium with glucose as a carbon and energy source. These conditions reduce expression of the *cob* operon, which contains most of the B_{12} synthetic genes and a transport system for cobalt (34). It seemed possible that induction of the cobalamin biosynthetic genes would accelerate processing of CysG enzyme products and correct the B_{12} defect of the mutants by mass action. Therefore, we grew cells anaerobically on pyruvate with fumarate as an electron acceptor and provided propanediol as an inducer of the *cob* operon. Under these conditions, the *cob* operon is induced 300-fold compared to the level during anaerobic growth on glucose (1, 8).

Cells were tested for growth on NCE pyruvate-fumarate propanediol plates with and without cysteine, B_{12}, HC, and methionine (Table 4). The parental *metE* mutant (TT18449) were Sir$^+$ B_{12}^+ (Cys$^+$ Met$^+$) regardless of added growth supplement. Standard null mutants of the *cysG* locus were Sir$^-$ B_{12}^- (Cys$^-$ Met$^-$). Again, the two new *cysG* mutant classes displayed distinctive phenotypes.

Class I mutants (*cysG3324*, *3325*, and *3328*) became Sir$^-$ B_{12}^+ (Cys$^-$ Met$^+$) when grown with an induced *cob* operon (Table 4). Thus, induction of the *cob* operon (like cobalt addition) corrected the B_{12} defect and induced a cysteine auxotrophy in these mutants.

Class II mutants behaved differently. Under inducing conditions for the *cob* operon, they required either B_{12} or cysteine for growth. These mutants appear to be poised so that they can produce either siroheme or B_{12} but not both. This behavior could reflect either regulatory effects of added cysteine and B_{12} on the activity of the CysG enzyme or effects of mass action on competition for a limited pool of precursors (see Discussion).

Surprisingly, when grown with an induced *cob* operon, all mutants made both cysteine and methionine if provided with HC, which does not circumvent either the B_{12} or the siroheme defect (Table 4). This result can be explained in terms of the interrelatedness of the cysteine and methionine synthetic pathways (Fig. 4). When mutants are grown with an induced *cob* operon, they become Sir$^-$ B_{12}^+; their growth defect appears to result from a cysteine shortage. Growth is restored by adding methionine or cysteine, either of which can reduce the demands on a limited sulfide pool. Since *cob* operon induction made the mutants B_{12}^+, they can use added HC to make methionine, thus reducing the sulfide requirement of the cell (and alleviating the cysteine shortage). It should be noted that HC did not correct the Sir$^+$ B_{12}^- phenotype of mutants when glucose reduced *cob* operon expression; under these conditions, no B_{12} was made and HC could not be converted to methionine.

DISCUSSION

We describe five CysG mutants that are defective for B_{12} synthesis (B_{12}^-) but retain the ability to synthesize siroheme (Sir$^+$); the mutants fell into two phenotypic classes. All five mutants appeared Sir$^+$ B_{12}^-, as judged by their inability to perform B_{12}-dependent methione synthesis and their failure to induce the *eut* operon without exogenous B_{12}. For class I mutants (Sir$^+$ B_{12}^-), added cobalt corrected the B_{12} defect and induced a siroheme defect (Sir$^-$ B_{12}^+). This correction was reversed by adding iron in addition to cobalt. For class II mutants, the B_{12} defect (and the ability to synthesize siroheme) was not affected by cobalt. Induction of the *cob* operon causes class I mutants to become Sir$^-$ B_{12}^+, suggesting that mass action causes CysG substrates to flow preferentially toward B_{12}. Under these conditions, class II mutants can produce either siroheme or B_{12} but not both. These results suggest two conclusions: (i) the CysG enzyme catalyzes the first reaction of cobalamin synthesis, cobalt insertion; and (ii) the CysG enzyme may show regulatory responses that can direct the flow of UroIII to either siroheme or B_{12}, depending on growth conditions.

The phenotype of class I mutants suggests that these mutants are impaired for insertion of both metals and can be directed by growth conditions or relative metal concentrations to insert either iron or cobalt. On standard glucose minimal medium, class I mutants synthesize only siroheme; added cobalt shifts the balance toward B_{12} and away from siroheme. This balance is shifted back toward siroheme when iron is added in addition to cobalt. Induction of the cobalamin pathway (like cobalt addition) shifts the balance toward B_{12} and away from siroheme production; this could be due to mass action as B_{12} precursors are processed by the cobalamin biosynthetic pathway or to increased levels of intracellular cobalt due to induction of a cobalt transport system encoded by the *cob* operon (34). All of these phenotypes could be explained by competition between cobalt and iron (the B_{12} and siroheme pathways) for factor II. We suspect that class I mutant enzymes possess a reduced ability to insert both metals but have a greater defect in cobalt insertion.

The class II mutant phenotypes on glucose (Table 2) suggest that the mutant enzyme has a greater defect in cobalt insertion than class I mutants; their B_{12} defect is not corrected by exogenous cobalt. When the *cob* operon is expressed, mass action may allow the low residual ability to insert cobalt to provide for B_{12} synthesis. Under these conditions, the mutant enzyme can provide for either cysteine (siroheme) or methionine (B_{12}) synthesis but cannot do both. We think it likely that these phenotypes reflect mutant enzymes whose conformation is biased toward one of two regulatory states that can be assumed by the normal enzyme. Class II mutant enzyme may be preferentially in the iron-inserting (siroheme) conformation; its activity is not affected by added cobalt. Under conditions of *cob* operon expression, the presence of high cysteine may cause the enzyme to shift to the cobalt inserting (B_{12}) conformation. Conversely, when B_{12} is provided, it may cause a shift to the iron-inserting (siroheme) form. The addition of methionine (or HC) also appears to shift class II mutants in favor of siroheme production; the latter effects might be due to the

sparing of a limited cysteine supply by reducing flow to methionine.

Since the CysG enzyme is responsible for directing UroIII away from the heme pathway toward siroheme and B_{12}, it can potentially control the relative flow of UroIII into all three pathways. B_{12} is required by wild-type cells mainly for growth on propanediol or ethanolamine (9, 19, 30, 33, 42). Siroheme-dependent sulfite reduction is required for synthesis of cysteine, methionine, and other sulfur-containing metabolites whenever sulfate or sulfite is the sulfur source (5, 6). Thus, under particular growth conditions, it would be advantageous for the cell to direct synthesis preferentially toward either siroheme or B_{12}. The step which appears to commit intermediates to one pathway or the other is the point at which CysG inserts iron or cobalt. Thus, regulation of insertion activity via allosteric shifts in the enzyme which favor either cobalt or iron would be an effective method of controlling the relative amounts of siroheme and B_{12} that are produced. If the methylation reactions (or insertion of both metals) were inhibited, all UroIII would be available for heme synthesis.

The presented data suggest that the CysG enzyme catalyzes cobalt insertion (leading to B_{12} synthesis) as well as iron insertion (leading to siroheme). An alternative, less likely possibility cannot be eliminated. One could imagine that the CysG enzyme plays no role in cobalt insertion and performs only four reactions (two methylations, ring oxidation, and iron insertion). The described mutations might then reduce production of precorrin-2 (or factor II) and show a B_{12} deficiency because that is the first growth defect to become apparent as the levels of both siroheme and cobalamin are reduced. According to this model, the effect of added cobalt would be to stimulate the true cobalt insertion enzymes, shifting the competition for the common precursor away from siroheme. While this possibility cannot be eliminated, we think it unlikely since the homologous *E. coli* CysG enzyme has been shown to insert cobalt and it is hard to account for the different behaviors of the class I and class II mutants in terms of this alternative explanation. Since very little B_{12} is needed, one would expect a parallel reduction of both pathways to lead first to a cysteine requirement.

The CysG proteins of *E. coli* and *S. typhimurium* are of the same size and show 90% identity (95% similarity) over their entire lengths (45). In describing enzymological analysis of the *E. coli* enzyme, Spencer et al. (40) mentioned parenthetically that the enzyme could catalyze insertion of cobalt into factor II. The biological significance of this observation was uncertain since *E. coli* does not make B_{12} de novo and since any metal-inserting enzyme might be expected to show some ability to insert related metals. The results presented here provide in vivo evidence that the *Salmonella* CysG enzyme catalyzes the first reaction in B_{12} synthesis.

We have previously presented evidence that B_{12} synthesis was lost by a common ancestor of salmonellae and *E. coli* and was regained by the *Salmonella* lineage through horizontal transfer of the *cob* operon (23). It is likely that the CysG enzyme shared by salmonellae and *E. coli* evolved in a common ancestor that synthesized B_{12} and has not changed significantly since *E. coli* and salmonellae diverged. Thus, the cobalt insertion activity assayed for the *E. coli* enzyme may have been biologically relevant in some common ancestor of modern *E. coli* and salmonellae.

Two different pathways of B_{12} synthesis have been identified, both of which use precorrin-2 as a precursor. In the anaerobic pathway found in *S. typhimurium* and *Propionibacterium shermanii*, cobalt insertion occurs as one of the first enzymatic steps (4, 29, 36, 37) and (based on results presented here) is catalyzed by a multifunctional CysG enzyme which also acts as a UroIII methyl transferase to produce precorrin-2. In contrast, cobalt insertion occurs late in the aerobic pathway found in *Pseudomonas denitrificans* (13, 41) and is catalyzed by several proteins distinct from the UroIII methyl transferase. The UroIII methyl transferase of *P. denitrificans* (late cobalt insertion) is considerably shorter than that of *S. typhimurium* (early cobalt insertion), lacking 256 amino acids at the N-terminal end compared to the *E. coli*/*S. typhimurium* enzyme. The extra region of the *E. coli*/*S. typhimurium* enzyme is inferred to be responsible for siroheme synthesis, ring oxidation, and iron insertion (43) and, based on the presented results, for cobalt insertion in B_{12} synthesis by *S. typhimurium*. The UroIII methylase from *P. shermanii* (early cobalt insertion) also corresponds only to the C-terminal region of the *Salmonella* CysG enzyme (35), suggesting that it performs only the methyl transfer reactions. Thus, in this organism, early cobalt insertion may be catalyzed by a polypeptide distinct from the UroIII methylase.

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