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A Role for *Salmonella typhimurium cbiK* in Cobalamin (Vitamin B_{12}) and Siroheme Biosynthesis

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The role of *cbiK*, a gene found encoded within the *Salmonella typhimurium cob* operon, has been investigated by studying its in vivo function in *Escherichia coli*. First, it was found that *cbiK* is not required for cobalamin biosynthesis in the presence of a genomic *cysG* gene (encoding siroheme synthase) background. Second, in the absence of a genomic *cysG* gene, cobalamin biosynthesis in *E. coli* was found to be dependent upon the presence of $cobA_{P.\ denitrificans}$ (encoding the uroporphyrinogen III methyltransferase from *Pseudomonas denitrificans*) and *cbiK*. Third, complementation of the cysteine auxotrophy of the *E. coli cysG* deletion strain 302Δa could be attained by the combined presence of $cobA_{P.\ denitrificans}$ and the *S. typhimurium cbiK* gene. Collectively these results suggest that CbiK can function in fashion analogous to that of the N-terminal domain of CysG ($CysG^B$), which catalyzes the final two steps in siroheme synthesis, i.e., NAD-dependent dehydrogenation of precorrin-2 to sirohydrochlorin and ferrochelation. Thus, phenotypically $CysG^B$ and CbiK have very similar properties in vivo, although the two proteins do not have any sequence similarity. In comparison to CysG, CbiK appears to have a greater affinity for Co^{2+} than for Fe^{2+}, and it is likely that *cbiK* encodes an enzyme whose primary role is that of a cobalt chelatase in corrin biosynthesis.

Although cobalamins belong to the same prosthetic group family as other modified tetrapyrroles such as hemes, sirohemes, and chlorophylls (44), their increased structural complexity requires a greater biosynthetic effort, and thus the pathway for cobalamin biosynthesis involves upwards of 30 enzyme-catalyzed steps (2, 7, 16, 31, 35). From a mechanistic point of view, the most intriguing part of the pathway is the transformation of uroporphyrinogen III (uro'gen III) into cobyric acid, a molecular metamorphosis which had eluded, until very recently, the most ardent of investigators. Now the individual steps of this part of the pathway have been elucidated (3–5, 7, 11–13, 15, 16, 27, 38–43, 47) for the ambiguously named *Pseudomonas denitrificans* (10, 17), an aerobic soil bacterium which has been adapted for commercial vitamin B_{12} production (18).

In *P. denitrificans*, the transformation of uro'gen III into cobyric acid requires the concerted action of 13 enzymes (7, 16) (Fig. 1). The cobalamin biosynthesis route in *Propionibacterium freudenreichii* (*shermanii*) and *Salmonella typhimurium* (31) appears to be somewhat different from that elucidated for *P. denitrificans* (6, 7, 36). Biochemical investigations have shown that cobalamin biosynthesis in *S. typhimurium* occurs under anaerobic conditions (25), and cobalt appears to be inserted at an early stage in the pathway, at the level of precorrin-2 or -3 (30). These biosynthetic investigations are supported by the fact that *S. typhimurium* does not have proteins equivalent to *P. denitrificans* CobG, CobF, or CobN, -S, and -T (11–13, 31), the enzymes identified in oxidation, deacylation, and chelation, respectively. In fact, a direct comparison of the known *P. denitrificans* and *S. typhimurium* cobalamin biosynthetic genes shows that although there are many similar enzymes, there are also important and definite differences (30, 31). Thus, seven *S. typhimurium cbi* (cobinamide biosynthetic) genes (*cbiD*, *-G*, *-K*, *-M*, *-N*, *-O*, and *-Q*) have no equivalent in *P. denitrificans*, and a *P. denitrificans cob* gene (*cobW*) is absent in *S. typhimurium* (30, 31).

Recently a method of studying the function of the *S. typhimurium cbi* genes in *Escherichia coli* has been described (30). This method involved the cloning of the *S. typhimurium cobI* genes into compatible plasmids which were then transformed in *E. coli*, bestowing upon *E. coli* the new ability to biosynthesize cobalamins de novo. As *E. coli* appears not to possess the *cobI* genes (26), the lack of any background biosynthetic activity makes it an appropriate organism in which to study the action of these genes.

CysG has been shown to be essential for cobalamin biosynthesis in *S. typhimurium* (20), and a study of a large number of *S. typhimurium* mutants suggested that mutations which affected siroheme synthesis had a similar detrimental effect on cobalamin synthesis (20). CysG (also called siroheme synthase) is a multifunctional protein involved in the last three steps of siroheme biosynthesis, i.e., the transformation of uro'gen III into siroheme by (i) methylation of uro'gen III at positions 2 and 7, (ii) NAD-dependent dehydrogenation of precorrin-2 to produce sirohydrochlorin, and (iii) ferrochelation (29, 37, 45, 46, 48–50). Thus, CysG is envisaged as consisting of two main catalytic domains, $CysG^B$ and $CysG^A$; the latter, C-terminal, domain is responsible for the transmethylation of uro'gen III into precorrin-2, and the former, N-terminal, domain is responsible for the transformation of precorrin-2 into siroheme. Very recently, it has been shown that $cysG^A$ and $cobA_{P.\ denitrificans}$ are able to sustain cobalamin biosynthesis in *E. coli* in the absence of a genomic *cysG*, thereby suggesting that the CobI pathway requires precorrin-2 as the starting substrate for corrin synthesis (50).

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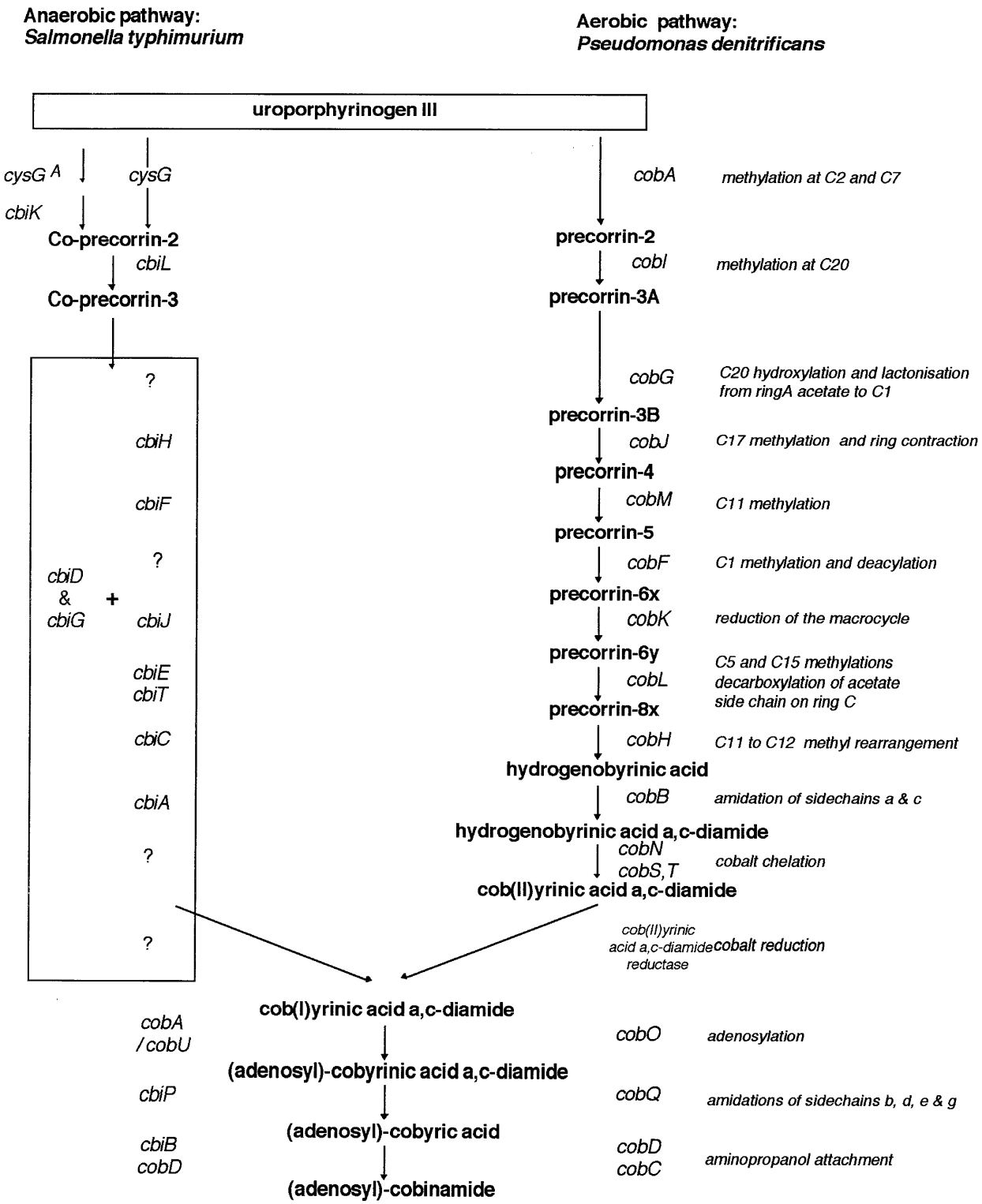

FIG. 1. Reactions and genetic requirements for the transformation of uro'gen III into adenosylcobinamide. The scheme highlights some of the similarities as well as the definitive differences between the pathways that are found in *S. typhimurium* and *P. denitrificans*. The major differences relate to the timing of cobalt insertion and the requirement for molecular oxygen.

Furthermore, it was observed that although $cysG^A$ or $cobA_{P.\ denitrificans}$ was unable to complement the cysteine auxotrophy of the *E. coli cysG* deletion mutant 302Δa, complementation was attained when the strain was further transformed with a plasmid containing the *S. typhimurium cobI* genes (50). The *E. coli cysG* deletion mutant 302Δa grew on minimal medium in the absence of cysteine if it was first transformed either with plasmids carrying the $cobA_{P.\ denitrificans}$ gene

and the *S. typhimurium cobI* genes or with the *E. coli cysG*^{A} and *S. typhimurium cobI* genes.

In this paper we identify the gene as *cbiK* and examine its role in both siroheme and cobalamin biosynthesis.

MATERIALS AND METHODS

Chemicals and reagents. Cyanocobalamin and most other chemicals were purchased from Sigma. Molecular biology reagents were purchased from Promega.

Media, growth conditions, and cobyric acid bioassays. All strains and plasmids used in this research are described in Table 1. Strains were routinely grown in Luria-Bertani broth or agar at 37°C. As necessary, antibiotics were added to final concentrations of 100 mg/liter for ampicillin and 35 mg/liter for chloramphenicol. For cobalamin production, strains were grown in minimal medium (NaCl, 0.5g/liter; Na_2HPO_4, 6 g/liter; NH_4Cl, 1 g/liter; glucose, 4 g/liter; $MgSO_4$, 2mM; $CaCl_2$, 0.1 mM) supplemented with 0.1 g of yeast extract per liter, 4.2 μM $CoCl_2$, antibiotics, and, if necessary, cysteine at 50 mg/liter. The culture media were inoculated with a bacterial suspension from Luria-Bertani plates and incubated for about 3 h at 37°C, at which stage 5-aminolevulinic acid (ALA) at 10 mg/liter and IPTG (isopropyl-β-D-thiogalactopyranoside) at 0.5 mM were added, and the culture was incubated for a further 20 h at 37°C. A layer of paraffin oil was placed on the top of the culture to produce anaerobic conditions. For cobalamin production by strains derived from LE *met*$^{+}$, conditions described previously (30) were used.

The cobalamin assays were tested with the indicator strain *S. typhimurium* AR3612 (*metE cysG*) as previously described (30). A standard curve with known quantities of cyanocobalamin was produced for each set of readings.

Construction of a mutation and a deletion in the *cbiK* gene of pAR8827. DNA techniques were performed as described previously (32). Two *cbiK* variants were derived from pAR8827 (containing the *S. typhimurium* genes *cbiA-C-D-E-T-F-G-H-J-K-L-M-N-Q-O-P*) as follows. A mutation was introduced into *cbiK* by restriction of one of the *Hin*dIII sites within *cbiK* on pAR8827 after a limited digestion with the restriction endonuclease. The overhangs were filled by the action of the Klenow fragment of DNA polymerase I and ligated with T4 DNA ligase. This produced pER125K^{M}, which contains an extra 4 bp introduced in the *cbiK* gene, and leads to a frameshift resulting in the reading of a stop codon at position 10046.

The second *cbiK* variant of pAR8827 was obtained from a digest of the two *Hin*dIII sites within *cbiK* at positions 10028 and 10082. After the restriction, the overhangs were filled in with the Klenow fragment and the ends were ligated with T4 ligase. The deletion of 54 bp in *cbiK* also introduced a frameshift such that the stop codon at position 10085 became in frame. This results in the translation of a truncated version of CbiK which contains 68 amino acids. The pAR8827 derivative with the *cbiK* deletion was called pER126K$^{\Delta}$.

Both variants of pAR8827, containing the modified *cbiK*, were confirmed by restriction analysis of the respective plasmids.

Complementation studies of the *E. coli cysG* deletion mutant 302Δa. Plasmids of interest were transformed into the *E. coli cysG* deletion mutant 302Δa. Complementation of the auxotroph was ascertained by growth of the transformed strain on a minimal medium plate and on a plate with cobalt chloride (up to a final concentration of 5 μM). The growth was compared to that on a control plate that had been supplemented with cysteine (50 mg/liter). All plates were incubated at 37°C for 24 to 30 h.

For growth curves, minimal medium was also used, and, as indicated, cobalt chloride was added to give a final concentration of 5 μM at the start of the exponential phase of growth.

UV-visible spectrophotometry. The tetrapyrrole-derived material which accumulated within the various bacterial strains was analyzed by UV-visible spectroscopy. The accumulated material was extracted from sonicated cell extracts by binding the chromophore onto DEAE resin. The resin was washed with 0.05 M Tris-HCl (pH 8.0), and the material was eluted from the resin by application of a solution of 1.0 M NaCl in the same buffer. The spectrum of the eluted material was recorded in a Hewlett-Packard 8452A diode array spectrophotometer over the range of 300 to 700 nm.

RESULTS

Mutation and deletion of *cbiK*. To try to elucidate the role of *cbiK* in cobalamin biosynthesis, two separate mutant variants of *cbiK* were constructed within pAR8827 (*cbiA-C-D-E-T-F-G-H-J-K-L-M-N-Q-O-P*). Initially, *cbiK*^{M} was produced by mutagenesis of one of the *Hin*dIII sites within *cbiK*, such that the mutant gene translated into a truncated 55-amino-acid version of CbiK. This version of CbiK was contained within plasmid pER125K^{M} (i.e., *cbiA-C-D-E-T-F-G-H-J-K*^{M}*-L-M-N-Q-O-P*). Second, *cbiK*$^{\Delta}$ was produced by deletion of the *Hin*dIII fragment within *cbiK*, giving rise to a shortened gene which encoded a further truncated version of CbiK, and was contained within pER126K$^{\Delta}$ (*cbiA-C-D-E-T-F-G-H-J-K*$^{\Delta}$*-L-M-N-Q-O-P*).

Deletion or mutation of *cbiK* has no effect on corrin biosynthesis in *E. coli* in a genomic *cysG*$^{+}$ background. The effect of the alterations in *cbiK* on cobyric acid biosynthesis in *E. coli* was investigated by transforming the appropriate plasmids into *E. coli* LE392. Transformation of strain LE392 with pER125K^{M} (containing the *S. typhimurium* genes *cbiA-C-D-E-T-F-G-H-J-K*^{M}*-L-M-N-Q-O-P*) gave strain ER125K^{M}. This strain was found to produce similar levels of cobyric acid (around 500 pmol/unit of optical density at 600 nm [OD_{600}] of bacteria) under inducing and noninducing conditions (i.e. in the presence and absence, respectively, of IPTG) (Table 2). This is akin to the amount of cobyric acid observed in AR8959 (LE392 transformed with pAR8827), which produced between 200 and 400 pmol/OD_{600} unit (30). Transformation of strain ER125K^{M} with a second, compatible, plasmid harboring the *cbiK* gene (pAR8580) gave ER125K^{M}K, and this strain produced comparable levels of cobyric acid under inducing and noninducing conditions (400 to 600 pmol/OD_{600} unit) (Table 2). When ER125K^{M} was transformed with a compatible plasmid containing the complete *cysG* gene of *E. coli* (pER108), giving ER125K^{M}G, cobyric acid levels under noninduced conditions were similar to those observed in ER125K^{M} and ER125K^{M}K but were significantly higher under inducing conditions for the *cysG* gene. The increased levels observed under inducing conditions may be due to the effect of increased *cysG* expression on corrin biosynthesis, as has been noticed before with AR8830 (LE392 transformed with pAR8827 and pAR8764), a result which has been attributed to an elevated level of precorrin-2 synthesis (30).

The effect of the *cbiK* deletion (*cbiK*$^{\Delta}$) was also investigated, and transformation of LE392 with pER126K$^{\Delta}$ (containing *S. typhimurium cbiA-C-D-E-T-F-G-H-J-K*$^{\Delta}$*-L-M-N-Q-O-P*) produced strain ER126K$^{\Delta}$. This strain made levels of cobyric acid matching those observed in ER125K^{M} under both inducing and noninducing conditions. Transformation of ER126K$^{\Delta}$ with a compatible plasmid containing *cbiK* (pAR8580) gave rise to ER126K$^{\Delta}$K, which, like ER125K^{M}K, produced similar cobyric acid levels under inducing and noninducing conditions. Transformation of ER126K$^{\Delta}$ with a plasmid containing *cysG* (pER108) gave ER126K$^{\Delta}$G, which had elevated cobyric acid levels under both inducing and noninducing conditions.

Cysteine biosynthesis in *E. coli* without *cysG*^{B}. The *E. coli cysG* deletion mutant 302Δa is unable to grow on minimal medium plates, as it cannot assimilate sulfide because the cells cannot make siroheme for the prosthetic group of sulfite reductase (21). Likewise, the cells cannot grow on minimal medium when transformed with *cysG*^{A} or *cobA*$_{P.\ denitrificans}$, as these genes encode for uro'gen methyltransferases which generate precorrin-2 rather than siroheme. The strain is, however, complemented by a complete *cysG* gene, as the CysG protein is a multifunctional protein and is able to perform all three enzymatic activities required to transform uro'gen III into siroheme, viz., methyl transfer, oxidation, and ferrochelation.

The role of the CysG protein in cobalamin biosynthesis was investigated by studying cobalamin biosynthesis in the *E. coli cysG* deletion mutant 302Δa (cells previously shown to be deficient in all functions of siroheme synthase [21, 48]). Curiously, when *E. coli* 302Δa cells were transformed with pAR8827 (*cbiA-C-D-E-T-F-G-H-J-K-L-M-N-Q-O-P*) together with a compatible plasmid containing the uro'gen methylase gene from *P. denitrificans* (*cobA*$_{P.\ denitrificans}$) (pER119) or *E. coli cysG*^{A} (data not shown), complementation of the cysteine auxotrophy was observed (Table 2); that is, the cells grew

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype	Description[a]	Reference
Strains			
AR3730	LE392 rendered *met*$^+$		30
302Δa	*E. coli cysG*; Nir^s Lac$^+$ CysG$^-$		21
ER123A	*E. coli cysG*(pAR8827)(pER119)	Contains all *S. typhimurium cbi* genes and *P. denitrificans cobA*	This work
ER125K^M	LE *met*$^+$(pER125K^M)	Contains all *S. typhimurium cbi* genes but with a mutation in *cbiK*	This work
ER125K^{M}G	LE *met*$^+$(pER125K^M)(pER108)	Contains all *S. typhimurium cbi* genes and *E. coli cysG* but with a mutation in *cbiK*	This work
ER125K^{M}K	LE *met*$^+$(pER125K^M)(pAR8580)	Contains all *S. typhimurium cbi* genes together with a mutant and normal copy of *S. typhimurium cbiK*	This work
ER125K^{M}p	LE *met*$^+$(pER125K^M)(pKK223.3)	Contains all *S. typhimurium cbi* genes but with a mutation in *cbiK*	This work
ER126K^Δ	LE *met*$^+$(pER126K^Δ)	Contains all *S. typhimurium cbi* genes but with a deletion in *cbiK*	This work
ER126K^{Δ}G	LE *met*$^+$(pER126K^Δ)(pER108)	Contains all *S. typhimurium cbi* genes and *E. coli cysG* but with a deletion in *cbiK*	This work
ER126K^{Δ}K	LE *met*$^+$(pER126K^Δ)(pAR8580)	Contains all *S. typhimurium cbi* genes together with a deleted and normal version of *S. typhimurium cbiK*	This work
ER126K^{Δ}p	LE *met*$^+$(pER126K^Δ)(pKK223.3)	Contains all *S. typhimurium cbi* genes but with a deletion in *cbiK*	This work
ER157K^M	*E. coli cysG*(pER125K^M)	Contains all *S. typhimurium cbi* genes but with a mutation in *cbiK*	This work
ER157K^{M}A	*E. coli cysG*(pER125K^M)(pER119)	Contains all *S. typhimurium cbi* genes and *P. denitrificans cobA* but with a mutation in *cbiK*	This work
ER157K^{M}AK	*E. coli cysG*(pER125K^M)(pER170)	Contains all *S. typhimurium cbi* genes and *P. denitrificans cobA* together with a mutant and normal copy of *S. typhimurium cbiK*	This work
ER157K^{M}G	*E. coli cysG*(pER125K^M)(pER108)	Contains all *S. typhimurium cbi* genes and *E. coli cysG* but with a mutation in *cbiK*	This work
ER172	*E. coli cysG*(pAR8086)(pER119)	Contains *P. denitrificans cobA*	This work
ER173	*E. coli cysG*(pAR8086)(pER170)	Contains *P. denitrificans cobA* and *S. typhimurium cbiK*	This work
ER182	*E. coli cysG*(pAR8086)(pER108)	Contains *E. coli cysG*	This work
ER185K^Δ	*E. coli cysG*(pER126K^Δ)	Contains all *S. typhimurium cbi* genes but with a deletion in *cbiK*	This work
ER185K^{Δ}A	*E. coli cysG*(pER126K^Δ)(pER119)	Contains all *S. typhimurium cbi* genes and *P. denitrificans cobA* but with a deletion in *cbiK*	This work
ER185K^{Δ}AK	*E. coli cysG*(pER126K^Δ)(pER170)	Contains all *S. typhimurium cbi* genes and *P. denitrificans cobA* together with a deleted and normal copy of *S. typhimurium cbiK*	This work
ER185K^{Δ}G	*E. coli cysG*(pER126K^Δ)(pER108)	Contains all *S. typhimurium cbi* genes and *E. coli cysG* but with a deletion in *cbiK*	This work
Plasmids			
pKK223.3		Overexpression vector derived from pBR322, with *tac* promoter	9
pAR8086	*lacI*^{q}	pACYC184 derived	30
pAR8580	*S. typhimurium cbiK*	pKK223.3 derived	30
pAR8827	*S. typhimurium cbiA-C-D-E-T-F-G-H-J-K-L-M-N-Q-O-P*	pACYC184 derived	30
pCR395	*P. denitrificans cobA*		33
pER108	*E. coli cysG*	Variant of pAR8414	30
pER119	*P. denitrificans cobA*	*Eco*RI-*Pst*I fragment of pCR395 cloned into pKK223.3	This work
pER125K^M	*S. typhimurium cbiA-C-D-E-T-F-G-H-J-K^M-L-M-N-Q-O-P*	Mutation at the *Hin*dIII 10028 site into *cbiK* gene of pAR8827 (TGA at position 10046)	This work
pER126K^Δ	*S. typhimurium cbiA-C-D-E-T-F-G-H-J-K^Δ-L-M-N-Q-O-P*	Deletion of 54 bp between *Hin*dIII sites (10028–10082) in *cbiK* gene of pAR8827 (TAA at position 10085)	This work
pER170	*P. denitrificans cobA* and *S. typhimurium cbiK*	*Eco*RI-*Sca*I fragment of pAR8580 cloned into pER119 cut partially by *Sal*I and *Sca*I	This work

[a] "All *S. typhimurium cbi* genes" means *cbiA-C-D-E-T-F-G-H-J-K-L-M-N-Q-O-P* and does not include *cbiB*.

TABLE 2. Cobyric acid synthesis in some of the strains used in this study and ability of plasmids to complement *E. coli cysG* deletion mutant 302Δa[a]

Strain	Parent strain	Gene in which plasmid pAR8827 carries mutation	Gene(s) in the second plasmid	Cobyric acid (pmol/OD) under:		Growth on:		
				Noninducing conditions	Inducing conditions	M9	M9 + $CoCl_2$	M9 + cysteine
ER125K^{M}p	LE *met*$^+$	*cbiK*^{M}		525	563			
ER125K^{M}K	LE *met*$^+$	*cbiK*^{M}	*cbiK*	563	394			
ER125K^{M}G	LE *met*$^+$	*cbiK*^{M}	*cysG*	600	>1,000			
ER126K$^\Delta$p	LE *met*$^+$	*cbiK*$^\Delta$		588	500			
ER126K$^\Delta$K	LE *met*$^+$	*cbiK*$^\Delta$	*cbiK*	650	475			
ER126K$^\Delta$G	LE *met*$^+$	*cbiK*$^\Delta$	*cysG*	988	>1,000			
ER157K^{M}A	302Δa (*cysG*)	*cbiK*^{M}	*cobA*	8	0.1	−	−	+++
ER157K^{M}AK	302Δa (*cysG*)	*cbiK*^{M}	*cobA*, *cbiK*	3	431	++	−	+++
ER157K^{M}G	302Δa (*cysG*)	*cbiK*^{M}	*cysG*	607	663	+++	++	+++
ER185K$^\Delta$A	302Δa (*cysG*)	*cbiK*$^\Delta$	*cobA*	0	0	−	−	+++
ER185K$^\Delta$AK	302Δa (*cysG*)	*cbiK*$^\Delta$	*cobA*, *cbiK*	14	482	++	−	+++
ER185K$^\Delta$G	302Δa (*cysG*)	*cbiK*$^\Delta$	*cysG*	536	544	+++	++	+++
ER123A	302Δa (*cysG*)	Normal	*cobA*	208	548	++	−	+++

[a] *cobA* is from *P. denitrificans*. Plasmid pAR8827 contains *cbiACDETFGHJKLMNQOP* of *S. typhimurium*. Cobalt was used at 4.2 or 5 μM. −, no growth; +, slow growth; +++, normal growth.

normally on minimal media in the absence of exogenous cysteine. This suggested that a protein encoded within the *cobI* region was capable of undertaking the oxidation and ferrochelation of precorrin-2.

To try to identify the responsible gene within the *cobI* region, the complementation experiment was repeated with a number of derivatives of pAR8827 which had had individual genes inactivated by either mutagenesis or deletion. When this systematic search was completed (data not shown), it was found that only plasmids with the modified *cbiK* constructs, pER125K^M and pER126K$^\Delta$, failed to complement the *cysG* strain in the presence of an active $cobA_{P.\ denitrificans}$ gene (pER119) (Table 2). Thus, when the *E. coli cysG* deletion mutant (302Δa) harboring pER119 ($cobA_{P.\ denitrificans}$) was further transformed with pER125K^M or pER126K$^\Delta$, it failed to grow on minimal medium plates. Conversely, when the *cysG* deletion mutant (302Δa) was transformed with a plasmid containing both $cobA_{P.\ denitrificans}$ and *cbiK* alone (pER170), complementation was observed (Table 3). These two results clearly identify CbiK as the protein conferring the CysG^B-like properties.

Effect of cobalt and other exogenous metals on complementation. The foregoing result suggested that CbiK was able to perform ferrochelation. To investigate whether CbiK had an affinity for other divalent metal ions, the effect of exogenous metals on the ability of *cbiK* to complement the *E. coli cysG* deletion mutant (302Δa) was studied. It was observed that the presence of exogenous Co^{2+} had a detrimental effect on growth of ER157K^{M}AK, ER185K$^\Delta$AK, and ER173 in the absence of cysteine, in that complementation did not occur if Co^{2+} was added to the minimal medium at concentrations above 0.5 μM (Tables 2 and 3). Growth curves for ER173, which is *E. coli* 302Δa (*cysG*) transformed with pER170 ($cobA_{P.\ denitrificans}$ and *cbiK*), gave a similar result in that growth of the culture was retarded when exogenous Co^{2+} (5 μM) was added (Fig. 2). The control experiment in which the *cysG* deletion mutant was transformed with only a plasmid containing the *cysG*$^+$ gene (pER108) did not show the same growth retardation in the presence of exogenous Co^{2+} (Fig. 2).

Other metals, such as Ni^{2+}, Fe^{3+}, Zn^{2+}, and Ba^{2+}, did not affect complementation (data not shown). Previous work on the cobalt chelatase in *P. denitrificans*, the CobN-S-T complex, has shown that the enzyme is inhibited by Ni^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+} (15). The significance of the Co^{2+} inhibition of complementation is that it suggests that, in comparison to CysG, CbiK has a preference for Co^{2+} over Fe^{2+}.

Effect of the *cbiK* mutation and deletion on cobalamin biosynthesis in a *cysG* deletion background. In a *cysG*$^+$ strain (LE392), we had observed that the mutation and deletion in the *cbiK* gene had little effect on cobyric acid synthesis (see above). However, when the experiments were performed with a *cysG* deletion mutant (302Δa) with a plasmid-borne $cobA_{P.\ denitrificans}$ gene, the *cbiK* mutations showed a strong phenotype. Thus, transformation of *E. coli* 302Δa with pER125K^M and pER119 ($cobA_{P.\ denitrificans}$) gave strain ER157K^{M}A, which produced barely detectable levels of cobyric acid (less than 10 pmol/OD_{600} unit) (Table 2). A similar observation was made when the *cysG* deletion mutant (302Δa)

TABLE 3. Growth of *E. coli* 302Δa (*cysG* deletion mutant) with plasmids containing combinations of *cobA*, *cbiK*, and *cysG* with different exogenous cobalt concentrations

Strain[a]	Gene(s) on plasmid (plasmid)	Growth on minimal medium with[b]:					
		No. addition	$CoCl_2$ at:				Cysteine
			0.005 μM	0.05 μM	0.5 μM	5 μM	
ER172	*cobA* (pER119)	−	−	−	−	−	+++
ER173	*cobA*, *cbiK* (pER170)	++	++	+	−	−	+++
ER182	*cysG* (pER108)	+++	+++	+++	+++	++	+++

[a] The parent strain was 302Δa.
[b] −, no growth; +, slow growth; +++, normal growth.

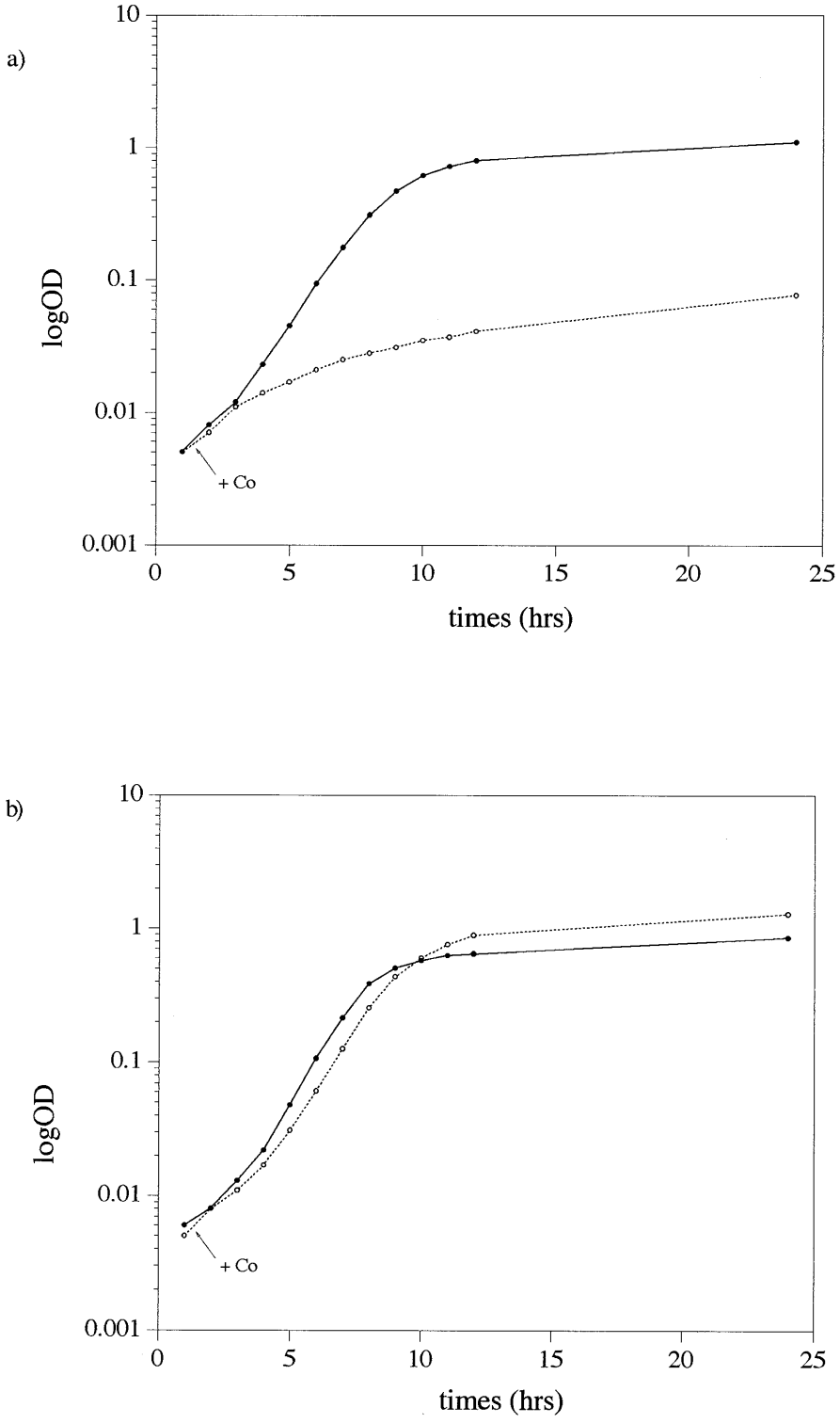

FIG. 2. Effect of cobalt on the growth of ER173 (*E. coli cysG* deletion mutant 302Δa overexpressing *P. dentitrificans cobA* and *S. typhimurium cbiK*) (a) and ER182 (302Δa overexpressing *E. coli cysG*) (b) in the presence (○) and absence (●) of exogenous cobalt. Cobalt was added to the cultures at a concentration of 5 μM at the start of the exponential phase.

was transformed with plasmids pER126K$^{\Delta}$ and pER119, giving strain ER185K$^{\Delta}$A; in this strain no cobyric acid synthesis could be detected (Table 2). In comparison, transformation of the *cysG* deletion mutant (302Δa) with plasmids pAR8827 and pER119 gave strain ER123A, which produced levels of cobyric acid very similar to those observed in the LE392 host (600 pmol/OD_{600} unit) (Table 2).

The inability to produce cobyric acid could be rectified in strains ER157K^{M}A and ER185K$^{\Delta}$A by transforming the strains further with plasmids containing either *cysG* or *cbiK*.

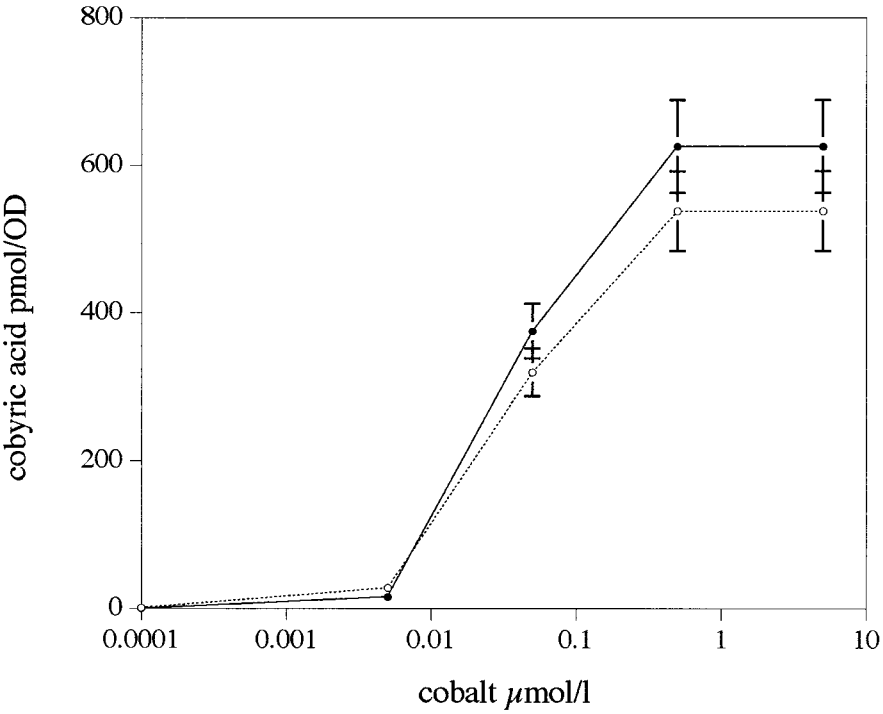

FIG. 3. Effect of exogenous cobalt on cobyric acid synthesis in ER185K$^{\Delta}$AK and ER185K$^{\Delta}$G. The strains were grown in minimal medium under anaerobic conditions in a range of different cobalt concentrations. The results show that ER185K$^{\Delta}$AK (○) and ER185K$^{\Delta}$G (●) produced very similar levels of cobyric acid under the conditions tested. Error bars indicate standard deviations.

Thus, by replacing the plasmid pER119 (*cobA*$_{P.\ denitrificans}$) in ER157K^{M}A with either pER170 (*cobA*$_{P.\ denitrificans}$ and *cbiK*) or pER108 (*cysG*), giving ER157K^{M}AK and ER157K^{M}G, respectively, the barely detectable levels of cobyric acid found in ER157K^{M}A were greatly increased (Table 2). The increase in cobyric acid levels was observed in strain ER157K^{M}AK only when transcription of the *cobA*$_{P.\ denitrificans}$ and *cbiK* genes was induced by IPTG (Table 2). Similarly, when plasmid pER119 (*cobA*$_{P.\ denitrificans}$) in ER185K$^{\Delta}$A is replaced with either pER170 (*cobA*$_{P.\ denitrificans}$ and *cbiK*) or pER108 (*cysG*), giving strains ER185K$^{\Delta}$AK and ER185K$^{\Delta}$G, respectively, the levels of cobyric acid are greatly increased, to levels found when LE392 was used as a host (Table 2). As with ER157K^{M}AK, the levels of cobyric acid are increased in ER185K$^{\Delta}$AK only when the strain is induced with IPTG; the inducer requirement probably reflects poor expression of *cobA*$_{P.\ denitrificans}$ and *cbiK* in pER170.

Effect of cobalt on cobyric acid synthesis in *cysG*- and *cbiK*-dependent strains. To try to see whether any difference between the requirement for CbiK or CysG with respect to cobyric acid synthesis could be observed, the effect of the exogenous Co^{2+} concentration on cobyric acid synthesis in the *cbiK*-dependent strain ER185K$^{\Delta}$AK and the *cysG*-dependent strain ER185K$^{\Delta}$G was investigated. A range of Co^{2+} concentrations from 0 to 5 μM was investigated. The results, shown in Fig. 3, suggest that there is no difference in vivo between the *cysG* and *cbiK* strains in regard to the effect of Co^{2+} on cobyric acid synthesis, as both strains produce similar levels of cobyric acid at the same exogenous Co^{2+} concentration.

UV-visible spectra of cell extracts from strains overexpressing CobA and both CobA and CbiK. The cell extracts of ER172 (CobA$_{P.\ denitrificans}$), ER173 (CobA$_{P.\ denitrificans}$ plus CbiK), and ER182 (CysG) after growth on media supplemented with ALA (10 mg/liter) and Co^{2+} (5 μM) were analyzed by UV-visible spectroscopy. The accumulated tetrapyrrole-derived material was extracted from the cell lysate by binding it onto an ion-exchange resin as described in Materials and Methods. The material from ER172 gave a spectrum that had an absorption maximum at 354 nm (Fig. 4a) and was indistinguishable from the spectrum of the trimethylpyrrocorphin, which has been reported to be a nonphysiological product of the CysG reaction (34, 46). The spectrum of the material isolated from ER182 (CysG) gave a spectrum with absorption maxima at 413 and 590 nm (Fig. 4b), which is consistent with the presence of cobalt isobacteriochlorin (cobalt sirohydrochlorin). There was no evidence of any overmethylated material. The spectrum from ER173 was consistent with the presence of both the trimethylpyrrocorphin and the cobalt isobacteriochlorin, as absorption maxima at 354, 413, and 590 nm were observed (Fig. 4c).

DISCUSSION

Previously we have described the construction of pAR8827 (*cbiA-C-D-E-T-F-G-H-J-K-L-M-N-Q-O-P*), which is a plasmid that contains all of the *S. typhimurium cbi* genes required to convert precorrin-2 into cobyric acid. When transformed into *E. coli* LE392, pAR8827 was found to endow the host bacterium (AR8959) with the ability to synthesize cobyric acid (30). By using this approach and a series of similar plasmids, it was possible to confirm the roles of a number of *cbi* genes within the *S. typhimurium cob* operon, including *cbiA*, *-B*, *-C*, *-D*, *-E*, *-F*, *-G*, *-J*, and *-P*. However, the functional status of *cbiK* as a *cobI* gene, a gene required for the transformation of precorrin-2 into cobinamide, was not investigated in those studies. The *cbiK* gene encodes a protein of 264 amino acids with no known function and does not have a *P. denitrificans* homolog. Thus, to determine whether *cbiK* belongs to the CobI path, two

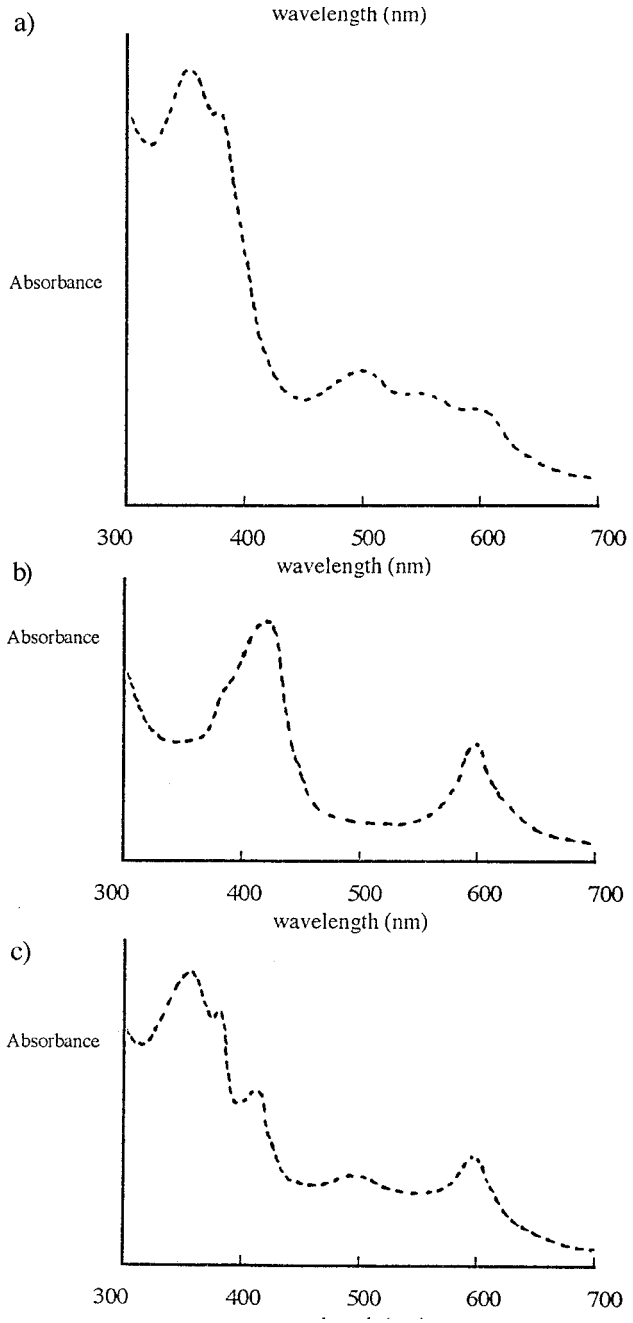

FIG. 4. UV-visible spectra of the accumulated chromophoric material isolated from lysates of ER172, an *E. coli cysG* deletion mutant which overexpresses the *P. denitrificans cobA* gene (a); ER182, an *E. coli cysG* deletion mutant which overexpresses the *E. coli cysG* gene (b); and ER173, an *E. coli cysG* deletion mutant which overexpresses the *P. denitrificans cobA* and the *S. typhimurium cbiK* genes (c). The strains were grown anaerobically on minimal medium supplemented with ALA and Co^{2+}.

mutant variants of *cbiK* were made and were termed $cbiK^{M}$ and $cbiK^{\Delta}$.

With the two mutant variants in hand, the effect of the mutations on cobalamin synthesis was investigated in *E. coli*. Mutants of *E. coli* LE392 which contained either $cbiK^{M}$ or $cbiK^{\Delta}$ together with *cbiA-C-D-E-T-F-G-H-J-L-M-N-Q-O-P* were grown, and the amount of cobyric acid produced by the cells was determined by bioassay. The conclusion from these studies was that mutations in *cbiK* had no detrimental effect on corrin synthesis in *E. coli*. Likewise, the addition of a normal *cbiK* gene in the strains to compensate for the presence of the mutated version had little effect on corrin synthesis, and equally, the presence of a plasmid encoding *cysG* had no net effect on cobalamin biosynthesis in comparison to that of the appropriate control strains. Thus, on this basis, in a $cysG^{+}$ background, *cbiK* does not appear to be a *cobI* gene, as it is not essential for cobalamin biosynthesis in *E. coli*. These results do not preclude the possibility that *E. coli* contains a genomic *cbiK* gene, although there is no evidence that *E. coli* contains any of the *cobI* genes (26).

The cysteine auxotrophy of the *E. coli* deletion mutant 302Δa can be overcome by transformation of the strain with plasmids pAR8827 (*cbiA-C-D-E-T-F-G-H-J-K-L-M-N-Q-O-P*) and pER119 ($cobA_{P.\ denitrificans}$) (50). This indicated that the product of a gene within the *cobI* operon was able to mimic $CysG^{B}$-like activities, that is, dehydrogenation and ferrochelation. To identify the gene responsible for these properties, a systematic screen of the effects of mutant variants of the *cobI* genes within pAR8827 on this complementation was undertaken. Rather surprisingly, we found that *cbiK* was the gene responsible. Thus, it was possible to clearly demonstrate that whereas a plasmid combination of pAR8827 (all of the *cobI* genes except *cbiB*) and pER119 ($cobA_{P.\ denitrificans}$) was able to complement 302Δa, combinations of $pER125K^{M}$ and pER119, and $pER126K^{\Delta}$ plus pER119, were unable to overcome the cysteine requirement of the host strain. In fact, complementation of the *E. coli cysG* deletion mutant 302Δa can be attained with a plasmid containing only $cobA_{P.\ denitrificans}$ and *cbiK*.

Although the plasmids with the combinations of $cobA_{P.\ denitrificans}$ and *cbiK* were able to complement *E. coli* 302Δa, the complementation was overcome by the addition of exogenous Co^{2+} to the minimal medium plates or to growing cultures. This suggests that the ferrochelation by CbiK is a surrogate reaction which is undertaken only in the absence of Co^{2+}. A plausible explanation for these observations is that CbiK is a cobalt chelatase of cobalamin biosynthesis but that in the absence of Co^{2+} it can act as a ferrochelatase in siroheme synthesis (substituting for $CysG^{B}$). Whether the ferrochelatase is effective under normal growth conditions in *S. typhimurium* remains to be established.

The chelatases responsible for heme, siroheme, bacteriochlorphyll, chlorophyll, and cobalamin synthesis have been largely identified. Ferrochelatase is the enzyme responsible for the synthesis of protoheme from protoporphyrin. Generally this enzyme has been found to have a molecular mass of between 35,000 and 45,000 Da (for a review, see reference 14). The ferrochelatase activity associated with siroheme synthesis was found to be resident within CysG, in the $CysG^{B}$ domain (31, 48). There is no similarity between heme ferrochelatase and CysG. The $CysG^{B}$ domain is composed of 202 amino acids and has a molecular mass of 22,000 Da (48). Magnesium chelation in bacteriochlorophyll synthesis is accomplished by the combined action of BchI, BchD, and BchH, which are able to convert protoporphyrin into Mg-protoporphyrin in an ATP-dependent process (8, 19). A similar complex is probably involved with magnesium chelation in chlorophyll synthesis (24). Finally, cobalt chelation in cobalamin biosynthesis in the aerobic bacterium *P. denitrificans* is accomplished by a complex formed between CobN, -S, and -T (15). As with magnesium chelation, ATP is required in the process. Similarity was found to exist between the primary structures of CobN and BchH, both of which contain around 1,200 amino acids with a subunit molecular mass of 130,000 Da, suggesting that magnesium

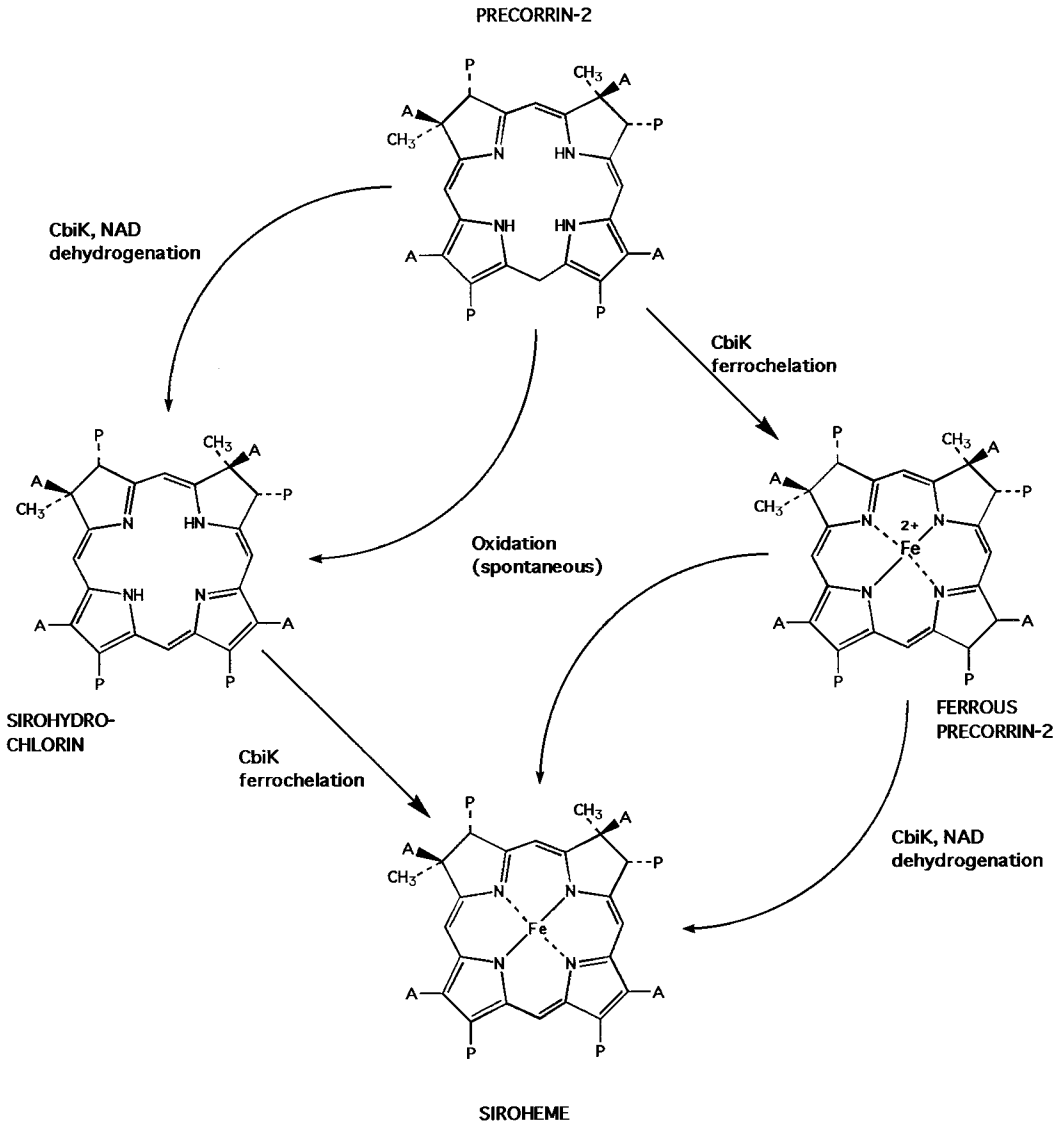

FIG. 5. Conceivable reactions for the transformation of precorrin-2 into siroheme by CbiK. There are a number of possible permutations which would allow the conversion of precorrin-2 into siroheme by CbiK. For instance, the protein could contain both dehydrogenase and chelatase activities, or the enzyme could act solely as a chelatase, with the oxidation of the macrocycle occurring spontaneously.

chelation may have evolved from the primordial cobalamin biosynthetic enzyme (23). Apart from the similarity between CobN and BchH, no other similarity between the chelatases is apparent. CbiK, which contains 264 amino acids and has a molecular mass of 29,000 Da, displays no similarity with any of the aforementioned chelatases.

The finding that *cbiK* was able to substitute for *cysG*^{B} prompted us to look at the production of cobyric acid in a *cysG* deletion background. It was found that when the *E. coli cysG* deletion mutant 302Δa was transformed with pER119 (*cobA*$_{P.\ denitrificans}$) and pAR8827 (*cbiA-C-D-E-T-F-G-H-J-K-L-M-N-Q-O-P*), essentially normal levels of cobyric acid were produced. However, when the plasmids harboring the mutated versions of *cbiK*, i.e., pER157K^{M} and pER185K$^{\Delta}$, were used in place of pAR8827, it was found that either cobyric acid production was greatly reduced or cobyric acid was not produced in measurable quantities. Cobyric acid production could be restored by addition of *cbiK* to the strain, thus demonstrating that the inhibition of cobyric acid synthesis was not due to a polar effect. Cobyric acid production could also be restored by addition of the complete *cysG* gene to the strain. These results clearly show that in a *cysG*^{B} minus background, *cbiK* is an essential *cobI* gene.

A UV-visible spectrum of the accumulated tetrapyrrole-derived material isolated from ER172 was found to be identical to the spectrum of the 2,7,12-trimethylpyrrocorphin. Thus, in vivo at least, it would appear that CobA$_{P.\ denitrificans}$ is able to overmethylate precorrin-2 in a manner similar to that of CysG (34, 46) and *P. freudenreichii* CobA (33). The spectrum of the material isolated from ER182 was found to be very similar to the spectrum of a cobalt isobacteriochlorin (cobalt sirohydrochlorin) (1). It had been reported previously that CysG can form this compound in vitro, although the experimental data was not presented (37). Cobalt sirohydrochlorin is thought to act as the prosthetic group for sulfite reductase in a family of primitive sulfite-reducing organisms, including *Desulfovibrio gi-*

gas and *Desulfovibrio desulfuricans* (22, 28). The spectrum of the material isolated from ER173 is a composite of the spectra obtained from ER172 and ER182; that is, it contains absorbance maxima consistent with the presence of both the trimethylpyrrocorphin and cobalt sirohydrochlorin. This result is in agreement with the role of CbiK as a cobalt chelatase at the level of precorrin-2 or sirohydrochlorin.

In conclusion, the work described in this report has shown that siroheme synthesis can be achieved in the *E. coli cysG* deletion mutant 302Δa by the presence of both $cobA_{P.\ denitrificans}$ (or $cysG^{A}$) and *cbiK*. Furthermore, we have demonstrated that in the presence of a genomic *cysG* background, *cbiK* is not essential for cobalamin biosynthesis. However, *cbiK* does have a role to play in corrin biosynthesis if $cysG^{B}$ is absent. CbiK appears to have a preference for cobalt chelation, as *cbiK*-dependent synthesis of siroheme is effectively inhibited by the presence of exogenous Co^{2+}. This is not the case with *cysG*, which complements the *cysG* cysteine auxotroph even in high concentrations of exogenous Co^{2+}.

CbiK could cause complementation in a number of ways. First, the gene could encode a protein with dehydrogenase and chelatase properties, like those associated with the N terminus of CysG ($CysG^{B}$). Alternatively, CbiK may encode solely a chelatase that ferrochelates sirohydrochlorin, a substrate which may be obtained from spontaneous oxidation of precorrin-2. Finally, CbiK may act as a chelator at the level of precorrin-2, producing ferrous precorrin-2 which may subsequently spontaneously oxidize to form siroheme. These various possibilities are shown in Fig. 5. With an in vivo system it is not possible to discriminate between these possibilities. However, the fact that the siroheme synthesis ability of CbiK can be easily prevented by the presence of low concentrations of exogenous Co^{2+} suggests that the preferred substrate for CbiK may be Co^{2+} rather than Fe^{2+}, implicating it in cobalamin rather than siroheme synthesis.

It has been shown that $CysG^{B}$ has an NAD-dependent dehydrogenase ability, and the protein contains a conserved dinucleotide binding site (48, 49). However, no such binding motif is present on CbiK. In fact, there is no similarity between the proteins at the primary-structure level.

Why does *S. typhimurium* contain both *cbiK* and *cysG*? CbiK appears to be designed to work with a CobA-type uro'gen methylase, and the presence of CysG negates the requirement for *cbiK*. An explanation for this apparent dichotomy may lie with the ideas put forward by Lawrence and Roth (26), who have suggested that the *cob* operon in *S. typhimurium* was reacquired by horizontal transfer. This would explain why *S. typhimurium* contains both CysG and CbiK, as both may have evolved separately prior to eventually ending up in the one organism.

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