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cobA Function Is Required for Both De Novo Cobalamin Biosynthesis and Assimilation of Exogenous Corrinoids in *Salmonella typhimurium*

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***Salmonella typhimurium* is able to synthesize cobalamin (B_{12}) under anaerobic growth conditions. The previously described cobalamin biosynthetic mutations (phenotypic classes CobI, CobII, and CobIII) map in three operons located near the *his* locus (minute 41). A new class of mutant (CobIV) defective in B_{12} biosynthesis was isolated and characterized. These mutations map between the *cysB* and *trp* loci (minute 34) and define a new genetic locus, *cobA*. The anaerobic phenotype of *cobA* mutants suggests an early block in corrin ring formation; mutants failed to synthesize cobalamin de novo but did so when the corrin ring is provided as cobyric acid dicyanide or as cobinamide dicyanide. Under aerobic conditions, *cobA* mutants were unable to convert either cobyric acid dicyanide or cobinamide dicyanide to cobalamin but could use adenosylcobyric acid or adenosylcobinamide as a precursor; this suggests that the mutants are unable to adenosylate exogenous corrinoids. To explain the anaerobic CobI phenotype of a *cobA* mutant, we propose that the *cobA* gene product catalyzes adenosylation of an early intermediate in the de novo B_{12} pathway and also adenosylates exogenous corrinoids. Under anaerobic conditions, a substitute function, known to be encoded in the main Cob operons, is induced; this substitute function can adenosylate exogenous cobyric acid and cobinamide but not the early biosynthetic intermediate. The *cobA* gene of *S. typhimurium* appears to be functionally equivalent to the *btuR* gene of *Escherichia coli*.**

The de novo biosynthesis of cobalamin (B_{12}) in *Salmonella typhimurium* occurs only under anaerobic growth conditions (15). Under aerobic conditions, B_{12} can be made only if the corrin ring is supplied exogenously. There are three distinct classes of (*cob*) mutants deficient in the synthesis of cobalamin. The Cob phenotypes are defined below, and the structures of adenosyl (Ado)-B_{12} and precursors used in this paper are presented in Fig. 1 and 2. (i) CobI mutants can make B_{12} if cobinamide (Cbi) is provided; they are inferred to be unable to synthesize the corrin ring. (ii) CobII mutants can make B_{12} only if 5,6-dimethylbenzimidazole (DMB) is provided; they are inferred to be blocked in DMB synthesis. (iii) CobIII mutants cannot make B_{12} even if both Cbi and DMB are provided; it is inferred that they cannot join these precursors to form B_{12}. If either compound is not a true intermediate, CobIII mutants could be blocked in converting either compound to a true intermediate.

The phenotypes of these mutants are corrected by providing vitamin B_{12} (CN-cobalamin). All previously known mutants with these phenotypes are located in three operons (CobI, CobII, and CobIII) mapping at 41 min on the linkage map of *S. typhimurium*; mutations in these operons are designated *cob* (15). Expression of these operons is repressed by B_{12} and by aerobic growth conditions and is stimulated by cyclic AMP (10).

The ability to synthesize or utilize B_{12} can be scored genetically by using various metabolic processes that require it. To monitor the presence of B_{12} without requiring adenosylation, we measure methionine-independent growth of strains with a *metE* mutation. This growth requires use of the B_{12}-dependent homocysteine methyltransferase, encoded by the *metH* gene (25). All the strains used carry *metE* mutations, and their methionine synthesis (via the MetH enzyme) depends on B_{12}. The MetH enzyme can use B_{12} provided as HO-B_{12} or CN-B_{12} and does not require Ado-B_{12} (25); cob(I)alamin is used as a transient recipient of the methyl group donated by N^5-methyltetrahydrofolate to homocysteine. To genetically monitor the presence of Ado-B_{12}, we measured the ability of cells to grow using ethanolamine as a carbon and/or nitrogen source. Ethanolamine utilization requires activity of the Ado-B_{12}-dependent enzyme ammonia-lyase, encoded by the *eutB* and *eutC* genes (21).

Since *S. typhimurium* cannot synthesize cobalamin de novo under aerobic conditions, *metE* mutants are methionine auxotrophs in the presence of oxygen. A *cob*$^+$ *metE* mutant can synthesize methionine (using the MetH enzyme) if the corrin ring is provided (cobyric acid [Cby], Cbi, or B_{12}). Thus, only corrin ring synthesis is defective under aerobic conditions; synthesis of DMB and joining of DMB to a corrinoid (presumably Ado-Cbi) can occur under both aerobic and anaerobic conditions.

The *cob* mutants described above were isolated as derivatives of *metE* mutants that are unable to synthesize B_{12} (and therefore methionine) when grown anaerobically. In the process of characterizing *cob* mutants, a class was found that is phenotypically distinct from those described above; mutations causing this phenotype map far from the other *cob* mutations. We describe here the genetic and nutritional characterization of these new mutants (*cobA*) and present evidence that they are blocked in adenosylation of corrinoid compounds. Results suggest that adenosylation is an obligatory, early step in de novo B_{12} synthesis and that exogenous corrinoids like Cby and Cbi are not true synthetic intermediates but must be adenosylated before they can be joined to DMB to form a complete B_{12} (Ado-B_{12}).

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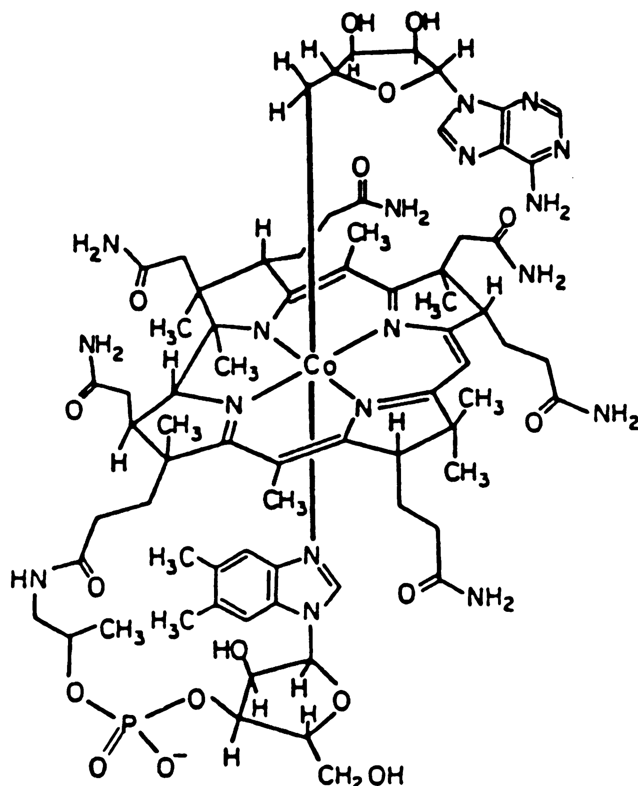

FIG. 1. Chemical structure of coenzyme B_{12} (Ado-B_{12}).

MATERIALS AND METHODS

Bacteria, media, and growth conditions. All bacterial strains used are derivatives of *S. typhimurium* LT2, and their genotypes are listed in Table 1. The genotypes and sources of *Escherichia coli* strains and plasmids used in this work are also listed in Table 1. Three derivatives of the specialized transducing bacteriophage Mu d1(Ap^r *lac*) were used in this study (5). Mu d1-8 is a conditionally defective transposon which will be referred in this report as Mu dA (12). Mu d2-8 is a derivative of Mu d1-8 which now forms protein fusion and will be referred as Mu dB (14). Phage Mu d11734(Km^r Lac^+) which carries the deletions of the necessary genes for transposition will be referred as Mu dJ (6). Nutrient broth (0.8% [wt/vol]; Difco Laboratories, Detroit, Mich.) containing NaCl (85 mM) was used as a complex medium. The E medium of Vogel and Bonner (24) supplemented with glucose (11 mM) and trace metals (3) was used as a minimal medium. The final concentration of cobalt in the medium was maintained at 1 μM. A no-nitrogen medium, NCN (20), was used to score the utilization of ethanolamine as a nitrogen source. For this purpose, Noble agar (1.5% [wt/vol]) was used to reduce the level of contaminating nitrogenated compounds. The following compounds were supplied at final concentrations of: magnesium, 1 mM; ethanolamine hydrochloride, 20 mM; glucose, 11 mM; and methionine, 0.5 mM. Experiments with liquid medium were performed in 125-ml flasks containing 5 ml of medium to maximize aeration. Cultures were incubated in a forced-air orbital incubator (Lab-Line) and shaken at 175 rpm. Cell growth was monitored with a Klett-Summerson colorimeter. Whenever added, the final concentration of antibiotics in the complex medium was as follows (micrograms per milliliter): tetracycline, 20; kanamycin, 50; ampicillin, 30; streptomycin, 2 mg/ml. In minimal medium, the concentration of antibiotics was as follows (micrograms per milliliter): tetracycline, 10; kanamycin, 125; ampicillin, 15; streptomycin, 2 mg/ml. Solid medium contained, unless otherwise stated, 1.5% Bacto-Agar (Difco). When the following compounds were present in the medium, the concentrations were tryptophan, 0.1 mM; uracil, 0.1 mM; cystine, 0.3 mM; 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal), 20 mg/liter; $Cbi(CN)_2$, 15 nM; B_{12} (CN or OH or CH_3 or Ado), 15 nM.

Anaerobic growth conditions. Studies requiring anaerobic incubation were performed inside an anaerobic chamber (model 1024; Forma Scientific) whose atmosphere contained N_2-H_2-CO_2 (90%:5%:5%). Anoxic liquid medium was prepared as previously described (10).

Genetic techniques. (i) P22-mediated transductions. Transductional crosses were performed with a derivative of bacteriophage P22 that contains the mutation HT *105/1*, which increases the frequency of generalized transduction (22, 23), and the mutation *int*-201, which prevents the formation of stable lysogens (24). All crosses were performed at a multiplicity of infection of approximately 1. A rapid procedure for obtaining phage lysates with this phage has been described previously (8). Crosses to be plated on kanamycin- or streptomycin-containing medium were first plated on drug-free medium to allow expression of the drug-resistant phenotype and then replica printed onto selective medium.

(ii) DNA isolation and transformation. Plasmid DNA isolation and transformation protocols were as described by Davis et al. (8).

(iii) Isolation of Tn*10* element inserted near the *cobA* locus. A pool of approximately 32,000 independent Tn*10* insertions was obtained as described elsewhere (8) and used as the donor in a transductional cross with strain TT10331 (*metE205 cobA28*::Mu dA) as the recipient. One strain carrying a Tn*10* element 60% cotransducible with the Mu dA insertion (strain TT13897) was isolated and used for preliminary Hfr mapping of *cobA*.

(iv) Direction of transcription of *cobA*. The direction of transcription of *cobA* was determined by the method of Hughes and Roth (13). A transduction cross is performed with approximately equal numbers of phage particles grown on two strains with each one carrying a Mu dA insertion. One insertion is in a gene whose direction of transcription is known (e.g., *hisF*:: Mu dA; strain TT7690 or TT7693), and the other insertion is in the *cobA* gene (TT10331 or JE1680). The mixed lysates are used to transduce a recipient (strain TR6583) to Ap^r. Prototrophic transductants arise only when two different fragments carrying the Mu dA insertions recombine with each other and then with the chromosome to generate a duplication with the hybrid Mu dA element at the join point. The resulting strain is phenotypically His^+ Cob^+ and carries a duplication of the material between the sites of the donor insertion mutations. These duplication-bearing transductants only arise if both donor insertions are in the same orientation. If the orientation of one Mu d fusion is known, the orientation of a second (unknown) Mu d can be determined. If the unknown Mu d is known to be fused to a particular transcript, the orientation of that transcript is thus determined. To determine the direction of transcription of *cobA* by the above technique, we converted *cobA343*::Mu dJ (TT12895) into *cobA343*::Mu dA (JE1680) by the method of Castilho et al. (6). This was done to increase the size of the shared homology and thus the frequency of recombination between two Mu d elements.

(v) Isolation of *cobA*::Tn*10d*(Tc) strain. A pool of approximately 40,000 independent Tn*10d*(Tc) insertions was pre-

TABLE 1. Strain list

Strain	Genotype	Source
Salmonella typhimurium		
TR122	*pyrF146 cysB12 trpA52*	
TR628	*pyrC7 strA1*(F' *ts*114 *lac*$^+$ *zzf-21*::Tn*10*)	
TR629	*pyrC7 strA1*(F' *ts*114 *lac*$^+$ *zzf-22*::Tn*10*)	
TR6583	*metE205 ara-9*	
TT7690	*hisF9951*::Mu dA	
TT7693	*hisF9954*::Mu dA	
TT10331	*metE205 ara-9 cobA28*::Mu dA	
TT10852	*metE205 ara-9 cob-24*::Mu dJ	
TT10857	*metE205 ara-9 cob-62*::Mu dJ	
TT10858	*metE205 ara-9 cob-66*::Mu dJ	
TT12895	*metE205 ara-9 cobA343*::Mu dJ	This study
JE1680	*metE205 ara-9 cobA343*::Mu dA	This study
JE1314	*metE2113*::Mu dJ DE*topA-trp* (*supX33*)	This study
TT13582	*metE205 ara-9 trp-2482*::Mu dA	This study
TT13584	*metE205 ara-9 cobA367*::Tn*10d*(Tc)	This study
TT13897	*metE205 ara-9 zdd-3672*::Tn*10*	This study
TT14266	*metE205 ara-9 trp-2451*::Tn*10*	This study
TT14298	*metE205 ara-9 cob-24*::Mu dJ *cobA367*::Tn*10d*(Tc)	This study
TT14299	*metE205 ara-9 cob-62*::Mu dJ *cobA367*::Tn*10d*(Tc)	This study
TT14300	*metE205 ara-9 cob-66*::Mu dJ *cobA367*::Tn*10d*(Tc)	This study
Escherichia coli strains and plasmids		
RK6725	(*argF-lac*)*U169 araD139 thi non relA1 rpsL150 metE70 deoC1 flbB5301 gyrA* Φ(*btuB-lac*)*481 btuR482*::Tn*10*Δ16Δ17	R. J. Kadner
RK6725(pMJF4)	*btuR*$^+$	R. J. Kadner
RK6725(pML46-1)	*btuR*$^+$	R. J. Kadner
RK6725(pML46-1)	*btuR*::Tn*1000*	R. J. Kadner
pJW80	*topA*$^+$	J. Wang via R. Menzel

pared as described elsewhere (9). This pool was used as the donor to transduce a tryptophan auxotroph (strain TT13582) to prototrophy on minimal medium supplemented with methionine. The tetracycline resistance and CobIII phenotypes of the resulting prototrophs were determined. Putative insertions in *cobA* were mobilized by transduction into a *metE cobA*$^+$ strain (strain TR6583), selecting tetracycline resistance and determining all three CobA phenotypes. Strain TT13584 was used in subsequent experiments.

(vi) Isolation of *cobA*::Mu dJ operon fusion. A pool of approximately 50,000 independent and random Mu dJ insertions was prepared as previously described (14). This pool was used as the donor to transduce a tryptophan auxotroph (strain TT14266) to prototrophy on minimal medium containing glucose, methionine, X-Gal, and kanamycin. Kanamycin-resistant (Km^r) transductants were replica printed onto minimal medium containing glucose, kanamycin, X-Gal, EGTA, and either Cbi(CN)$_2$ and DMB or CN-B$_{12}$. Strain TT12895 (Km^r X-Gal$^+$) was unable to grow aerobically on minimal medium supplemented with Cbi(CN)$_2$ and DMB. This strain was used in subsequent experiments.

^{57}Co labeling experiments. $^{57}CoCl_2$ (carrier-free, ~7 Ci/mg) was obtained from ICN Biomedicals, Inc. Cells (1 ml) from full-density cultures grown anaerobically in E minimal medium with glycerol (22 mM) and fumarate (10 mM) were centrifuged for 10 min at room temperature. The pellet was suspended in 1 ml of 50 mM KP$_i$ (pH 7.0) and centrifuged again for 10 min at room temperature. The pellet was suspended in 40 μl of 50 mM sodium borate buffer (pH 9.0) containing KCN (20 μg/ml). The cell suspension was boiled for 15 min in a water bath, the cells were pelleted by centrifugation, and the supernatant was saved. Samples (2 to 4 μl) of these supernatants were spotted on high-performance silica gel plates (Sigma Chemical Co., St. Louis, Mo.) and developed with a mixture of a *sec*-butanol-isopropanol-H_2O-ammonia (30:40:25:2) system containing KCN (10 μg/ml). The developing time was 3 to 4 h. Authentic Cbi(CN)$_2$, CN-B$_{12}$, and Cby(CN)$_2$ were used as standards and were spotted on the same plates in separate lanes. Autoradiographs were obtained with Kodak XAR-5 film (8 by 10 in.) after 48 h of exposure. R_f values were determined based on the mobility of Cbi(CN)$_2$ (R_{Cbi}).

β-Galactosidase activity assays. β-Galactosidase activity was assayed by the method of Miller (19) as described elsewhere (10).

Chemicals. Ado-Cbi, Ado-Cby, and Cby(CN)$_2$ were generous gifts from Rhône-Poulenc Santé, 94403 Vitry sur Seine Cedex, France. All other B$_{12}$ derivatives were purchased from Sigma. All compounds were used without further purification.

RESULTS

Phenotype of *cobA* mutants. The first *cobA* mutants were isolated from *metE* mutant parent strains by identifying derivatives that were unable to synthesize B$_{12}$ (and therefore methionine) under anaerobic conditions. Further phenotypic characterization of *cobA* mutants was done by scoring their ability to synthesize methionine aerobically and anaerobically when various corrinoid compounds were provided; these tests score synthesis of cobalamin without requiring Ado-B$_{12}$. Ability to synthesize Ado-B$_{12}$ was assessed by the ability of the mutants to grow with ethanolamine as a nitrogen source (Eut/N) aerobically or anaerobically when various forms of B$_{12}$ were provided in the medium. All the many *cobA* mutants tested showed the phenotypes described below.

(i) Anaerobic phenotypes. Table 2 (top) presents the MetH

TABLE 2. Phenotypes of *cobA* mutants

Condition and relevant genotype[a]	MetH phenotype with indicated medium supplement[b]						Eut(N) phenotype with indicated medium supplement[c]			
	No addition	$Cby(CN)_2$	Ado-Cby	$Cbi(CN)_2$	Ado-Cbi	$CN\text{-}B_{12}$	No addition	$CN\text{-}B_{12}$	$Me\text{-}B_{12}$	$Ado\text{-}B_{12}$
Anaerobic										
metE	+	+	+	+	+	+	+	+	+	+
metE cobA	−	+	+	+	+	+	−	+	+	+
Aerobic										
metE	−	+	+	+	+	+	+	+	+	+
metE cobA	−	−	+	−	+	+	−	−	−	+

[a] Strains assayed are TR6583 (*metE cobA*$^+$) and TT12895 (*metE cobA*).
[b] A MetH$^+$ phenotype denotes growth observed on solid minimal glucose medium containing the indicated supplement. Compounds $CN\text{-}B_{12}$ and $Cbi(CN)_2$ were added to the medium at 15 and 18 nM, respectively; response to all other compounds was determined by observing growth around a filter paper disk containing 3 μl of a 4 nM solution of each compound. +, Requires intracellular B_{12}.
[c] A Eut(N)$^+$ phenotype denotes growth observed on solid NCN medium containing glucose as the sole carbon source and ethanolamine (20 mM) as the sole nitrogen source. All corrinoids were present in the medium at 15 nM. +, Requires intracellular $Ado\text{-}B_{12}$.

and Eut/N phenotypes seen for cells growing on glucose medium without oxygen. Under these conditions, wild-type (*cobA*$^+$) cells could perform de novo synthesis of B_{12} forms including $Ado\text{-}B_{12}$; this is shown by the Met$^+$ and Eut/N$^+$ phenotypes observed even without any added corrinoids. The *cobA* mutant could not synthesize B_{12} de novo but could convert any of the provided corrinoids to B_{12}, as judged by the MetH$^+$ phenotype seen when Cby, Cbi, or cobalamin was provided with or without the Ado moiety. The Eut$^+$ phenotype seen anaerobically demonstrates that *cobA* mutants can produce complete $Ado\text{-}B_{12}$ cofactor from any of the corrinoid precursors including $CN\text{-}B_{12}$. These results suggest that the defect of *cobA* mutants under anaerobic conditions is an early block in corrin ring synthesis preceding Cby (or Ado-Cby) in the pathway.

(ii) Aerobic phenotypes. Table 2 (bottom) presents the MetH and Eut/N phenotypes observed during growth on glucose in the presence of oxygen. Under these conditions, de novo B_{12} production was not expected, since even wild-type (*cobA*$^+$) cells cannot make B_{12} aerobically. Wild-type cells could, however, convert any of the provided corrinoids to B_{12} as judged by the Met$^+$ phenotypes observed. These corrinoids could be converted by *cobA*$^+$ cells to $Ado\text{-}B_{12}$, as demonstrated by the Eut/N$^+$ phenotype. In contrast, the *cobA* mutant failed to synthesize B_{12} from either Cby (the fully decorated corrin ring) or from Cbi (same ring as Cby but with the aminopropanol side chain added) (Fig. 1 and 2). The mutant could synthesize B_{12} if the adenosylated form of either one of the precursors was provided (Ado-Cby or Ado-Cbi). Two conclusions can be drawn from these results. (i) The *cobA* gene product is required under aerobic conditions for assimilation of nonadenosylated precursors; and (ii) adenosylation of these intermediates is a prerequisite of their conversion to B_{12}. Note that the MetH$^+$ phenotype does not itself require the formation of $Ado\text{-}B_{12}$. Thus, the requirement of *cobA* mutants for adenosylated precursors suggests that the completion of cobalamin can only be accomplished with adenosylated precursors.

The Eut/N$^+$ phenotype of *cobA* mutants was observed

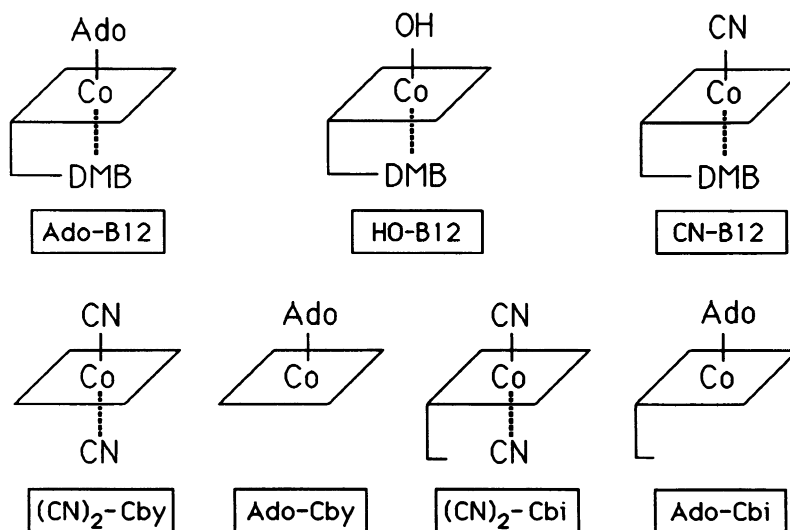

FIG. 2. Sketches of compounds used. The boxlike structure represents the fully decorated corrin ring seen in Fig. 1 for $Ado\text{-}B_{12}$. The bracket below the ring represents the aminopropanol side chain and ribose moiety through which DMB is attached to the ring. The structure of DMB can be seen as part of the $Ado\text{-}B_{12}$ molecule in Fig. 1.

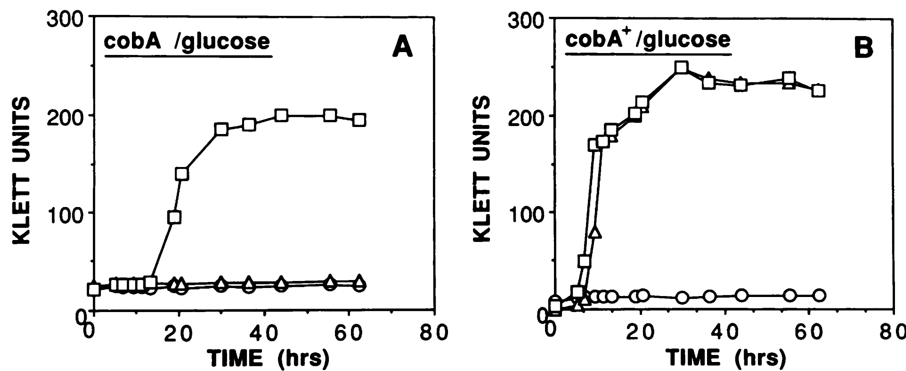

FIG. 3. Phenotype of a *cobA* mutant in liquid culture. Symbols: □, Ado-B_{12}; △, CN-B_{12}; ○, no addition. For both experiments, ethanolamine was the sole source of nitrogen. The concentrations of the supplements to the medium and other culture conditions are described in Materials and Methods. The medium contained methionine to minimize selection for *metE*$^+$ revertants. Overnight nutrient broth cultures of strains TR6583 (*cobA*$^+$) and TT12895 (*cobA*) were used as inocula. Shown are the growth response to Ado-B_{12} and to CN-B_{12} of a *cobA* mutant (A) and the *cobA*$^+$ parent strain (B).

when Ado-B_{12} was provided but not when CN-B_{12} or methyl (Me)-B_{12} was provided. The inability to use ethanolamine (with CN-B_{12} or Me-B_{12}) was based on growth observed on plates after 12 h of incubation; at this time, the growth difference between the wild type and the *cobA* mutant was very clear. However, after extended incubation on plates (36 h), some growth of the *cobA* mutant was noted.

The slight growth seen on plates aerobically appeared to be due to a fraction of cells that were limited in their access to oxygen. To test this possibility, we grew the cells in liquid medium under strong aeration with glucose as the carbon and energy source (Fig. 3A). Under these conditions, a *cobA* mutant was unable to utilize ethanolamine as a nitrogen source unless Ado-B_{12} was provided (compare Fig. 3A and B); the growth seen on plates was not observed during aeration of liquid cultures, even after prolonged incubation (65 h). Similar results were obtained when HO-B_{12} or Me-B_{12} was used as a source of B_{12} (data not shown). We conclude that under truly aerobic conditions, *cobA* mutants are defective in adenosylation of B_{12}. The residual growth of *cobA* mutants on plates is presumed to be due to some cells becoming anoxic with the concomitant expression of an alternative function that suppresses the *cobA* defect. This is expected since under anaerobic conditions (described above) *cobA* mutants show no Eut defect when growing on CN-B_{12}.

Aerobic growth of the *cobA* mutant cells on ethanolamine-glucose with provided Ado-B_{12} is twofold slower than that for wild type (μ = 0.3 doublings h^{-1} compared with μ = 0.63 doublings h^{-1}, respectively). This may be due to poor transport of Ado-B_{12} (C. Grabau and J. R. Roth, unpublished data).

Map location of *cobA*. After preliminary conjugation crosses showed that the *cobA* mutations map near the *trp* locus, a three-factor transduction cross was performed to refine the mapping. P22 transducing phage grown on a *cobA343*::Mu dJ insertion (TT12895) was used as donor in crosses with a recipient strain (TR122) carrying three mutations in the region of interest (*pyrF146*, *cysB12*, and *trpA52*). Minimal medium containing tryptophan and uracil was used to select for Cys$^+$ transductants. A total of 993 Cys$^+$ transductants were analyzed for the frequency of cotransduction of the Mu dJ (Km^r), *pyrF*$^+$, and *trp*$^+$ markers. The results of this cross are summarized in Table 3.

From these results, we conclude that *cobA* is located between *cysB* and the tryptophan biosynthetic operon (*trp*), with *cobA* being closer to *trp* (75% cotransduction frequency) than to *cysB* (33% cotransduction frequency). This position was confirmed by checking the phenotypes of several *trp* deletion mutations. Deletion (DE) *supX33*, which has a right endpoint within the *trp* operon and extends leftward out of the *trp* operon into the *topA* locus, has a CobA phenotype. Another deletion (DE*trp-43*) extends rightward from *trp* but retains CobA$^+$ function (4).

We demonstrated that *cobA* is distinct from the topoisomerase gene (*topA*) by Southern hybridization. A plasmid carrying a wild-type copy of the *topA* gene (pJW80, obtained from James Wang via Rolf Menzel) was used as a probe and hybridized to genomic DNA from a *cobA*$^+$ strain, a strain carrying a *cobA* insertion mutation (*cobA343*::Mu dJ), and strain carrying *supX33* (a generous gift from Paul Margolin). For genomic DNA cut with *Bgl*II, there was no difference between the band of hybridizing material seen in wild type and in the *cobA* insertion mutant, demonstrating that the regions of hybridization do not include the site of the *cobA* insertion. The hybridization pattern observed with *supX33* DNA clearly altered the band that corresponded to *topA*, demonstrating that in fact the probe carries material in this region. Further evidence that *cobA* is distinct from *topA* was obtained by complementation tests. When introduced into a *cobA* mutant, plasmid pJW80 failed to correct the CobA phenotype of the strain.

Direction of transcription of *cobA*. The direction of transcription of *cobA* was determined by transduction crosses

TABLE 3. Three-factor cross[a]

Phenotype of selected (Cys$^+$) transductants[b]				No. of exhanges	No. of recombinants	Relative frequency (% total)
PyrF	CysB	Km	Trp			
+	+	r	+	2 (donor type)	201	20
+	+	s	+	4	13	1
+	+	r	−	2	90	9
+	+	s	−	2	373	38
−	+	r	+	2	168	17
−	+	s	+	4	10	1
−	+	r	−	2	38	4
−	+	s	−	2	100	10

[a] Donor (TT12895): *pyrF*$^+$ *cysB*$^+$ *cobA343*::Mu dJ (Km^r) *trp*$^+$. Recipient (TR122): *pyrF cysB cobA*$^+$ (Km^s) *trp*.

[b] Cys$^+$ transductants were selected on E minimal medium supplemented with glucose, 11 mM; tryptophan, 0.1 mM; and uracil, 0.1 mM. Markers are listed horizontally according to the inferred map order.

TABLE 4. Direction of transcription of *cobA*[a]

Donor(s)	Nutritional requirement of selected Ap^r transductants (no.)		
	His⁻	B_{12}^- (MetH⁻)	None
hisF9951::Mu dA (Lac⁻)	100	0	0
hisF9954::Mu dA (Lac⁺)	100	0	0
cobA343::Mu dA (Lac⁺)	0	100	0
cobA28::Mu dA (Lac⁻)	0	100	0
hisF9951::Mu dA (Lac⁻) × *cobA343*::Mu dA (Lac⁺)	77	23	0
hisF9951::Mu dA (Lac⁻) × *cobA28*::Mu dA (Lac⁻)	80	12	8
hisF9954::Mu dA (Lac⁺) × *cobA343*::Mu dA (Lac⁺)	61	35	4
hisF9954::Mu dA (Lac⁺) × *cobA28*::Mu dA (Lac⁻)	85	15	0

[a] All transductional crosses were performed with a multiplicity of infection of about 1. Ap^r transductants were selected on nutrient broth medium containing ampicillin (30 μg/ml) and then replica printed to minimal medium containing Cbi (18 nM), glucose (11 mM) as the carbon source, and ampicillin (15 μg/ml).

TABLE 5. Effect of *cobA* on regulation of *cob* operons[a]

Strain	Relevant genotype	β-Galactosidase level (Miller units)	
		$-B_{12}$	$+B_{12}$
TT10852	*metE cobI-lac*	800	160
TT14298	*metE cobI-lac cobA*	780	200
TT10857	*metE cobII-lac*	690	150
TT14299	*metE cobII-lac cobA*	740	150
TT10858	*metE cobIII-lac*	680	90
TT14300	*metE2 cobIII-lac cobA*	670	200

[a] Strains were grown anaerobically on E minimal medium supplemented with glycerol (22 mM), fumarate (10 mM), and methionine (0.5 mM). When added to the medium, the concentration of B_{12} (CN-cobalamin) was 15 nM. Cultures in the early log phase were used in the assays. Assay conditions are described in Materials and Methods. These results are the average of duplicate measurements.

involving Mu d-*lac* insertions as described in Materials and Methods (13). Duplications were generated between a *cobA*::Mu dA insertion and a *his*::Mu dA insertion. The Lac⁺ *his*::Mu dA insertion generated prototrophic duplications only with a Lac⁺ *cobA*::Mu dA insertion. Conversely, the Lac⁻ *his*::Mu dA insertion generated duplications only with a Lac⁻ *cobA*::Mu dA insertion. Duplication formation requires that the two inserts be in the same orientation in the chromosome; the Lac phenotype of each insertion depends on whether the insertion is oriented so as to fuse transcription of the included *lac* operon to the target transcript. Since only insertions with similar Lac phenotypes formed fusions (Table 4), we conclude that *cobA* transcription is in the same direction as that of the *his* operon, which is know to be clockwise.

Accumulation of ^{57}Co-labeled intermediate. Wild-type cells produce B_{12} de novo under anaerobic growth conditions. A *cobA* mutant grown under these conditions accumulated a cobalt-containing intermediate that was chromatographically distinct from Cby and from Cbi. This was determined by adding ^{57}Co to cells growing anaerobically on glycerol, with fumarate as an electron acceptor. Cells were harvested and extracted under conditions that would convert all forms of B_{12} into CN-B_{12} (see Materials and Methods). The extract was subjected to thin-layer chromatography, and the cobalt-labeled compounds were visualized by autoradiography. Under the conditions used, mobilities of standards compared with that of Cbi (R_{Cbi}) were 0.85 for $Cby(CN)_2$ and 0.68 for CN-B_{12}. In the *cobA* mutants, an unknown intermediate accumulated whose R_{Cbi} was 0.22. The chemical identity of this compound is not known, but it seems clear that this compound is neither Cbi nor Cby. Experiments are currently being undertaken to identify this compound. It could be an early intermediate or a breakdown product of such an intermediate.

Effect of *cobA* on regulation of *cob* genes. It seemed possible that the phenotype of *cobA* mutants might be due to effects on regulation of expression of the CobI, CobII, and CobIII operons. Therefore, we tested the effect of a *cobA* mutation on the expression of *cob*::*lac* operon fusions to each of the three operons. Cell were grown anaerobically on glycerol as a carbon source and fumarate as an electron acceptor. These are conditions under which the operons show maximal expression in *cobA*⁺ strains (10). Both the level of transcription of *cob* genes and the repressive effect of B_{12} remained basically unaffected in a *cobA* mutant (Table 5). A possible exception was the *cobIII* operon, for which a small increase in the level of transcription was seen in the *cobA* mutant.

Relationship between *cobA* of *S. typhimurium* and *btuR* of *E. coli*. In *E. coli*, mutations called *btuR* were isolated as being constitutive for expression of the B_{12} transport gene *btuB* (17). The *btuR* gene maps at a point in the *E. coli* chromosome corresponding to the position of *cobA* in the *Salmonella* chromosome. It was proposed that *btuR* might encode a repressor of transcription of the outer membrane receptor of B_{12} coded by the *btuB* locus. Recently, Lundrigan and Kadner (18) have found that Ado-B_{12} pools in *btuR* mutants are strongly reduced in size; they suggest that the effect of *btuR* on *btuB* expression is due to this reduction and that Ado-B_{12} is the effector molecule for control of *btuB*. These observations are in agreement with the Eut/N phenotype of a *cobA* mutant. Several tests were performed to compare the *btuR* and *cobA* functions. We found that F'123, which carries the entire *cysB* to *trp* region of *E. coli*, complements a *cobA* mutation in *S. typhimurium*. We also found that a plasmid carrying a 900-base-pair *btuR* insert (pMJF4) from *E. coli* complements *cobA* mutants of *S. typhimurium* (this *btuR*⁺ plasmid was provided by R. Kadner). This plasmid also complemented a strain carrying a deletion spanning from *topA* to *trp* (strain JE1314). This result suggests that only the *cobA* gene is missing in this strain. A Tn*1000* insertion in the *btuR* coding sequences eliminated *cobA* complementation. On the basis of these complementation data, we conclude that the *cobA* locus of *S. typhimurium* and the *btuR* locus of *E. coli* perform equivalent functions and that they are involved in the adenosylation of corrinoids.

DISCUSSION

On the basis of the phenotype of *cobA* mutants, we conclude that the product of this gene plays two key roles in the biosynthesis of B_{12} in *S. typhimurium*. We propose that the CobA protein acts to adenosylate several corrinoid compounds as diagramed in Fig. 4. The unique function of *cobA* is to act on an early intermediate in the de novo synthesis of Cbi under anaerobic conditions. The adenosylation of this intermediate seems to be a prerequisite for the synthesis of Cbi (CobI pathway). Thus, the end product of this branch of the synthetic pathway is proposed to be Ado-Cbi (rather than Cbi). Apparently, Ado-Cbi is required

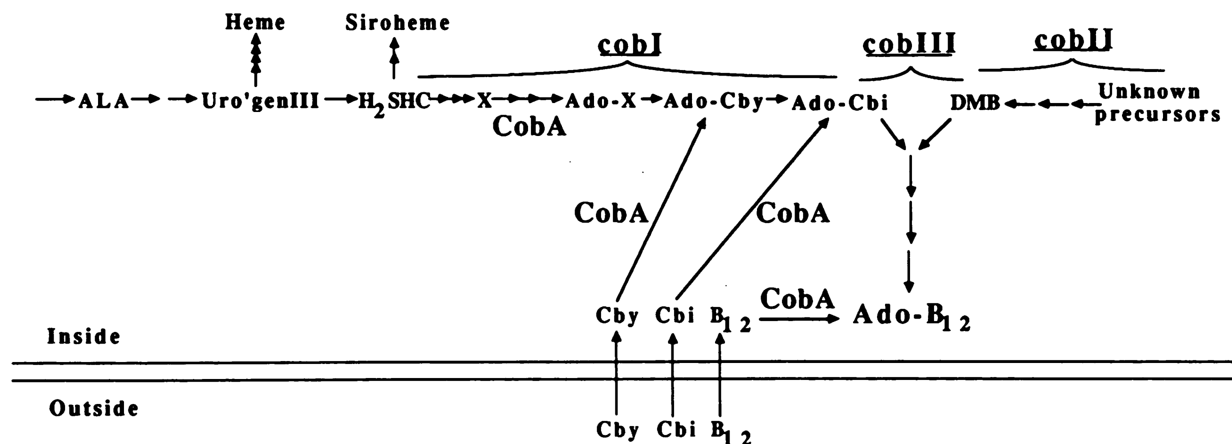

FIG. 4. Proposed roles of *cobA* in the B_{12} biosynthetic pathway. The *cobA* gene product is proposed to be involved in the adenosylation of a de novo intermediate of the CobI pathway (Cbi biosynthesis) and in the assimilation of exogenous corrinoids. Under anaerobic conditions, adenosylation of exogenous corrinoids can be performed by a function encoded within the main Cob operons. De novo biosynthesis requires *cobA* function under both aerobic and anaerobic conditions.

for the subsequent reactions that link DMB to the corrin ring, forming B_{12}.

We propose that the *cobA* function can also act to adenosylate corrinoids provided exogenously. Mutants for *cobA* only show a defect in adenosylation of exogenous corrinoids when tested under aerobic conditions. Under anaerobic conditions, the ability of *cobA* mutants to utilize nonadenosylated corrinoids is restored (Table 2). This correction of the *cobA* phenotype appears to be due to expression of a gene within the main *cob* operons near the *his* locus. Two preliminary pieces of evidence support the existence and map location of this substitute function. Regulatory mutants have been isolated which express the CobI, CobII, and CobIII operons at a high level under aerobic conditions (1, 2). These constitutive mutations act to allow *cobA* mutations to use nonadenosylated corrinoids aerobically (data not shown). Apparently, the regulatory mutation allows aerobic expression of the gene for the substitute function. The second line of evidence is that mutations mapping in the main Cob operons destroy the ability of *cobA* mutants to use nonadenosylated corrinoids under anaerobic conditions.

The fact that the *cobA* locus (34 min) maps outside of the reported *cob* clusters (41 min) may reflect the importance of assimilating exogenous corrinoids under aerobic conditions. The biosynthetic operons, especially the *cobI* operon which is involved in synthesis of the corrinoid ring, are repressed in the presence of O_2. Perhaps the location of the *cobA* gene outside of the main operons reflects the need to express this gene under aerobic conditions to permit assimilation of corrinoids.

In discussing the function of the *cobA* gene, we concluded only that it is required for adenosylation of several B_{12}-related substrates. Several reactions are involved in the adenosylation process, and the *cobA* locus could encode any or all of them. The three possible functions are (i) a reductase which reduces Co^{3+} to Co^{2+}; (ii) a reductase that reduces Co^{2+} to Co^{1+}; and (iii) the actual transfer of the Ado group with the concomitant formation of the carbon-cobalt bond. In other organisms, these functions are provided by three distinct proteins (27). The data presented here indicate the participation of the *cobA* function in adenosylation of corrinoids.

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LITERATURE CITED

1. **Andersson, D. I., and J. R. Roth.** 1989. Mutations affecting regulation of cobinamide biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **171**:6726–6733.
2. **Andersson, D. I., and J. R. Roth.** 1989. Redox regulation of the genes for cobinamide biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **171**:6734–6739.
3. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
4. **Blume, A. J., and E. Balbinder.** 1966. The tryptophan operon of *Salmonella typhimurium*. Fine structure analysis by deletion mapping and abortive transduction. Genetics **53**:577–592.
5. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: *in vivo* probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA **76**:4530–4533.
6. **Castilho, B. A., P. Olfson, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. J. Bacteriol. **158**:488–495.
7. **Chumley, F. G., R. Menzel, and J. R. Roth.** 1978. Hfr formation directed by Tn*10*. Genetics **91**:639–655.
8. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Elliot, T., and J. R. Roth.** 1988. Characterization of Tn*10d*-Cam: a transposition-defective Tn*10* specifying chloramphenicol resistance. Mol. Gen. Genet. **213**:332–338.
10. **Escalante-Semerena, J. C., and J. R. Roth.** 1987. Regulation of cobalamin biosynthetic operons in *Salmonella typhimurium*. J. Bacteriol. **169**:2251–2258.
11. **Frey, B., J. McCloskey, W. Kersten, and H. Kersten.** 1988. New function of vitamin B_{12}: cobamide-dependent reduction of ep-

oxyqueuosine to queuosine in tRNAs of *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **170**:2078–2082.

12. **Hughes, K. T., and J. R. Roth.** 1984. Conditionally transposition-defective derivative of Mu*d*1(Ap *lac*). J. Bacteriol. **159**: 130–137.
13. **Hughes, K. T., and J. R. Roth.** 1985. Directed formation of deletions and duplications using Mu*d*(Ap, *lac*). Genetics **109**: 263–282.
14. **Hughes, K. T., and J. R. Roth.** 1988. Transitory *cis* complementation: a method for providing transposition functions to defective transposons. Genetics **119**:9–12.
15. **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. **170**:2078–2082.
16. **Jeter, R. M., and J. R. Roth.** 1987. Cobalamin (vitamin B_{12}) biosynthetic genes of *Salmonella typhimurium*. J. Bacteriol. **169**:3189–3198.
17. **Lundrigan, M. D., L. C. DeVeaux, B. J. Mann, and R. J. Kadner.** 1987. Separate regulatory systems for repression of *metE* and *btuB* by vitamin B_{12} in *Escherichia coli*. Mol. Gen. Genet. **206**:401–407.
18. **Lundrigan, M. D., and R. J. Kadner.** 1989. Altered cobalamin metabolism in *Escherichia coli btuR* mutants affects *btuB* gene regulation. J. Bacteriol. **171**:154–161.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Ratzkin, P., and J. Roth.** 1978. Cluster of genes controlling proline degradation in *Salmonella typhimurium*. J. Bacteriol. **133**:744–754.
21. **Roof, D. M., and J. R. Roth.** 1988. Ethanolamine utilization in *Salmonella typhimurium*. J. Bacteriol. **170**:3855–3863.
22. **Schmieger, H.** 1971. A method for detection of phage mutants with altered transducing ability. Mol. Gen. Genet. **100**:378–381.
23. **Schmieger, H., and H. Backhaus.** 1973. The origin of DNA in transducing particles in P22 mutants with increased transduction frequencies (HT-mutants). Mol. Gen. Genet. **120**:181–190.
24. **Scott, J. F., J. R. Roth, and S. W. Artz.** 1975. Regulation of the histidine operon does not require *hisG* enzyme. Proc. Natl. Acad. Sci. USA **75**:5021–5025.
25. **Taylor, R. T., and H. Weisbach.** 1971. N^5-methyltetrahydrofolate-homocysteine methyltransferases, p. 121–165. *In* P. D. Boyer (ed.), The enzymes, vol. 9. Academic Press, Inc., New York.
26. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithase of *Escherichia coli*: partial purification, and some properties. J. Biol. Chem. **218**:97–106.
27. **Walker, G. A., S. Murphy, and F. M. Huennekens.** 1969. Enzymatic conversion of vitamin B_{12a} to adenosyl-B_{12}; evidence for the existence of two separate reducing systems. Arch. Biochem. Biophys. **134**:95–102.