JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 753–767 Vol. 178, No. 3
0021-9193/96/$04.00+0

Salmonella typhimurium Cobalamin (Vitamin B_{12}) Biosynthetic Genes: Functional Studies in *S. typhimurium* and *Escherichia coli*

EVELYNE RAUX,[1]† ANNE LANOIS,[1] FLORENCE LEVILLAYER,[1] MARTIN J. WARREN,[2] EDWARD BRODY,[1] ALAIN RAMBACH,[3] AND CLAUDE THERMES[1]*

Centre de Génétique Moléculaire, Laboratoire associé à l'Université Pierre et Marie Curie, Centre National de la Recherche Scientifique, 91198 Gif sur Yvette,[1] and CHROMagar, 75006 Paris,[3] France, and Department of Molecular Genetics, Institute of Ophthalmology, London EC1V 9EL, United Kingdom[2]

Received 2 March 1995/Accepted 6 November 1995

In order to study the *Salmonella typhimurium* cobalamin biosynthetic pathway, the *S. typhimurium cob* operon was isolated and cloned into *Escherichia coli*. This approach has given the new host of the *cob* operon the ability to make cobalamins de novo, an ability that had probably been lost by this organism. In total, 20 genes of the *S. typhimurium cob* operon have been transferred into *E. coli*, and the resulting recombinant strains have been shown to produce up to 100 times more corrin than the parent *S. typhimurium* strain. These measurements have been performed with a quantitative cobalamin microbiological assay which is detailed in this work. As with *S. typhimurium*, cobalamin synthesis is only observed in the *E. coli* cobalamin-producing strains when they are grown under anaerobic conditions. Derivatives of the cobalamin-producing *E. coli* strains were constructed in which genes of the *cob* operon were inactivated. These strains, together with *S. typhimurium cob* mutants, have permitted the determination of the genes necessary for cobalamin production and classification of *cbiD* and *cbiG* as *cobI* genes. When grown in the absence of endogenous cobalt, the oxidized forms of precorrin-2 and precorrin-3, factor II and factor III, respectively, were found to accumulate in the cytosol of the corrin-producing *E. coli*. Together with the finding that *S. typhimurium cbiL* mutants are not complemented with the homologous *Pseudomonas denitrificans* gene, these results lend further credence to the theory that cobalt is required at an early stage in the biosynthesis of cobalamins in *S. typhimurium*.

The coenzyme form of vitamin B_{12}, either deoxyadenosylcobalamin (50) or methylcobalamin (47), is used as a cofactor in a number of enzyme-catalyzed reactions and is able to donate a large chemical potential upon its protein counterpart by utilizing the central cobalt ion to perform chemical athletic feats (25). Cobalamins are some of the most complex nonpolymeric molecules biosynthesized in the cell and are present in organisms belonging to the three kingdoms eubacteria, archaebacteria, and eucaryotes. Higher plants seem not to need cobalamins, while animals require vitamin B_{12} in their diet. In humans, a deficiency of vitamin B_{12} causes the condition pernicious anemia and is also thought to be a risk factor in neural tube defects (30). It is likely that the ability to biosynthesize the vitamin de novo was present in the ancestor of eubacteria and archaebacteria (26, 28, 55) but was lost in the transition to multicellular organisms.

The complete biosynthesis of adenosyl cobalamin requires about 30 enzymes for a coenzyme required in vanishingly small quantities and with many highly labile intermediates. The structure of the coenzyme forms of cobalamin can be thought of in terms of three major parts (Fig. 1). Firstly, there is the tetrapyrrole-derived corrin ring structure which, on its own, is referred to as cobinamide. This molecule is derived from uroporphyrinogen III by a series of reactions including eight *S*-adenosyl-L-methionine-dependent methylations, ring contraction, cobalt chelation, decarboxylation, amidations, and 1-amino-2-propanol attachment. The genes which encode the enzymes that perform these reactions are referred to as the *cobI* genes. Secondly, there is the dimethylbenzimidazole (DMB) nucleotide which straddles the southern hemisphere of the molecule and forms a loop between the aminopropanol side chain and the central cobalt ion. The genes encoding the enzymes responsible for the biosynthesis of the DMB nucleotide are referred to as the *cobII* genes. Finally, the attachment of the corrin ring to the DMB as well as the addition of the upper coordinating ligand for the cobalt, either an adenosyl or a methyl group, are encoded by the *cobIII* genes (29). *Salmonella typhimurium* mutants unable to make cobalamins are divided into these three classes: the *cobI* mutants are unable to make cobinamide, the *cobII* mutants are unable to make DMB, and finally the *cobIII* mutants are unable to ligate the components.

Among the eubacteria, the ability to make cobalamins does not fall into any obvious pattern. Thus, there are bacteria which require vitamin B_{12} for growth and are able to make it de novo (e.g., *Bradyrhizobium japonicum*), bacteria that require vitamin B_{12} for growth and are apparently unable to make the coenzyme de novo (e.g., *Lactobacillus leichmannii*), and bacteria which neither have an auxotrophic dependency on the vitamin nor make the vitamin (6). Recently, it was discovered that *S. typhimurium* is able to make cobalamin de novo, but only under anaerobic conditions (29). Although it contains some cobalamin-dependent enzymes, there would appear to be no obvious selection pressure on *S. typhimurium* to retain the ability to make cobalamins, as cobalamin mutants live in the absence of the coenzyme; thus, why *S. typhimurium* makes vitamin B_{12} and makes it only under anaerobic conditions is still a conundrum (12). The closely related *Escherichia coli* also contains some cobalamin-dependent enzymes, but it neither synthesizes nor requires exogenous vitamin B_{12} for growth (4, 6, 31). *E. coli* can, however, convert cobinamide into the co-

* Corresponding author. Phone: 69 82 38 28. Fax: 69 82 33 54.
† Present address: Department of Molecular Genetics, Institute of Ophthalmology, London EC1V 9EL, United Kingdom.

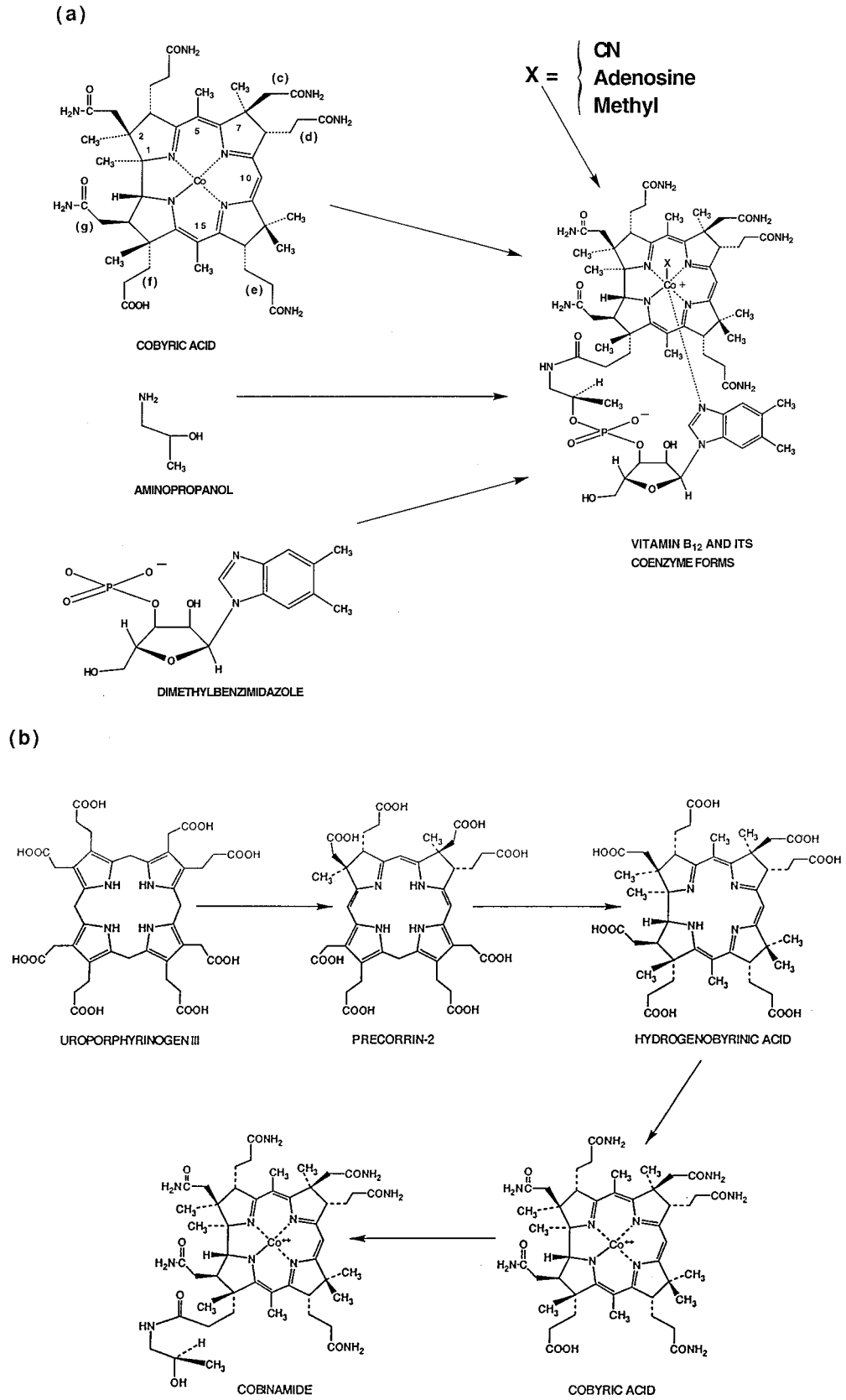

FIG. 1. (a) The structure of vitamin B_{12} and its coenzyme forms can be envisaged as a jigsaw of four major components, cobyric acid, aminopropanol, DMB, and either the cyano, methyl, or adenosyl group. (b) Some of the intermediates formed during the biosynthesis and elaboration of the corrin macrocycle are shown.

balamin forms, and must therefore have the ability to make the DMB nucleotide and to paste the different components together to give the final macromolecule (24, 27). In genetic terms, therefore, *E. coli* possesses the *cobII* and *cobIII* genes (18). However, it does not appear to possess the ability to synthesize the corrin macrocycle and thus to possess the *cobI* genes, and it is likely that this ability has been lost by *E. coli* as a relatively recent evolutionary development.

The biosynthetic pathway for cobalamins also differs between cobalamin-producing bacteria. In the last 15 years, many *Pseudomonas denitrificans cob* genes have been localized at four different loci and have been sequenced. Their functions have been established in many extensive enzymological studies (8–10, 15, 19–23). In *S. typhimurium*, the analogous *cob* genes have also been mapped and sequenced (44), and the *cobI* genes are given the prefix *cbi*. The majority of the *S. typhimurium cob* genes are clustered at 41 min on the chromosome, and they form part of a cobinamide operon with the genes organized in the order *cobI*, *cobIII*, and *cobII* (29). The *P. denitrificans cob* genes and their functions are listed in Table 1 together with the analogous *S. typhimurium cbi* genes. Sequence comparisons with the *P. denitrificans cob* genes have allowed the assignment of functions to 12 of the *S. typhimurium cbi* genes. However, eight genes of the *S. typhimurium* operon have no similar gene in *P. denitrificans* (*cbiD*, *-G*, *-J*, *-K*, *-M*, *-N*, *-O*, and *-Q* [44]) and 10 *P. denitrificans cob* genes have no homolog in *S. typhimurium* (*cobC*, *-E*, *-F*, *-G*, *-K*, *-N*, *-S*, *-T*, and *-W* [44] to which must be added the cob(II)yrinic acid *a*,*c*-diamide reductase gene [10]). However, it seems likely that *cobK* and *cbiJ* are in fact similar (see below). These comparative genome studies, together with observations made with *Propionibacterium shermanii* (5, 34, 35) and other species, suggest that there is more than one pathway for corrin biosynthesis (11, 48). The differences relate to the timing of the cobalt insertion and the method of ring contraction. Thus, in *S. typhimurium* the cobalt may be inserted at an early stage of the biosynthesis, before the synthesis of precorrin-3, as in *Propionibacterium shermanii* (5, 34, 35), rather than at a later stage, after the synthesis of hydrogenobyrinic acid *a*,*c*-diamide, as it has been shown in *P. denitrificans* (22).

In this work, a number of *S. typhimurium cob* mutants are defined by functional complementation. It is also demonstrated that it is possible to confer de novo cobalamin-biosynthesizing ability to *E. coli* by transferring 20 genes of the *S. typhimurium cob* operon. We show that the relevant genes for cobalamin biosynthesis are translated into proteins and that these recombinant strains produce elevated levels of certain corrin intermediates which can be purified and analyzed. Mutants of the model *E. coli* cobalamin producer, in which one or several genes of the *S. typhimurium cob* operon have been deleted or inactivated, allowed the identification of *cob* genes which are necessary for corrin production, including *cbiD* and *cbiG*. Cumulatively, these studies also suggest that the early steps of corrin biosynthesis in *S. typhimurium* are different from those in *P. denitrificans*, with cobalt being chelated at an early stage during the synthesis of precorrin-2 or -3.

MATERIALS AND METHODS

Chemicals and reagents. Cyanocobalamin, dicyanocobinamide, and most other chemicals and reagents were purchased from Sigma. Cobyric acid and cobyrinic acid were prepared by acid hydrolysis of cyanocobalamin (13). Other materials were purchased from manufacturers as follows: restriction enzymes, Appligene; Sequenase T7 DNA polymerase, U.S. Biochemicals; Packagene in vitro packaging system, calf intestinal alkaline phosphatase, and T4 DNA ligase, Promega; pKK223.3 and Klenow fragment of DNA polymerase I, Pharmacia; M13mp18 and M13mp19, Boehringer; pACYC184, New England Biolabs; [^{35}S]L-methionine and ^{35}S-dATP Amersham; ampholytes, Bio-Rad.

Bacterial strains, media, and growth conditions. *S. typhimurium* MS1868 was a gift from T. Elliot (Birmingham, Ala.), and *S. typhimurium* TN2540 was a gift from C. G. Miller (40); all *S. typhimurium* strains mentioned in this report were derived from these two strains (Table 2). All *E. coli* strains containing plasmids carrying fragments of the *cob* operon were derivatives of LE392; their genotypes are listed in Table 2. Strains were routinely grown in Luria-Bertani broth or Luria-Bertani agar at 37°C. As necessary, antibiotics were added to final concentrations as indicated: tetracycline, 15 mg/liter; ampicillin, 100 mg/liter; and chloramphenicol, 30 mg/liter. For cobalamin production, *E. coli* and *S. typhimurium* MS1868-derived strains were grown on minimum medium (NaCl, 0.5 g/liter; Na_2HPO_4, 6 g/liter; KH_2PO_4, 3 g/liter; NH_4Cl 1 g/liter; glucose, 4 g/liter; $MgSO_4$, 2 mM, $CaCl_2$ 0.1 mM), while *S. typhimurium* TN2540-derived strains were grown on yeast extract (10 g/liter); all strains were grown under anaerobic conditions, unless otherwise stated, with $CoCl_2 \cdot 6H_2O$ (1 mg/liter) and, if necessary, 1-amino-2-propanol (AP) (0.05%, vol/vol), 5,6-DMB (25 mg/liter), methionine (50 mg/liter), cysteine (50 mg/liter), IPTG (isopropyl-β-D-thiogalactopyranoside) (50 μM), 5-aminolevulinic acid (5-ALA) (10 mg/l), and antibiotics. Anaerobic conditions were produced by placing a layer of paraffin oil on top of the culture medium.

Isolation and classification of *S. typhimurium cob* mutants. *S. typhimurium* AR2156 and TN2540 were mutagenized with nitrosoguanidine. The *cob* mutants were detected by the methods outlined by Bobik et al. (12). The mutants were classified as *cobI*, *cobII*, or *cobIII* by their ability to grow in the presence of added cobinamide (0.05 nmol/liter), DMB (0.05 nmol/liter), or vitamin B_{12} (0.05 nmol/liter).

Construction of an *S. typhimurium* genomic library. *S. typhimurium* AR2155 DNA was partially digested with *Sau*3AI, and 20- to 25-kb fragments were purified after separation by agarose gel electrophoresis, cloned into the *Bgl*II site of the cosmid pLA2917 (Table 2), and encapsidated in vitro following the Packagene instructions. The library was used to infect *S. typhimurium cob* strains AR2718 and AR2719. One cosmid which complemented both these mutants was isolated and was labelled pAR3062.

Sequencing of pAR3062. Complete restriction mapping of pAR3062 was performed. After digestion with the appropriate restriction enzymes, small fragments of DNA (~1 kb) from the cosmid were cloned in M13mp18 and M13mp19 phages. The single-stranded DNA was sequenced following the method of Sanger et al. (46), with Sequenase T7 DNA polymerase (dITP was used when needed), and the complete sequence of the insert of pAR3062 was obtained (from the *Eco*RI site at position 666 to the *Eco*RI site at position 15531, according to the numbering system used for the *cobI-III-II* region as given in the GenBank under accession no. L12006).

Construction of plasmids containing one or several *cbi* genes. DNA fragments containing one or several *cbi* genes were cloned into pKK223.3, resulting in the construction of the following plasmids: pAR8720 (*cbiA**); pAR8296 (*cbiB*); pAR8591 (*cbiCDETFGHJ*); pAR8533 (*cbiD*); pAR8598 (*cbiET*); pAR8477 (*cbiF*); pAR8606 (*cbiFGH*); pAR8313 (*cbiH*); pAR8283 (*cbiHJ*); pAR8580 (*cbiK*); pAR8547 (*cbiL*); pAR8603 (*cbiMN*); pAR8607 (*cbiNO*); pAR8602 (*cbiQ*); pAR8531 (*cbiP*); pAR8449 (*cobUST*); and pAR8405 (*cobST*). The restriction sites used for the construction of these plasmids are indicated in Table 2. The plasmids were used for functional complementation studies, subcloning, and protein expression.

Cobyric acid and cobalamin quantitative bioassay. (i) Preparation of bioassay plate. The cobyric acid and cobalamin quantitative bioassay is based on the fact that *S. typhimurium metE* cells are reliant upon their alternative B_{12}-dependent methionine synthase, encoded by *metH*, for the biosynthesis of methionine. Two different *S. typhimurium metE cob* mutants were used as indicator strains (*S. typhimurium* AR3612 *cysG metE* and *S. typhimurium* AR2680 *cbiB metE*) to estimate the unknown corrin contents of various samples. The bioassay plates containing the indicator strains were prepared from lawns of the appropriate bacteria. The bacterial growth was scraped from an overnight minimum medium plate containing methionine, washed in 0.9% (wt/vol) NaCl, mixed with 500 ml of minimum medium agar, and poured into plates (cysteine was added to the medium for the *cysG metE* indicator strain).

(ii) Preparation of bacteria for estimation of corrin biosynthesis. To estimate the level of cobalamin production in various strains, the bacteria of interest were grown under anaerobic conditions in minimum medium supplemented with antibiotics, centrifuged, resuspended in water to an optical density of 8 when measured at 600 nm (OD_{600}) and sonicated. A 10-μl droplet of the cell extract solution was added on the surface of the bioassay plate containing the *S. typhimurium cysG* or *cbiB metE* strain and incubated for 18 h at 37°C. The extent of the indicator strain's growth on the bioassay plate, circumventing the sample application point, was dependent upon the amount of corrin in the sample. To generate a standard curve, samples containing 0.01, 0.1, 1, 10, and 40 pmol of vitamin B_{12} were also assayed for each set of experiments.

Modification of individual genes for improved protein production. (i) Modification of *cbiA* RBS. An *Mlu*I fragment restricted from pAR3062 and containing *cbiAB* was cloned into the *Sma*I site of M13. The cloned *cbiAB* fragment was used for the subsequent manipulations. The ribosome binding site (RBS) of *cbiA* was modified by site-directed mutagenesis to give the sequence 5′ AGGAG(7 × A)ATG. The altered gene is referred to as *cbiA**. The PCR was used to amplify *cbiA**, and the 5′ M13 universal primer and a specific 3′ primer were used to change the coupled *cbiA* stop codon and the *cbiB* methionine codon ATGA to

TABLE 1. Reactions and genetic requirements for transformation of uroporphyrinogen III into adenosylcobalamin[a]

P.d. B12 pathway	(1) Gene nomenclature		(3) existence of mutants		(5) effect of mutant on *S.t. metE cysG* indicator strain		(7) effect of mutant on *S.t. metE cbiB* indicator strain	
	P.d.	***S.t.***	*S.t.*	*E.c* + *S.t. cob*	*S.t.*	*E.c* + *S.t. cob*	*S.t.*	*E.c* + *S.t. cob*
uroporphyrinogen III								
↓ *methylation at C2 and C7*	*cobA*	*cysG*	yes	yes	−	−	−	−
precorrin-2								
↓ *methylation at C20*	*cobI*	*cbiL*	yes	no	−		−	
precorrin-3A								
↓ *C20 hydroxylation and lactonisation from ringA acetate to C1*	*cobG*	?						
precorrin-3B								
↓ *C17 methylation and ring contraction*	*cobJ*	*cbiH*	no	no				
precorrin-4								
↓ *C11 methylation*	*cobM*	*cbiF*	yes	no	−		−	
precorrin-5								
↓ *C1 methylation and deacetylation*	*cobF*	?						
precorrin-6x								
↓ *reduction of macrocycle*	*cobK*	*cbiJ*	yes	yes	−	−	−	−
precorrin-6y								
↓ *C5 and C15 methylations decarboxylation of acetate side chain on ring D*	*cobL*	*cbiE* *cbiT*	no no	yes no		−		−
precorrin-8x								
↓ *methyl rearrangement*	*cobH*	*cbiC*	no	yes		−		−
hydrogenobyrinic acid								
↓ *amidation of sidechain a & c*	*cobB*	*cbiA*	yes	yes	−	−	−	−
hydrogenobyrinic acid a,c-diamide								
↓ *cobalt chelation*	*cobN* *cobS,T*	?						
cob(II)yrinic acid a,c-diamide								
↓ *cobalt reduction*	*cob(II)yrinic acid a, c- diamide reductase*	?						
cob(I)yrinic acid a,c-diamide								
↓ *adenosylation*	*cobO*	*cobA* / *cobU*						
(adenosyl)-cobyrinic a,c-diamide								
↓ *amidations of sidechains b, d, e & g*	*cobQ*	*cbiP*	yes	no	−		−	
(adenosyl)-cobyric acid								
↓ *aminopropanol attachment*	*cobD* *cobC*	*cbiB* ?	yes	yes	+	+	−	−
(adenosyl)-COBINAMIDE								
↓ *cobinamide kinase and guanylyl transferase*	*cobP*	*cobU*	no	yes		+		+
(adenosyl)-COBINAMIDE-GTP								
DMB → ↓ *DMB nucleotide transferase*	*cobU*	*cobT*	yes	yes	+	+	+	+
phosphoribosyl ↗ ↓ *cobalamin synthase*	*cobV*	*cobS*	yes	yes	+	+	+	+
(adenosyl)-COBALAMIN								

function	Gene		existence of mutants		effect of mutant on *S.t. metE cysG* indicator strain		effect of mutant on *S.t. metE cbiB* indicator strain	
	P.d.	***S.t.***	*S.t.*	*E.c* + *S.t. cob*	*S.t.*	*E.c* + *S.t. cob*	*S.t.*	*E.c* + *S.t. cob*
?	?	*cbiD*	yes	yes	−	−	−	−
?	*cobE* ?	*cbiG*	no	yes		−		−
?	?	*cbiK*	no	no				
?	?	*cbiM*	no	yes		+		+
cobalt transport	? ? ?	*cbiN* *cbiO* *cbiQ*	no no no	yes yes yes		+ + +		+ + +

[a] The table also indicates mutants which have been defined and whether the mutants produce growth on either of the two vitamin B_{12} indicator strains. The second part of the table lists the genes which are found in *S. typhimurium* but not in *P. denitrificans*. Note that the mutations in *cbiD* and *cbiG* both lead to a cessation of cobalamin production. *P.d.*, *P. denitrificans*; *S.t.*, *S. typhimurium*; *E.c.*, *E. coli*. +, cobyric acid or cobinamide production; −, no production of cobyric acid or cobinamide; ?, uncertain or unknown.

TABLE 2. Strains and plasmids used in this study

Strain, plasmid, or cosmid	Genotype and/or phenotype	Description	Reference or source
S. typhimurium strains			
MS1868	*S. typhimurium* Leu$^-$		Gift from T. Elliot
TN2540	*S. typhimurium lamB*$^+$ *metE551 metA22 ilv-452 trpB2 hisC527* (AM) *galE496 xyl-404 rpsL 120 flaA66 hsdL6 hsdSA29* (r$^-$ m$^+$)		Gift from C. G. Miller (40)
AR2154	MS1868 Leu$^+$		This work
AR2155	MS1868 Leu$^+$ Nal$^+$		This work
AR2156	MS1868 Leu$^+$ Sm^r		This work
S. typhimurium cob mutant strains			
AR2201	MS1868 Leu$^+$ Sm^r *cobT* (*cobII* mutant)	Complemented by pAR8405	This work
AR2206	MS1868 Leu$^+$ Sm^r *cbiB* (*cobI* mutant)	Complemented by pAR8296	This work
AR2709	TN2540 *cbiP* (*cobI* mutant)	Complemented by pAR8531	This work
AR2718	TN2540 *cbiB* (*cobI* mutant)	Complemented by pAR8296	This work
AR2719	TN2540 *cbiA* (*cobI* mutant)	Complemented by pAR8720	This work
AR2720	TN2540 *cbiF* (*cobI* mutant)	Complemented by pAR8477	This work
AR3609	TN2540 *cbiJ* (*cobI* mutant)	Complemented by pAR8283, not by pAR8313	This work
AR3618	TN2540 *cbiJ* (*cobI* mutant)	Complemented by pAR8283, not by pAR8313	This work
AR3619	TN2540 *cbiF* (*cobI* mutant)	Complemented by pAR8477	This work
AR3620	TN2540 *cbiF* (*cobI* mutant)	Complemented by pAR8477	This work
AR3621	TN2540 *cbiF* (*cobI* mutant)	Complemented by pAR8477	This work
AR3622	TN2540 *cbiF* (*cobI* mutant)	Complemented by pAR8477	This work
AR3624	TN2540 *cbiF* (*cobI* mutant)	Complemented by pAR8477	This work
AR3625	TN2540 *cbiB* (*cobI* mutant)	Complemented by pAR8296	This work
AR3626	TN2540 *cbiJ* (*cobI* mutant)	Complemented by pAR8283, not by pAR8313	This work
AR3627	TN2540 *cobS* (*cobIII* mutant)	Complemented by pAR8405	This work
AR3628	TN2540 *cobS* (*cobIII* mutant)	Complemented by pAR8405	This work
AR3632	TN2540 *cbiJ* (*cobI* mutant)	Complemented by pAR8283, not by pAR8313	This work
AR3633	TN2540 *cbiF* (*cobI* mutant)	Complemented by pAR8477	This work
AR3635	TN2540 *cbiF* (*cobI* mutant)	Complemented by pAR8477	This work
AR3637	TN2540 *cbiF* (*cobI* mutant)	Complemented by pAR8477	This work
AR3711	TN2540 *cbiL* (*cobI* mutant)	Complemented by pAR8547	This work
AR3713	TN2540 *cbiP* (*cobI* mutant)	Complemented by pAR8531	This work
AR3714	TN2540 *cbiP* (*cobI* mutant)	Complemented by pAR8531	This work
AR3717	TN2540 *cbiL* (*cobI* mutant)	Complemented by pAR8547	This work
AR3718	TN2540 *cbiD* (*cobI* mutant)	Complemented by pAR8533	This work
AR3719	TN2540 *cbiP* (*cobI* mutant)	Complemented by pAR8531	This work
AR3720	TN2540 *cbiA* (*cobI* mutant)	Complemented by pAR8720	This work
AR3728	TN2540 *cbiD* (*cobI* mutant)	Complemented by pAR8533	This work
S. typhimurium indicator strains[a]			
AR2680	MS1868 Leu$^+$ Sm^r *cbiB metE*	Used in assays for cobinamide	This work
AR3612	MS1868 Leu$^+$ Sm^r *cysG metE*	Used in assays for cobyric acid	This work
E. coli strains			
LE392	*E. coli* F$^-$ e14$^-$ (mcrA$^-$) *hsdR514* (r_K^- m_K^-) *supE44 supF58 lacY1* or *D*(*lacIZY*)*6 galK2 galT22 metB1 trpR55*		45
AR3730	LE392 *met*$^+$	Rendered *met*$^+$ with P1	This work
AR8654	LE392 *met*$^+$(pAR8086)		This work
Cobinamide-producing *E. coli* strains			
AR8639	LE392 *met*$^+$ (pAR8633)(pAR8600)		This work
AR8640	LE392 *met*$^+$ (pAR8626)(pAR8613)	Contains all *cbi* genes but *cbiM*, *-N*, *-Q*, and *-O*	This work
AR8752	LE392 *met*$^+$ (pAR8686)(pAR8736)		This work
AR8981	LE392 *met*$^+$ (pAR3062)		This work
Cobyric-acid producing *E. coli* strains			
AR8829	LE392 *met*$^+$ (pAR8827)	Contains all *cbi* genes but *cbiB*	This work
AR8830	LE392 *met*$^+$ (pAR8827)(pAR8414)	Contains all *cbi* genes but *cbiB*	This work
AR8959	LE392 *met*$^+$ (pAR8827)(pKK223.3)	Contains all *cbi* genes but *cbiB*	This work
Non-cobyric acid-producing *E. coli* strains[b]			
AR8688	LE392 *met*$^+$ (pAR8686)	Contains all *cbi* genes but *cbiE* and *-A*	This work

Continued on following page

TABLE 2—*Continued*

Strain, plasmid, or cosmid	Genotype and/or phenotype	Description	Reference or source
AR8866	LE392 *met*$^+$ (pAR8686)(pAR8720)	Contains all *cbi* genes but *cbiE*	This work
AR8905	LE392 *met*$^+$ (pAR8903)(pAR8834)	Contains all *cbi* genes but *cbiB* and *-J*	This work
AR8965	LE392 *met*$^+$ (pAR8086)(pAR8000)	Contains all *cbi* genes but *cbiA*	This work
AR8984	LE392 *met*$^+$ (pAR8658)(pAR8976)	Contains all *cbi* genes but *cbiB* and *-G*	This work
AR8985	LE392 *met*$^+$ (pAR8658)(pAR8977)	Contains all *cbi* genes but *cbiB* and *-D*	This work
AR8986	LE392 *met*$^+$ (pAR8658)(pAR8978)	Contains all *cbi* genes but *cbiB* and *-C*	This work
Plasmids and cosmids			
pACYC184		Carries the origin of replication from plasmid p15A	16
pHM3	*E. coli cysG*		Gift from J. Cole (32)
pKK223.3		Chloramphenicol- and tetracycline-resistant overexpression vector derived from pBR322, with *tac* promoter, ampicillin resistant	14
pLA2917		λ nonlytic cosmid	2
pAR3062	*pduBAF-pocR-cbiABCDETFGHJKLMN QOP-cobUST*	21.5-kb *S. typhimurium* (AR2155) *Sau*3AI fragment cloned into the *Bgl*II site of pLA2917	This work
pAR8000	*cbiBCDETFGHJKLMNQOP-cobUST*	Partial *Pst*I digest of pAR3062 (from *Pst*I 1606) cloned into *Pst*I site of pKK223.3	This work
pAR8086	*lacI*^{q}	pACYC184 with *lacI*^{q} in *Eco*RV site	This work
pAR8283	*cbiHJ*	*Eco*RI-*Hin*dIII fragment cloned into pKK223.3	This work
pAR8296	*cbiB*	*Nru*I fragment (2531–3701) cloned into pKK223.3	This work
pAR8313	*cbiH*	*Eco*RI 8207-*Pst*I 9594 fragment cloned into pKK223.3	This work
pAR8405	*cobST*	*Eco*RI 15531-*Sau*3AI fragment cloned into pKK223.3	This work
pAR8414	*E. coli cysG**	Cloned into *Eco*RI-*Hin*dIII sites of pKK223.3 (modified RBS, AGGAGGTTTTACATG)	This work
pAR8477	*cbiF*	*Cla*I 6241-*Eco*RI 8207 fragment cloned into pKK223.3	This work
pAR8449	*cobUST*	*Dra*I 13997-*Sau*3AI fragment cloned into *Sma*I site of pKK223.3	This work
pAR8531	*cbiP*	*Sal*I 13423-*Eco*RI 15531 fragment cloned into pKK223.3	This work
pAR8533	*cbiD*	*Stu*I 4261-*Xmn*I 5676 fragment cloned into pKK223.3	This work
pAR8547	*cbiL*	*Dde*I 10583-*Pst*I 11432 fragment cloned into pKK223.3	This work
pAR8572	*cbiA*B*	*Mlu*I fragment (1219–3706) cloned into *Sma*I site of pKK223.3 (modified RBS, AGGAGAAAAAAAATG)	This work
pAR8575	*cbiA*B/cysG**	*Bam*HI fragment of pAR8572 cloned into *Bam*HI site of a variant of pAR8629	This work
pAR8580	*cbiK*	*Pst*I 9594-*Hin*dIII 11219 fragment cloned into pKK223.3	This work
pAR8591	*cbiCDETFGHJ*	*Dde*I 3620-*Hin*dIII 10028 fragment of cosmid pAR3062 cloned into *Sma*I site of pKK223.3	This work
pAR8597	*cbiKLMNQOP*	*Bgl*II 9509-*Eco*RI 15531 digest of cosmid pAR3062 cloned into *Sma*I site of pKK223.3	This work
pAR8598	*cbiET*	*Sal*I 5370-*Nar*I 6596 fragment cloned into pKK223.3	This work
pAR8600	*cbiCDETFGHJ-cbiKLMNQOP*	*Bam*HI fragment of pAR8597 cloned into *Hin*dIII site of pAR8591	This work
pAR8602	*cbiQ*	*Bst*XI 13027-*Dra*I 13997 fragment cloned into pKK223.3	This work
pAR8603	*cbiMN*	*Hin*dIII 11219-*Bst*XI 13027 fragment cloned into pKK223.3	This work
pAR8606	*cbiFGH*	*Xmn*I 6400-*Nsi*I 9308 fragment cloned into pKK223.3	This work
pAR8607	*cbiNO*	*Sma*I 11717-*Sal*I 13423 fragment cloned into pKK223.3	This work
pAR8613	*cbiCDETFGHJKL*	Partial *Bst*XI 3450-*Nsi*I 11476 fragment of pAR3062 cloned into *Sma*I-*Pst*I sites of pKK223.3	This work
pAR8626	*cbiA* B-cysG*-cbiP*	Ptac-*cbiP* fragment cloned into *Sma*I site of pAR8575	This work
pAR8629	*cysG**	*Bam*HI-*Sca*I fragment of pAR8414 cloned into *Nru*I-*Bam*HI sites of pAR8086	This work
pAR8633	*cysG*-cbiA*B*	*Bam*HI-*Sca*I fragment of pAR8572 cloned into *Bam*HI site of pAR8629	This work
pAR8634	*cbiE*^{ic}	Cloned into *Eco*RI-*Hin*dIII-cut pKK223.3	This work
pAR8636	*cbiB**	Cloned into *Eco*RI-*Hin*dIII-cut pKK223.3 (modified RBS: AGGAGAAAAAAAATG)	This work
pAR8655	*cbiB* CD*	*Nsi*I 3638-*Xmn*I 5676 digest of pAR3062 cloned into *Nsi*I-*Xba*I sites of pAR8636	This work
pAR8658	*cysG**	Variant of pAR8629	This work
pAR8667	*cbiE*^{i}*TFGHJKLMNQ*	*Sac*I 6079-*Sna*BI 13389 digest of pAR3062 cloned into *Sac*I-*Pst*I sites of pAR8634	This work
pAR8670	*cysG*OP*	*Bst*XI 13027-*Eco*RI 15531 digest of pAR3062 cloned into *Sma*I site of a variant of pAR8658	This work
pAR8683	*cbiB*CD-cysG*OP*	*Bam*HI-*Sph*I digest of pAR8655 cloned into *Bam*HI site of pAR8670	This work

Continued on following page

TABLE 2—*Continued*

Strain, plasmid, or cosmid	Genotype and/or phenotype	Description	Reference or source
pAR8686	*cbiB*CD-cbiE^{i}TFGHJKLMNQ-cysG*OP*	*Sph*I digest of pAR8667 cloned into *Bam*HI site of pAR8683	This work
pAR8720	*cbiA**	Cloned from *Mlu*I 1219 site to the *cbiA** stop codon into *Eco*RI site of pKK223.3 after PCR	This work
pAR8736	*cbiA*E*	*Nde*I 5228-*Cla*I 6241 fragment cloned into *Sma*I site of pAR8720	This work
pAR8903	*cbiA*-cbiCDETFGH-cbiKLMNQOP*	Mutation at the *Bgl*II 9509 site into *cbiJ* gene of pAR8827	This work
pAR8814	*cbiA*-cbiCDETFGHJ-cbiKLMNQOP*	Partial *Bam*HI-*Sca*I digest of pAR8600 cloned into pAR8720 cut with *Bam*HI-*Sca*I	This work
pAR8827	*cbiA*-cbiCDETFGHJ-cbiKLMNQOP*	*Sph*I-*Sca*I fragment of pAR8814 cloned into *Sph*I-*Nru*I sites of pAR8086	This work
pAR8976	*cbiA*-cbiCDETFHJ-cbiKLMNQOP*	Deletion of 417 bases between *Bst*BI sites (7774–8211) into *cbiG* gene of pAR8814	This work
pAR8977	*cbiA*-cbiCETFGHJ-cbiKLMNQOP*	Mutation at the *Mlu*I 4608 site into *cbiD* gene of pAR8814 (TAA at position 4619)	This work
pAR8978	*cbiA*-cbiDETFGHJ-cbiKLMNQOP*	Mutation at the *Mlu*I 3706 site into *cbiC* gene of pAR8814	This work

[a] Strains used for B_{12} bioassays.
[b] *E. coli* strains which have plasmids with mutations in *cbi* genes and do not produce cobyric acid.
[c] Gives inactive gene product.

ATAA. The PCR product was subsequently cloned into pKK223.3, resulting in the construction of plasmid pAR8720 (Table 2).

(ii) Modification of the *E. coli cysG* RBS. The *E. coli cysG* gene was cloned from the plasmid pHM3 (29) (Table 2) into M13. The RBS was changed to 5′ AGGAGGTTTTACATG by site-directed mutagenesis, and the resultant modified gene, *cysG**, was cloned into the *Eco*RI-*Hin*dIII sites of pKK223.3, yielding pAR8414.

(iii) Modification of *cbiB* RBS. The *Nru*I fragment from position 2531 to 3701 of pAR3062 was cloned into the *Sma*I site of M13. A new *cbiB* RBS was generated by site-directed mutagenesis to give the sequence 5′ AGGAG(7 × A) ATG. The modified gene, *cbiB**, was cloned into pKK223.3, producing pAR8636.

(iv) Modification of *cbiE*. A *Sal*I-*Cla*I fragment from position 5373 to 6241 of pAR3062 was cloned into the *Sma*I site of M13. A modified RBS of AGGAG(7 × A)ATG was introduced into the gene by site-directed mutagenesis. The modified gene, *cbiE^i*, was found to be inactive, presumably because of a further mutation that had been introduced into the protein.

Construction of derivatives of pAR3062 for cobalamin overproduction. In order to improve expression of the *cbi* genes present in the 14,119 bp of the *cobI* region, the *cbi* genes were placed under the control of three *tac* promoters, positioned at regular intervals throughout the sequence (Fig. 2). To achieve this goal, three plasmids had to be used: pAR8720, which contains *cbiA**, described previously; pAR8591, which contains *cbiCDETFGHJ*; and pAR8597, containing *cbiKLMNQOP*. The plasmid pAR8591 was constructed by cloning the *cbiCDE TFGHJ* fragment, obtained from a partial digest of *Dde*I and *Hin*dIII (bases 3620 to 10028) into the *Sma*I site of pKK223.3. Plasmid pAR8597 was constructed by cloning the *Bgl*II-*Eco*RI fragment (bases 9509 to 15531) into the *Sma*I site of pKK223.3. The *ptac-cbiKLMNQOP* fragment of pAR8597 was isolated after a *Bam*HI digest and cloned into pAR8591 which had been cut with *Hin*dIII. The resulting plasmid, pAR8600, has the following arrangement: *ptac-cbiCDETF GHJ-ptac-cbiKLMNQOP*. This plasmid was digested with *Sca*I and partially digested with *Bam*HI to isolate the double *ptac-cbi* gene fragment and was cloned into the *Bam*HI and *Sca*I sites of pAR8720. This new plasmid, named pAR8814, contains *ptac-cbiA*-ptac-cbiCDETFGHJ-ptac-cbiKLMNQOP*. Thus, pAR8814 contains all the *cbi* genes except *cbiB*.

This triple *ptac-cbi*-T1T2 terminator fragment was subcloned from pAR8814 into pAR8086, which contains the p15A origin of replication (16), which is compatible with the ColE1 replication origin of pKK223.3. To increase the amount of precorrin-2 available for B_{12} biosynthesis, the plasmid containing the modified *cysG*, pAR8414, was transferred along with pAR8827 into *E. coli*.

Another plasmid, pAR8686, harboring three *tac* promoters and the *cbi* genes, but in an order different from that described for pAR8827, was constructed with the same rationale. This large plasmid was assembled from plasmids pAR8667, pAR8655, and pAR8670. The construction of these plasmids is briefly outlined in Table 2.

Introduction of mutations in *cbiC*, *cbiD*, *cbiG*, and *cbiJ* to inactivate the gene products. In order to introduce a mutation into *cbiC*, pAR8814 was partially digested with *Mlu*I so that the plasmid was restricted once at position 3706 and the overhangs were filled by the action of the Klenow fragment of DNA polymerase I and ligated with T4 DNA ligase. This procedure produced pAR8978, which contains a frameshift in *cbiC*. In a similar fashion, a mutation was introduced into *cbiD* of pAR8814 at position 4608 after this plasmid was restricted by *Mlu*I, filled, and ligated. This produced pAR8977, which contains a frameshift in *cbiD*. For mutation of *cbiG*, pAR8814 was digested with *Bst*BI at positions 7774 and 8211 and ligated with T4 DNA ligase, resulting in a deletion of 437 bp. The mutated plasmid was named pAR8976. For mutation of *cbiJ*, plasmid pAR8827 (which contains the same genes as pAR8814 but in the pACYC184 vector) was partially digested with *Bgl*II at position 9509. After filling in and ligation, pAR8903 was produced which contains a frameshift in *cbiJ*.

Two-dimensional protein gels. Samples were prepared as follows. Bacterial cultures were grown anaerobically to an OD_{600} of 0.15, at which stage IPTG was added (final concentration, 0.5 mM). After 60 min, 10 to 20 μCi of [^{35}S]L-methionine per ml of culture was added and incubated for a further hour. The cells were centrifuged and resuspended in lysis buffer. Two-dimensional gels,

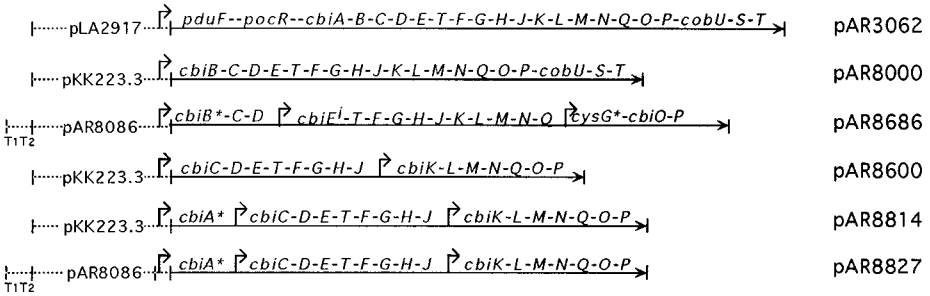

FIG. 2. Linear diagrammatic representation of some plasmid constructs used in this study. ↱, *tac* promoter.

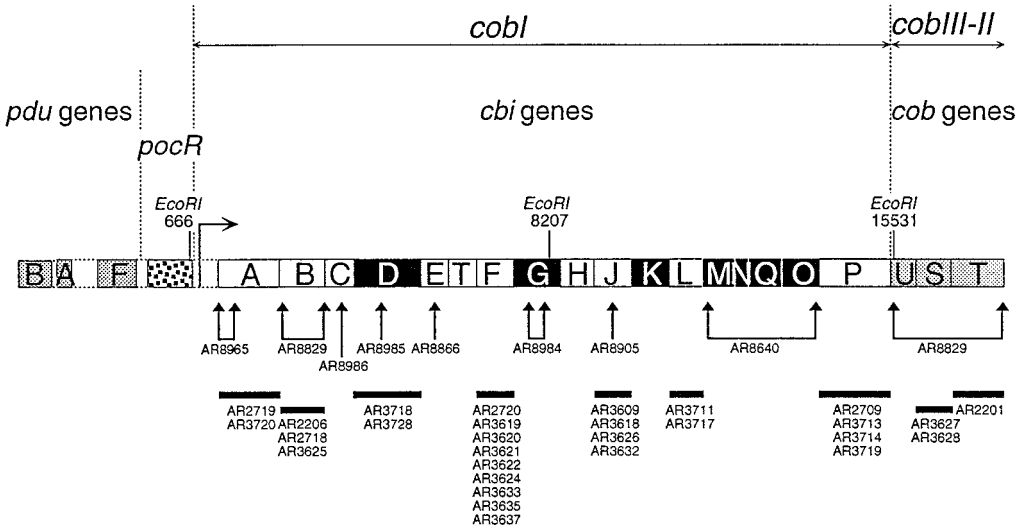

FIG. 3. Genetic organization of the *S. typhimurium cobI-III-II* region. Genes shown in white boxes have similar genes in *P. denitrificans*. Genes shown in black boxes have no *P. denitrificans* homologs. Shaded boxes represent the propanediol utilizing (*pdu*) and *cobIII-II* genes. The DNA fragments used to complement the *S. typhimurium* mutants are indicated with bold lines. The associated mutant strains are shown underneath. The arrows designate the *S. typhimurium* genes which have been inactivated or deleted in the indicated *E. coli* strains harboring the *S. typhimurium cob* genes.

buffers, and running conditions were prepared and run as described by O'Farrell (36, 37).

Other methods. The methyl esters of the precorrins were prepared after DEAE Sephadex A-25 extraction of the intermediates from sonicated cell extracts and esterification in methanol-H_2SO_4 (95:5). The methyl esters were separated by either thin-layer or high-performance liquid chromatography on a C_{18} reverse-phase column as previously described (54). A Hewlett-Packard 8452A diode array spectrophotometer was used to record UV and visible spectra.

RESULTS

Isolation of *S. typhimurium cob* mutants. *S. typhimurium* AR2156 and TN2540 were mutagenized and screened for mutations in their capacity to biosynthesize cobalamins as described in Materials and Methods. The mutants were classified as *cobI*, *cobII*, or *cobIII* by their ability to grow in the presence of added cobinamide, DMB, or cyanocobalamin. Twenty-six *cobI*, one *cobII*, and two *cobIII* mutants were obtained, and these are listed in Table 2.

Isolation and analysis of a DNA fragment containing the *S. typhimurium cob* region. An *S. typhimurium* AR2155 genomic library was produced in the cosmid pLA2917 as described in Materials and Methods. By its ability to confer cobalamin biosynthesis on the *S. typhimurium cobI* mutants AR2718 and AR2719 and the *cobII* mutant AR2201, a single cosmid containing the *cobI-III-II* region was identified from this library. This cosmid was named pAR3062 and was found to contain a 21.5-kb insert, from which an extensive restriction map was generated. The insert was digested into fragments of ~1 kb, and these were subcloned into M13. This facilitated the complete sequencing of the *cobI* region, which was found to be identical to that reported by Roth et al. (44). As well as containing the *cobI-III-II* region, the cloned fragment also contained 4 kb of DNA upstream of the *cobI* region, including the promoter and the *pocR* gene (43).

Analysis of the 29 isolated *S. typhimurium cob* mutants. The *cobI-III-II* region contains 20 open reading frames (44). Of them, 13 have been assigned as *cob* genes by sequence comparison with the *P. denitrificans cob* genes (44) (Table 1), among which the genes *cbiF*, *cbiL*, *cobS*, *cobT*, and *cobU* have also had their function formally identified by enzymatic studies (17, 38, 39, 42, 51). The function of the seven remaining open reading frames (*cbiD*, *-G*, *-K*, *-M*, *-N*, *-O*, and *-Q*) are unknown, although those of *cbiN*, *-O*, *-Q* were proposed to be involved in cobalt transport (44). In order to genetically define the 29 isolated *cob* mutants, the cloned *S. typhimurium cobI-III-II* region was restricted, where possible, into fragments containing single genes, which were subsequently cloned behind the *tac* promoter of pKK223.3. These plasmids are listed in Table 2 and were used to transform the *S. typhimurium* mutants.

Confirmation that *S. typhimurium* homologs *cbiA*, *-B*, *-F*, *-J*, *-L*, and *-P* and *cobS* and *-T* are *cob* genes and evidence that *cbiD* is a *cobI* gene. First, the 26 *S. typhimurium cobI* mutants were transformed by the individually cloned *cbi* genes and tested for corrin production (see Materials and Methods). The cloned *cbiA* was found to complement two mutants, *cbiB* was found to complement three mutants, *cbiF* was found to complement nine mutants, *cbiL* was found to complement two mutants, and *cbiP* was found to complement four (Fig. 3 and Table 2). Four *cbiJ* mutants were identified on the basis that the mutants were complemented by plasmid pAR8283, which contains *cbiH* and *-J*, but were not complemented by pAR8313, which contains *cbiH* alone. Thus, the prediction, which was based on sequence similarity, that *S. typhimurium cbiA*, *-B*, *-J*, and *-P* are cobalamin biosynthetic genes has been confirmed by functional complementation. These complementations defined 24 of the 26 isolated *S. typhimurium cobI* mutants. The two remaining *cobI* mutants were identified as *cbiD* mutants, as they were complemented by pAR8533 (Table 2). There is no *P. denitrificans* homolog for *cbiD*, and this is the first direct evidence that *cbiD* is a *cobI* gene. The cloned *S. typhimurium cobT* complemented the *S. typhimurium cobII* mutant, and the cloned *cobS* complemented the two *cobIII* mutants. No mutants were found to be complemented by DNA fragments containing *cbiC*, *cbiG*, *cbiH*, *cbiK*, *cbiMNQO*, or *cbiET*.

Quantitative detection of cobyric acid and cobalamin. To aid in the detection of cobyric acid and cobalamin, a well-known, simple microbiological assay for vitamin B_{12} (6) was adapted with *S. typhimurium metE* strains. The modified assay with *S. typhimurium metE cysG* (AR3612) and *S. typhimurium metE cbiB* (AR2680) (Table 2) could be used to estimate

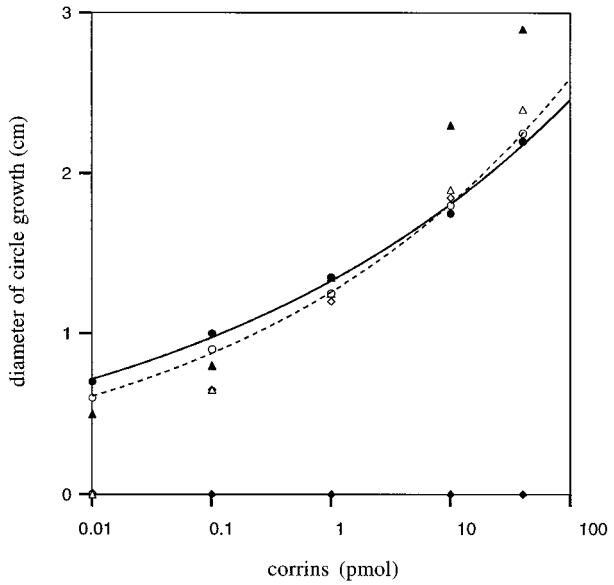

FIG. 4. Standard curve from indicator strains AR3612 and AR2680. A plot of the amount of cyanocobalamin or cobyric acid used to elicit the measured growth response produces a curve as shown. The test is able to detect as little as 10 fmol of cobalamin. ○, cyanocobalamin on AR3612; ●, cyanocobalamin on AR2680; △, cobinamide on AR3612; ▲, cobinamide on AR2680; ◇, cobyric acid on AR3612; ◆, cobyric acid on AR2680.

cobalamin levels but could also be used to discriminate between cobyric acid and cobinamide amounts. When AR2680 (*metE cbiB*) is incorporated into a minimal medium plate or AR3612 (*metE cysG*) is incorporated into a minimal medium plate containing cysteine, no growth is observed, although the bacteria remain dormant in the agar for up to 3 weeks. However, application of a vitamin B_{12} solution to either of the indicator strains results in growth of the dormant bacterial lawn circumventing the application point. The extent of growth around the application point is dependent upon the amount of vitamin B_{12} that is added. The correlation between growth size and vitamin B_{12} quantity permits a standard curve to be constructed which can be used to estimate the concentrations of unknown cobalamin solutions with a satisfying reproducibility of ±10% with both indicator strains. Growth of the *metE cysG* indicator strain was observed with the addition of vitamin B_{12}, cobinamide, and cobyric acid but not with cobyrinic acid (Fig. 4). Growth of the *metE cbiB* strain was observed with addition of vitamin B_{12} and cobinamide but not cobyric acid. This result confirms that CbiB is required for the conversion of cobyric acid into cobinamide, but it also means that the two indicator strains can be used to discriminate between solutions of cobyric acid and cobinamide. Addition of cobyrinic acid to either strain did not stimulate growth. As cobyrinic acid is thought to be an intermediate in the biosynthesis of cobalamins, this would suggest that the intermediate is not taken up by the cells. The results of the analysis of cell extracts from various *S. typhimurium cob* mutants (Table 2) on the bioassay plates were consistent with the observations described above: growth was not observed with extracts of *cysG*, *cbiL*, *cbiF*, *cbiJ*, *cbiA*, and *cbiP* mutants which produce precursors prior to cobyric acid (Table 1, column 5), probably because the biosynthetic intermediates were not taken up by the indicator strains; the peripheral side chain acid groups of the modified tetrapyrrole would appear to have to be amidated for effective transport by the cells. On the other hand, growth was observed with cell extracts from *cbiB*, *cobT*, and *cobS* mutants, which produce cobyric acid and later intermediates. To ensure that mixtures of cobyric acid and vitamin B_{12} did not interfere with the bioassay to give erroneous results, growth of both indicator strains was measured in the presence of fixed amounts of vitamin B_{12} and variable amounts of cobyric acid. As expected, the diameter of growth area with the *metE cysG* indicator corresponded to the amount of the most abundant of these two compounds (Fig. 5). Cobyric acid had no effect on the diameter of growth of the *metE cbiB* indicator.

The *S. typhimurium cob* DNA fragment confers de novo cobalamin biosynthesis ability on *E. coli*. When cosmid pAR3062 was transformed into *E. coli*, producing AR8981, it conferred upon the host bacterium the ability to make cobinamide and thus cobalamin (Table 3). Extracts of AR8981 produced growth on both indicator strains, but the circle of growth was greater with the *metE cysG* indicator; the amount of cobyric acid produced in this strain was greater than that of cobinamide or later intermediates. Increased quantities of cobyric acid and intermediates which occur after cobyric acid on the cobalamin pathway could be obtained from AR8981 by addition of exogenous 5-ALA to the growth medium. This is because addition of exogenous 5-ALA leads to greater flux through the tetrapyrrole biosynthetic pathway. It was noted that extracts of the parent strain *S. typhimurium* AR2155 produced growth circles of the same diameter on the two indicator strains (Table 3), demonstrating that cobyric acid does not accumulate in *S. typhimurium*.

Construction and analysis of an *E. coli* strain with elevated cobyric acid levels. Overproducing strains were constructed in order to allow chemical and biochemical studies of the various intermediates on the cobalamin pathway. To remove the known regulatory elements of *cbiA* (41) and to enhance the translation of the gene product, *cbiA* was modified by deletion of the upstream region and by incorporation of a consensus RBS by site-directed mutagenesis and PCR (the new gene, *cbiA**, was cloned in pKK223.3, yielding pAR8720; see Materials and Methods and Table 2). The resulting *cbiA** gene produced high

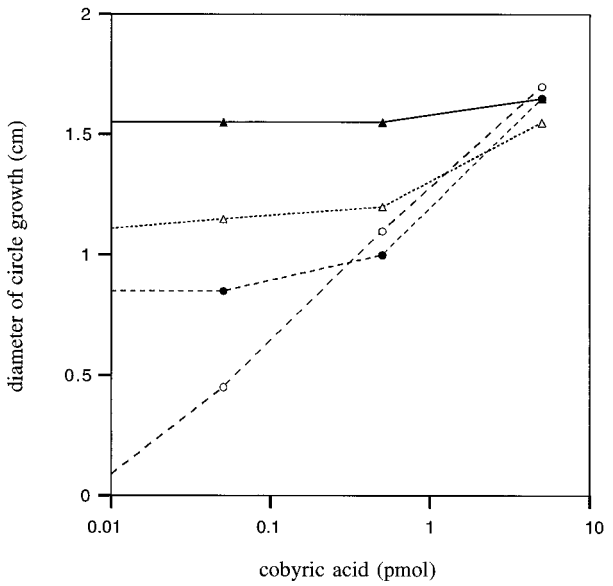

FIG. 5. Competing effects of cyanocobalamin and cobyric acid on strain AR3612. Growth of the indicator strain is produced by both compounds on an equal basis. Symbols for amount of cyanocobalamin added: ○, 0 pmol; ●, 0.05 pmol; △, 0.5 pmol; ▲, 5.0 pmol.

TABLE 3. Corrin production[a]

Strain	Growth condition	Amt of corrin (nmol/OD_{600} unit) detected after growth in medium[b]					
		M9		M9 + AP + 5-ALA		M9 + AP + 5-ALA + IPTG	
		Cobyric acid	Cobinamide	Cobyric acid	Cobinamide	Cobyric acid	Cobinamide
AR2155	Aerobic			ND[c]	ND		
	Anaerobic	0.01	0.01	0.02	0.004	0.01	0.004
AR8981	Aerobic			ND	ND		
	Anaerobic	0.04	ND	0.1	0.01	0.2	0.01
AR8959	Aerobic			ND	ND		
	Anaerobic	0.1	ND	0.4	ND	0.2	ND
AR8830	Aerobic			ND	ND		
	Anaerobic	0.8	ND	0.8	ND	0.5	ND
AR8639	Aerobic			ND	ND		
	Anaerobic	0.9		0.9	ND	0.2	0.03

[a] Corrin production was monitored under a number of conditions as described in Materials and Methods. All experiments were performed several times and the mean values are presented.
[b] The indicator strains for cobyric acid and cobinamide were AR3612 and AR2680, respectively.
[c] ND, not detectable.

levels of CbiA protein as adjudged by two-dimensional polyacrylamide gel electrophoresis (PAGE) (data not shown). The *S. typhimurium* DNA fragment in pAR3062 was further restricted and modified so that three *tac* promoters were introduced at regular intervals between the genes. This was accomplished by a complex cloning procedure which is described in Materials and Methods and is also outlined in Table 2. Such manipulations yielded pAR8827, a derivative of pACYC184, and contained the following gene order: *ptac-cbiA*-ptac-cbiC DETFGHJ-ptac-cbiKLMNQOP*. This plasmid was transformed into *E. coli* to give strain AR8959, and analysis of the extracts of this strain showed that it was able to produce levels of cobyric acid between 0.1 and 0.4 nmol per OD_{600} unit. Transformation of pAR8827 into an *E. coli* strain harboring a compatible plasmid, containing a copy of the *E. coli cysG* gene with an improved RBS (*cysG** on pAR8414), was performed to improve corrin production and gave the strain AR8830. The *cysG** mutant was produced from a plasmid-borne *cysG* by directed mutagenesis of the RBS (Materials and Methods and Table 2), resulting in high levels of CysG protein production as adjudged by sodium dodecyl sulfate-PAGE. Strain AR8830, harboring both *ptac-cysG** and *ptac-A*-ptacCDETFGHJ-ptac KLMNQOP*, was found to produce cobyric acid up to 0.8 nmol per OD_{600} unit; addition of IPTG to the bacterial culture did not lead to any further enhancement of corrin production, even though the cells were always maintained in the presence of the $lacI^q$ repressor, which keeps a basal transcriptional level in the absence of IPTG. The 20-fold enhancement of cobyric acid production of AR8830 (harboring *cysG** and the three-*ptac* construct) compared with that of AR8981 (harboring the original cosmid) in the absence of exogenous 5-ALA reflects the effect of the modified *cysG** gene, which leads to higher levels of precorrin-2 (52) and to the transcriptional activation mediated by the replacement of the original promoter and regulatory regions by the strong *tac* promoter. This together with the copy number of the plasmid ensures a higher level of cobyric acid production.

The proteins encoded by the genes on pAR8827 were examined by one- and two-dimensional PAGE (see Materials and Methods). A typical gel corresponding to *E. coli* AR8688 carrying the plasmid pAR8686 demonstrates that the products of all the cloned genes, except for the products of *cbiM*, *cbiN*, and *cbiQ*, are clearly visible (Fig. 6). The least abundant among the visible proteins appears to be CbiB, which is required to convert cobyric acid into cobinamide by aminopropanol attachment. The lack of CbiB expression is concurrent with the observation that in strains containing all the *cobI* genes, such as AR8981 and AR8639 (Table 2), the levels of cobyric acid are higher than the levels of later intermediates (Table 3). This does not preclude the fact that there could be more than one polypeptide involved in cobinamide formation from cobyric

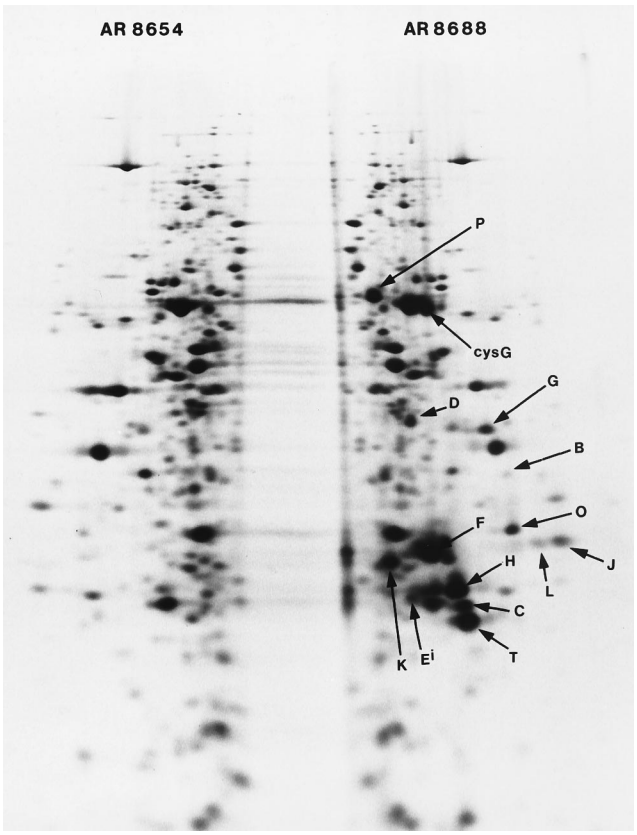

FIG. 6. Autoradiograph of a two-dimensional protein gel. The strains AR8654 (control) and AR8688 were analyzed as described in the text (Materials and Methods). Letters refer to the corresponding Cbi proteins.

acid (8, 44). The proteins shown in Fig. 6 were extracted from cells grown under anaerobic conditions, but the proteins were also expressed when cultures were grown under aerobic conditions (data not shown). Thus, expression of the cloned *cobI* genes is not regulated by anaerobicity.

Corrins are produced de novo in *E. coli* only under anaerobic conditions. The corrin-producing *E. coli* strains were analyzed for corrin biosynthesis after growth under aerobic and anaerobic conditions. Experiments were performed with strains carrying the *cob* genes with their natural promoter (AR8981) and with the *tac* promoter (AR8959, AR8830, and AR8639), but it was observed that in all cases corrins were produced only when the cultures were made anaerobic (Table 3). Thus, the recombinant corrin-producing *E. coli* strains have the same cobalamin biosynthetic phenotype as *S. typhimurium* (29) in that de novo biosynthesis of cobalamin occurs only under anaerobic conditions.

Construction of *cob* mutants in the cobalamin-producing *E. coli* strains. The construction of recombinant *E. coli* strains capable of making cobyric acid with cloned *S. typhimurium cob* genes permitted the construction of defined mutations in these genes on the plasmids. This allowed us to further investigate the role of a number of genes found in the *cobI* region, which were analyzed in the recombinant *E. coli* strains to see the effect of the mutations on corrin biosynthesis. A series of such plasmids, containing the *S. typhimurium cob* region in which one or several genes were either absent or inactivated by mutagenesis, were constructed. Mutations or deletions were introduced into *cbiC*, *cbiD*, *cbiE*, *cbiG*, *cbiJ*, and *cbiMNOQ*. Transformation of the plasmids containing the mutations and deletions into *E. coli* produced a series of cobalamin mutants (Fig. 3 and Table 2).

(i) Construction of *cbiC*, *cbiE*, and *cbiJ* mutants. The analysis of the *S. typhimurium cob* mutants showed that 26 *cobI* mutants were complemented by DNA fragments containing *cbiA*, *-B*, *-D*, *-F*, *-J*, *-L* and *-P* genes (see above), establishing that these are *cobI* genes. No *S. typhimurium* mutant was found to be complemented by a fragment containing *cbiC*, which was proposed to be homologous to the *P. denitrificans cobH* gene (44). To overcome this, an *E. coli cbiC* mutant (strain AR8986; see Materials and Methods) was generated and found to produce no detectable amount of corrins when extracts of this strain were analyzed on both indicator strains (Table 1). This result strongly suggests that *cbiC* is a *cobI* gene but does not rule out the possibility that the mutation introduced into *cbiC* may have a polar effect. However, as cell extracts of the *cbiC* strain AR8986 did not promote any growth on the indicator strain (<10 fmol) this polar effect would have to reduce the amount of cobyric acid biosynthesis by more than 1,000-fold (Fig. 4). A protein analysis of a whole-cell extract of AR8986 by two-dimensional PAGE was performed and showed that the mutation did not prevent the expression of genes situated downstream of *cbiC*, thus indicating that there was no strong polar effect due to the mutation.

During the modification of *cbiE* to improve the translation of its gene product (see Materials and Methods), a derivative of pAR3062 which, when transformed into *E. coli* (AR8866), produced no detectable corrin (Table 1), was obtained and designated pAR8686. Presumably, the reason why AR8866 did not produce any corrin was that a mutation had been accidentally introduced into *cbiE* on pAR8686, producing *cbiE*^{i}. When the unmodified *cbiE* was introduced into this strain, full corrin production was restored. This fortuitous event has, however, allowed the identification of *cbiE* as a *cobI* gene.

Although we had isolated an *S. typhimurium cbiJ* mutant, we further confirmed the role of *cbiJ* as a *cobI* gene by introducing a mutation into *cbiJ* in the plasmid pAR8903 by restriction, end filling, and ligation of the *Bgl*II site located in the gene (see Materials and Methods). When this plasmid was transformed into *E. coli*, corrin biosynthesis was not observed (Table 1). A comparison by the FASTA program between the CbiJ and the *P. denitrificans* CobK gave scores of 159 (initn) and 203 (opt). This was significantly higher than the best match by the MIPS database, and thus *cbiJ* would appear to be similar to the *P. denitrificans cobK* (also noted in references 8 and 42). This similarity was not found in reference 44, possibly because of the wrong orientation of this gene sequence in the database.

```
cbiG   1 ..............................................  200

cbiG 201 LPELDALICVSLRNDLPELPVPHWKLVPQR....VVAGIGCRRDTPFPLL 246
                        :| .: .  . :    :| |:|| | || . :
cobE   1 ..................MPSGQHSAQTTKAGAGLVLGLGCERRTPAEEV 32
                           .        .        .        .
     247 ATLLARQLEAQKLDPLALKAIGSVTLKKGEPGLIQLASCCRVPFKTFTAE 296
           .| .|.|... :.| .|: ::|:. : :||::: |    ||   :.|.
      33 IALAERALADAGVAPGDLRLVASLDARAEEPAILAAAQHFAVPAAFYDAA 82
              .        .        .        .        .
     297 ALREFEHHFPG.SGFVRKTVGVGSVSGPAAWLLS..QGQLLGETLREQGV 343
           .| . . :::. |::|   .|. :|.:.||:: .  :: |:.:.: .. .
      83 TLEAEASRLANPSEIVFAYTGCHGVAEGAALVGAGREAVLIVQKIVSAHA 132
                 .        .
     344 TITLGVAH..............  351
           | .|:.:
     133 TAALAGPATLRAEKRIQAAEAV 154
```

FIG. 7. Sequence alignment of *S. typhimurium cbiG* and *P. denitrificans cobE* gene products. The GAP program was used for this sequence comparison.

(ii) *S. typhimurium cbiD* and *cbiG* are *cobI* genes. The role of *cbiD* as a *cobI* gene was also further confirmed by the mutation of a plasmid-borne *cbiD* as described in Materials and Methods. The mutation of *cbiD* (pAR8977) led to a frameshift in the gene and produced a truncated protein of 262 amino acids. After this construct was cloned into *E. coli* together with the plasmid containing *cysG**, strain AR8985, which contained all the functional genes required for cobyric acid biosynthesis except for *cbiD*, was obtained. Examining extracts of this strain on the microbiological assay showed that AR8985 was unable to produce cobyric acid. This result confirmed that *cbiD* is a *cobI* gene.

No *S. typhimurium* mutant was found to be complemented by *cbiG*. Thus, an *E. coli* variant was produced by deletion of 417 bases of *cbiG* to give pAR8976 (see Materials and Methods and Table 2). When this plasmid was transformed into *E. coli* (generating AR8984), corrin production was undetectable by analysis of the cell extract by the microbiological assay.

Two-dimensional PAGE analyses of extracts of *E. coli* strains carrying mutations in *cbiD* and *cbiG* genes (AR8985 and AR8984) were performed. They showed that CbiE, CbiT, and CbiF, which are encoded downstream of *cbiD*, were visible as well as CbiH and CbiJ, which are encoded downstream of *cbiG* (data not shown). This indicated that there was no strong polar effect due to the mutations. These results established that *cbiD* and *cbiG* are both *cobI* genes. A computer search with the BLAST, FASTA, and GAP programs highlighted a sequence similarity between a region of the CbiG protein (amino acid 231 to 298) and the *P. denitrificans* CobE protein (amino acid 17 to 84) (Fig. 7). This suggests that the second half of the *cbiG* gene is similar to part of the *P. denitrificans cobE* gene; the function of CobE is still unknown even though the problem of the biosynthesis of vitamin B_{12} in *P. denitrificans* has apparently been solved (8).

(iii) *S. typhimurium cbiM*, *-N*, *-Q*, and *-O* are not required for de novo cobalamin biosynthesis in *E. coli*. No *S. typhimurium cob* mutants were complemented by DNA fragments containing *cbiM*, *-N*, *-Q*, and *-O*. These open reading frames show no

similarity to any *P. denitrificans cob* gene, but *cbiN*, *-P*, and *-O* are thought to be involved in cobalt transport (44). An *E. coli* strain harboring the complete *S. typhimurium cob* operon but in which the *cbiM*, *-N*, *-Q*, and *-O* genes had been deleted was analyzed for corrin production (AR8640 in Table 2). This strain was still able to make cobyric acid and cobinamide, producing levels similar to those found in AR8639 (Table 3). The possibility that one or several of these genes could be located on the *E. coli* genome remains; furthermore, the concentration of cobalt used in the culture conditions may compensate for the absence of active transport proteins (44). However, the fact that the *E. coli* strain produces 40 to 90 times more cobyric acid than the parent *S. typhimurium* suggests that under the experimental conditions used, *cbiM*, *-N*, *-Q*, and *-O* are not essential for corrin production in *E. coli.*

The *P. denitrificans cobI* gene cannot replace its *S. typhimurium* equivalent. The second methyltransferase which methylates precorrin-2 at position 20 is encoded by *cbiL*. DNA fragments carrying the *P. denitrificans* gene equivalent, *cobI*, were inserted into various plasmids (53). Two *S. typhimurium cbiL* mutants, AR3711 and AR3717 (Table 2), were transformed with these plasmids, but the resulting strains were not complemented for cobalamin production. One of these plasmids, pCR333 (53), cloned into *E. coli* was able to produce large quantities of the CobI protein, which was active in vitro; the protein was shown to convert precorrin-2 into precorrin-3 with good yields in experiments performed with *S. typhimurium* total cellular extracts as described in reference 42. The simplest interpretation is that the *P. denitrificans* CobI protein cannot efficiently replace its *S. typhimurium* counterpart protein, the *cbiL* gene product.

Absence of cobalt causes accumulation of precorrin-2 and -3. Further evidence for an early role of cobalt in cobalamin biosynthesis in the *S. typhimurium* pathway came from the analysis of accumulated intermediates in AR8830, the strain which produced one of the highest levels of cobyric acid, when it was grown in the absence of cobalt. When the medium was not supplemented with endogenous cobalt, corrin biosynthesis in this strain was reduced by a factor of 200. Analysis of the cytosolic contents of the cells after esterification, extraction, and separation as described in Materials and Methods led to the detection of the oxidized forms of both precorrin-2 and -3. The oxidized forms are referred to as factor II and III, respectively, and in this case they were isolated as their dilactone hexamethyl esters. The oxidized intermediates were identified on the basis of their UV and visible spectra, with maxima at A_{376} and A_{407} and mass spectra as follows: factor III dilactone, M + 1 = 957; factor II dilactone, M + 1 = 945) (Fig. 8 and data not shown) (7, 33). When AR8830 was grown in the presence of cobalt, no such intermediates could be detected but high levels of cobyric acid were obtained (Table 3; also see above). The accumulation of factors II and III in the absence of cobalt is thus similar to what has been observed with *Propionibacterium shermanii* (7, 8, 33).

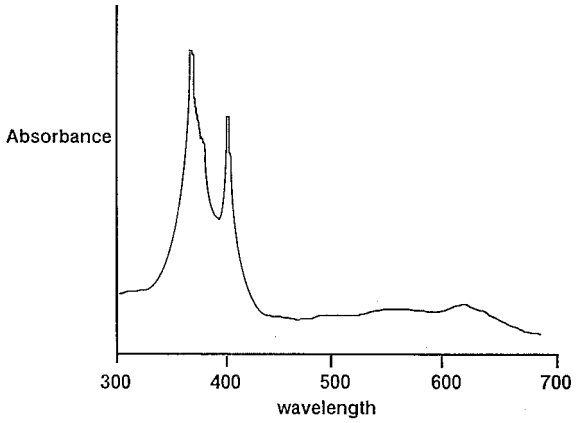

FIG. 8. UV and visible spectrum of a sample of factor II dilactone hexamethyl ester after extraction and purification from AR8639 cells grown in the absence of exogenous cobalt. The spectrum is identical to that reported by others (7, 33). No such intermediate was extracted when the cells were grown in the presence of cobalt.

DISCUSSION

A cosmid (pAR3062) carrying the *S. typhimurium* cobalamin genes was identified from a library of *S. typhimurium* genomic DNA by its ability to complement *S. typhimurium cob* mutants. The cosmid was isolated and restricted, and the *cobI* region was sequenced. When *E. coli* was transformed with this cosmid it was found that the cosmid endowed the bacterium with the ability to produce cobalamin. The levels of cobalamin in this *E. coli* strain were found to be similar to those produced by the parent *S. typhimurium* strain. The level of corrin production in AR8981 must reflect a level of expression of the cloned *cob* genes similar to that of the level of the chromosomal *S. typhimurium cob* genes. A rapid quantitative method for the measurement of cobalamin in the *E. coli* strains which was able to discriminate between cobyric acid and cobinamide levels was devised. The microbiological analysis could not, however, be used on intermediates produced prior to cobyric acid, presumably because the intermediates are not transported into the cell. The microbiological assay was found to be both sensitive and fairly rapid, but for chemical analysis of the cobalamin intermediates a much greater quantity of intermediate needed to be produced. For this reason, plasmids containing various combinations of the *S. typhimurium cob* region under the control of the *tac* promoter were made and were used to try to improve cobalamin biosynthesis in *E. coli.*

The construction of one such plasmid (pAR8827) and its transformation into *E. coli* (AR8830) did indeed allow the production of far greater quantities of cobyric acid. The level of cobyric acid produced in this strain reflects several aspects of the cloning procedures, including the promoter type, the copy number of the plasmid, and the increased activity of an overexpressed *cysG* gene, which encodes a branchpoint enzyme on the pathway. By diverting the majority of uroporphyrinogen III, the common progenitor to all modified tetrapyrroles, towards corrin construction, the enhanced CysG level increases flux along the cobalamin pathway (52).

Several derivatives of the *E. coli* cobalamin-producing strains in which genes of the *cob* operon were inactivated were constructed. The study of corrin production in these mutant strains, together with complementation studies of *S. typhimurium cob* mutants, demonstrated that, in vivo, at least 12 genes of this operon are necessary for cobalamin production. Ten of these genes have a *P. denitrificans* equivalent, whereas the other two, *cbiD* and *cbiG*, have no *P. denitrificans* sequence homologs. However, sequence similarity between the second half of *cbiG* and the *P. denitrificans cobE* gene was found. The significance of this finding is unclear as, although it is known that mutations in *cobE* lead to a cobalamin-negative phenotype in *P. denitrificans*, no function has yet been ascribed to the CobE protein. The studies reported here show that *cobS* and *cobT* are *cobIII* and *cobII* genes, respectively, and that *cbiA*, *-B*, *-C*, *-D*, *-E*, *-F*, *-G*, *-J*, *-L*, and *-P* are *cobI* genes. The functional status of *cbiK*, which has no homolog in *P. denitrificans*, and

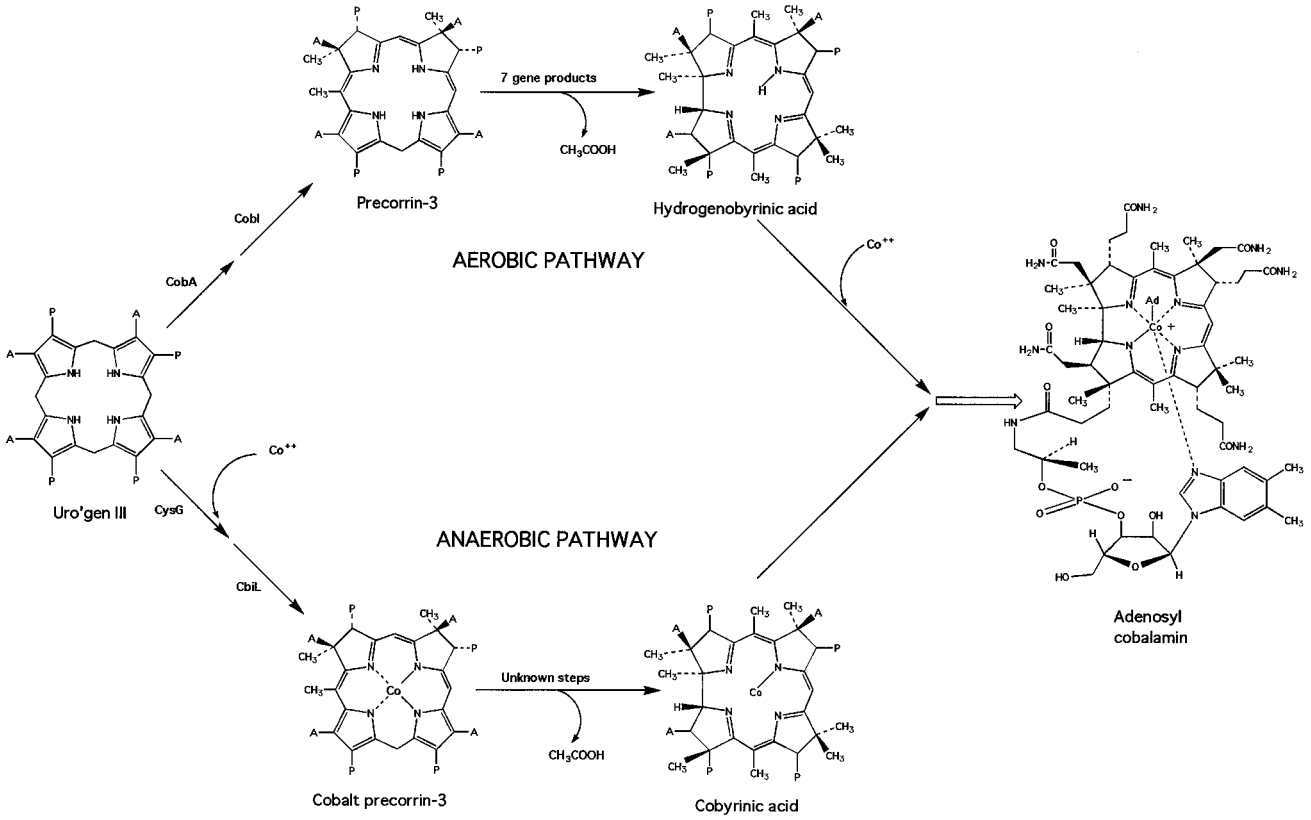

FIG. 9. The two distinct pathways for cobalamin biosynthesis. Uro'gen, uroporphyrinogen.

those of *cbiT* and *cbiH*, which are similar to *cobL* and *cobJ*, still need to be addressed.

One of the intriguing factors about cobalamin biosynthesis in *S. typhimurium* is that it occurs only under anaerobic conditions (29), and it is interesting that vitamin B_{12} production also only proceeds under anaerobic conditions when the *cob* operon has been cloned into *E. coli*. In *S. typhimurium*, transcription of the operon is controlled by cyclic AMP, the Arc system, and the *pocR* gene product (1, 12, 43, 44). In the *E. coli* cobalamin-producing strains, aerobic restriction of cobalamin biosynthesis must be mediated in some way other than by transcription initiation, since the upstream regulatory DNA regions and promoter have been removed. Furthermore, PAGE analysis has revealed that the encoded proteins of the cloned fragments are expressed both aerobically and anaerobically (data not shown). The anoxic dependency of cobalamin biosynthesis could be due to the instability of some of the intermediates on the pathway which are known to be sensitive to oxygen, or it could be due to regulation of an enzyme involved in the cobalamin pathway still present in the *E. coli* genome. Such an enzyme may be required for cobalt reduction. This result is therefore consistent with observations previously made by others (3, 43) with a mutant *S. typhimurium* strain which was able to express the genes of the *cob* operon under aerobic conditions but with no resulting cobalamin production.

When grown in the absence of exogenous cobalt, cultures of the corrin-producing *E. coli* AR8830 accumulated factors II and III, the oxidized forms of precorrin-2 and -3, respectively, indicating that cobalt is required for continuance of the pathway beyond precorrin-3. Furthermore, the *P. denitrificans cobI* gene does not complement *S. typhimurium cbiL* mutants, even though cell extracts contain active CobI protein. Both these observations are consistent with the hypothesis that the true substrate for CbiL is cobalt–precorrin-2, a substrate which may not be recognized by CobI of *P. denitrificans*. Likewise, the synthesis of precorrin-3 rather than cobalt–precorrin-3 would lead to a product that is not recognized by the proceeding enzyme in the pathway. Two such distinct pathways for the early part of corrin biosynthesis are outlined in Fig. 9. It is interesting that the CbiL protein does not make precorrin-3 from precorrin-2 with good yields in vitro (data not shown and reference 42). Thus, the *S. typhimurium* vitamin B_{12} biosynthetic pathway, like the pathway in *Propionibacterium shermanii* requires cobalt to be inserted at an early stage (5, 8, 11, 35, 48, 49).

It is also noteworthy that two *P. denitrificans cobI* genes, *cobF* and *cobG*, have no *S. typhimurium* homologs. The enzymes derived from these genes are known to be intricately involved in the ring contraction process during corrin formation. The enzymes in *P. denitrificans* are required in the order *CobI*, *-G*, *-J*, *-M*, *-F*, *-K*, *-L*, *-H* (references 8 and 23 and Table 1). It is possible that in *S. typhimurium* the CbiL protein, possibly in association with the *cbiD* and *cbiG* gene products, performs the early steps in a way different from that which has been observed with *P. denitrificans*. Thus, cobalt may be inserted at an early stage in the biosynthesis of the corrin ring to, for instance, aid in the ring contraction process as has been suggested by Scott et al. (48, 49). In this respect, it should be noted that *S. typhimurium* does not contain homologs to the *P. denitrificans cobN*, *-S*, and *-T*, which are required for the late chelation of cobalt.

These results demonstrate the importance of *E. coli* cobal-

amin-producing strains in elucidating the function of the individual *S. typhimurium cob* genes. Furthermore, this approach also allows the differences with other bacterial cobalamin pathways to be studied by exchanging homologous *cob* genes and analyzing the cobalamin production.

ACKNOWLEDGMENTS

We thank M. Ouhammouch for invaluable help in sequencing experiments and Y. d'Aubenton Carafa for friendly and constant help with computer work.

This work was supported by a research contract from Technogram-CNRS. Financial support from BBSRC is gratefully acknowledged by M.J.W.

REFERENCES

1. **Ailion, M., T. A. Bobik, and J. Roth.** 1993. Two global regulatory systems (Crp and Arc) control the cobalamin/propanediol regulon of *Salmonella typhimurium*. J. Bacteriol. **175:**7200–7208.
2. **Allen, L. N., and R. S. Hanson.** 1985. Construction of broad-host-range cosmid cloning vectors: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. J. Bacteriol. **161:**955–962.
3. **Andersson, D. I., and J. R. Roth.** 1989. Mutations affecting regulation of cobinamide biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **171:**6726–6733.
4. **Baker, H., H. Franck, I. Pasher, H. Sobotka, H. A. Nathan, S. H. Hunter, and S. A. Aaronson.** 1960. Elaboration of vitamin B12 by *Escherichia coli*. Experientia **16:**187.
5. **Balachandran, S., R. A. Vishwakarma, S. M. Monaghan, A. Prelle, N. P. J. Stamford, F. J. Leeper, and A. R. Battersby.** 1994. Pulse labelling experiments concerning the timing of cobalt insertion during vitamin B_{12} biosynthesis. J. Chem. Soc. Perkin Trans. I **1:**487–491.
6. **Beck, W. S.** 1982. Biological and medical aspects of vitamin B_{12}, p. 1–30. *In* D. Dolphin (ed.), B_{12}, vol. 2. John Wiley & Sons, New York.
7. **Bergmann, K.-H., R. Deeg, K. D. Gneuss, H.-P. Kriemler, and G. Muller.** 1977. Gewinnung von Zwischenprodukten der Cobyrinsäure-Biosynthese mit Zellsuspensionen von *Propionibacterium shermanii*. Hoppe-Seyler's Z. Physiol. Chem. **358:**1315–1323.
8. **Blanche, F., B. Cameron, J. Crouzet, L. Debussche, D. Thibaut, M. Vuilhorgne, F. J. Leeper, and A. R. Battersby.** 1995. Vitamin B_{12}: how the problem of its biosynthesis was solved. Angew. Chem. Int. Ed. Engl. **34:**383–411.
9. **Blanche, F., A. Famechon, D. Thibaut, L. Debussche, B. Cameron, and J. Crouzet.** 1992. Biosynthesis of vitamin B_{12} in *Pseudomonas denitrificans*: the biosynthetic sequence from precorrin-6y to precorrin-8x is catalyzed by the *cobL* gene product. J. Bacteriol. **174:**1050–1052.
10. **Blanche, F., L. Maton, L. Debussche, and D. Thibaut.** 1992. Purification and characterization of cob(II)yrinic acid *a*,*c*-diamide reductase from *Pseudomonas denitrificans*. J. Bacteriol. **174:**7452–7454.
11. **Blanche, F., D. Thibaut, L. Debussche, R. Hertle, F. Zipfel, and G. Müller.** 1993. Parallels and decisive differences in vitamin B_{12} biosynthesis. Angew. Chem. Int. Ed. Engl. **32:**1651–1653.
12. **Bobik, T., A. M. Ailion, and J. R. Roth.** 1992. A single regulatory gene integrates control of vitamin B_{12} synthesis and propanediol degradation. J. Bacteriol. **174:**2253–2266.
13. **Bonnett, R., J. M. Godfrey, and D. G. Redman.** 1969. Cobyric acid and related compounds from vitamin B12. J. Chem. Soc. Sect. C Org. Chem. **1969:**1163–1166.
14. **Brosius, J., and A. Holy.** 1984. Regulation of ribosomal RNA promoters with a synthetic lac operator. Proc. Natl. Acad. Sci. USA **81:**6929–6933.
15. **Cameron, B., K. Briggs, S. Pridmore, G. Brefort, and J. Crouzet.** 1989. Cloning and analysis of genes involved in coenzyme B_{12} biosynthesis in *Pseudomonas denitrificans*. J. Bacteriol. **171:**547–557.
16. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
17. **Chen, P., M. Ailion, N. Weyland, and J. Roth.** 1995. The end of the *cob* operon: evidence that the last gene (*cobI*) catalyzes synthesis of the lower ligand of vitamin B_{12}, dimethylbenzimidazole. J. Bacteriol. **177:**1461–1469.
18. **Collins, C., D. Gutman, and J. Isaza.** 1993. EMBL database, reference no. L25054. European Molecular Biology Laboratory, Heidelberg, Germany.
19. **Crouzet, J., B. Cameron, L. Cauchois, S. Rigault, M.-C. Rouyez, F. Blanche, D. Thibaut, and L. Debussche.** 1990. Genetic and sequence analysis of an 8.7-kilobase *Pseudomonas denitrificans* fragment carrying eight genes involved in transformation of precorrin-2 to cobyrinic acid. J. Bacteriol. **172:**5980–5990.
20. **Crouzet, J., L. Cauchois, F. Blanche, L. Debussche, D. Thibaut, M.-C. Rouyez, S. Rigault, J.-F. Mayaux, and B. Cameron.** 1990. Nucleotide sequence of a *Pseudomonas denitrificans* 5.4-kilobase DNA fragment containing five *cob* genes and identification of structural genes encoding *S*-adenosyl-L-methionine:uroporphyrinogen III methyltransferase and cobyrinic acid *a*,*c*-diamide synthase. J. Bacteriol. **172:**5968–5979.
21. **Crouzet, J., S. Levy-Schil, B. Cameron, L. Cauchois, S. Rigault, M.-C. Rouyez, F. Blanche, L. Debussche, and D. Thibaut.** 1991. Nucleotide sequence and genetic analysis of a 13.1-kilobase-pair *Pseudomonas denitrificans* DNA fragment containing five *cob* genes and identification of structural genes encoding cob(I)alamin adenosyltransferase, cobyric acid synthase, and bifunctional cobinamide kinase-cobinamide phosphate guanylyltransferase. J. Bacteriol. **173:**6074–6087.
22. **Debussche, L., M. Couder, D. Thibaut, B. Cameron, J. Crouzet, and F. Blanche.** 1992. Assay, purification, and characterization of cobaltochelatase, a unique complex enzyme catalyzing cobalt insertion in hydrogenobyrinic *a*,*c*-diamide during coenzyme B_{12} biosynthesis in *Pseudomonas denitrificans*. J. Bacteriol. **174:**7445–7451.
23. **Debussche, L., D. Thibaut, B. Cameron, J. Crouzet, and F. Blanche.** 1993. Biosynthesis of the corrin macrocycle of coenzyme B_{12} in *Pseudomonas denitrificans*. J. Bacteriol. **175:**7430–7440.
24. **Dellweg, H., E. Becher, and K. Bernhauer.** 1956. Über die biosynthetische Verwerbarkeit von Benzimidazolcarbonsaüren und verwandten Verbindungen. Biochem. Z. **328:**88.
25. **Drennan, C. L., S. Huang, J. T. Drummond, R. G. Matthews, and M. L. Ludwig.** 1994. How a protein binds B12: a 3.0 A X-ray structure of B_{12}-binding domains of methionine synthase. Science **266:**1669–1674.
26. **Eschenmoser, A.** 1988. Vitamin B_{12}: experiments concerning the origin of its molecular structure. Angew. Chem. Int. Ed. Engl. **27:**5–39.
27. **Ford, J. E., E. Holdsworth, and S. K. Kon.** 1955. The biosynthesis of vitamin B_{12}-like compounds. Biochem. J. **59:**86.
28. **Gilbert, W.** 1986. The RNA world. Nature (London) **319:**618.
29. **Jeter, R. M., B. M. Olivera, and J. R. Roth.** 1984. *Salmonella typhimurium* synthesizes cobalamin (vitamin B_{12}) de novo under anaerobic growth conditions. J. Bacteriol. **159:**206–213.
30. **Kirke, P. N., A. M. Mooly, L. E. Daly, H. Burke, D. G. Weir, and J. M. Scott.** 1993. Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects. QJM **86:**703–707.
31. **Kisliuk, R. L., and D. D. Woods.** 1960. Interrelationships between folic acid and cobalamin in the synthesis of methionine by extracts of *Escherichia coli*. Biochem. J. **75:**467–477.
32. **Macdonald, H., and V. J. Cole.** 1985. Molecular cloning and functional analysis of *cysG* and *NirB* genes of *E. coli* K12, two closely linked genes required for NADH-dependent reductase activity. Mol. Gen. Genet. **200:**328–334.
33. **Muller, G., R. Deeg, K. D. Gneuß, G. Gunzer, and H.-P. Kriemler.** 1979. On the methylation process in cobyrinic acid biosynthesis, p. 279–291. *In* B. Zagalak and W. Friedrich (ed.), Vitamin B_{12}. Walter de Gruyter, Berlin.
34. **Müller, G., K. Hlineny, E. Savvidis, F. Zipfel, J. Schiedl, and E. Schneider.** 1990. On the methylation process and cobalt insertion in cobyrinic acid biosynthesis, p. 281–298. *In* T. O. Baldwin (ed.), Chemical aspects of enzyme biotechnology. Plenum Press, New York.
35. **Müller, G., F. Zipfel, K. Hlineny, E. Savvidis, R. Hertle, U. Traub-Eberhard, A. I. Scott, H. J. Williams, N. J. Stolowich, P. J. Santander, M. Warren, F. Blanche, and D. Thibaut.** 1991. Timing of cobalt insertion in vitamin B_{12} biosynthesis. J. Am. Chem. Soc. **113:**9893–9895.
36. **O'Farrell, P.** 1975. High resolution of 2-dimension electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
37. **O'Farrell, P.** 1977. High resolution of 2-dimension electrophoresis of basic as well as acidic proteins. Cell **12:**1133–1142.
38. **O'Toole, G. A., and J. C. Escalante-Semerena.** 1993. *cobU*-dependent assimilation of nonadenosylated cobinamide in *cobA* mutants of *Salmonella typhimurium*. J. Bacteriol. **175:**6328–6336.
39. **O'Toole, G. A., M. R. Rondon, and J. C. Escalante-Semerena.** 1993. Analysis of mutants of *Salmonella typhimurium* defective in the nucleotide loop of cobalamin. J. Bacteriol. **175:**3317–3326.
40. **Palva, E. T., and P. Liljeström.** 1981. Cosmid cloning and transposon mutagenesis in *Salmonella typhimurium* using phage λ vehicles. Mol. Gen. Genet. **181:**153–157.
41. **Richter-Dahlfors, A. A., and D. I. Andersson.** 1992. Cobalamin (vitamin B_{12}) repression of the Cob operon in *Salmonella typhimurium* requires sequences within the leader and the first translated open reading frame. J. Mol. Biol. **6:**743–749.
42. **Roessner, C. A., M. J. Warren, P. J. Santander, B. A. Atshaves, S. I. Ozaki, N. J. Stolowich, K. Iada, and A. I. Scott.** 1992. Expression of 9 *Salmonella typhimurium* enzymes for cobinamide synthesis. FEBS Lett. **301:**73–78.
43. **Rondon, M. R., and J. C. Escalante-Semerena.** 1992. The *poc* locus is required for 1,2-propanediol-dependent transcription of the cobalamin biosynthetic (*cob*) and propanediol utilization (*pdu*) genes of *Salmonella typhimurium*. J. Bacteriol. **174:**2267–2272.
44. **Roth, J. R., J. G. Lawrence, M. Rubenfield, S. Kieffer-Higgins, and G. M. Church.** 1993. Characterization of cobalamin (vitamin B_{12}) biosynthetic genes of *Salmonella typhimurium*. J. Bacteriol. **175:**3303–3316.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

46. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
47. **Schneider, Z., and A. Stroinski.** 1987. Methylcobamide-dependent reactions, p. 259–266. *In* Z. Schneider and A. Stroinski (ed.), Comprehensive B_{12}. Walter de Gruyter, Berlin.
48. **Scott, A. I.** 1994. On the duality of mechanism of ring contraction in vitamin B_{12} biosynthesis. Heterocycles **39:**471–476.
49. **Scott, A. I., C. A. Roessner, N. J. Stolowich, J. B. Spencer, C. Min, and S. I. Ozaki.** 1993. Biosynthesis of vitamin B12. Discovery of the enzymes for oxidative ring contraction and insertion of the fourth methyl group. FEBS Lett. **331:**105–108.
50. **Stroinski, A.** 1987. Adenosylcobamide-dependent reactions, p. 226–259. *In* Z. Schneider and A. Stroinski (ed.), Comprehensive B_{12}. Walter de Gruyter, Berlin.
51. **Trzebiatowski, J. R., G. A. O'Toole, and J. C. Escalante-Semerena.** 1994. The *cob*T gene of *Salmonella typhimurium* encodes the NaMN:5,6-dimethylbenzimidazole phosphoribosyltransferase responsible for the synthesis of N^1-(5-phospho-α-D-ribosyl)-5,6-dimethylbenzimidazole, an intermediate in the synthesis of the nucleotide loop of cobalamin. J. Bacteriol. **176:**3568–3575.
52. **Warren, M. J., E. L. Bolt, C. A. Roessner, A. I. Scott, J. B. Spencer, and S. C. Woodcock.** 1994. Gene dissection demonstrates that the *Escherichia coli cysG* gene encodes a multifunctional protein. Biochem. J. **302:**837–844.
53. **Warren, M. J., C. A. Roessner, S. Ozaki, P. J. Santander, N. J. Stolowich, and A. I. Scott.** 1992. Enzymatic synthesis and structure of precorrin-3, the trimethyldipyrrocorphin intermediate in vitamin B_{12}. Biochemistry **31:**603–609.
54. **Warren, M. J., C. A. Roessner, P. J. Santander, and A. I. Scott.** 1990. The *Escherichia coli cysG* gene encodes *S*-adenosylmethionine-dependent uroporphyrinogen III methylase. Biochem. J. **265:**725–729.
55. **White, H. B., III.** 1976. Coenzymes as fossils of an earlier metabolic state. J. Mol. Evol. **7:**101.