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A *Salmonella typhimurium* Cobalamin-Deficient Mutant Blocked in 1-Amino-2-Propanol Synthesis

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Salmonella typhimurium **synthesizes cobalamin (vitamin B_{12}) when grown under anaerobic conditions. All but one of the biosynthetic genes (*cob*) are located in a single operon which includes genes required for the production of cobinamide and dimethylbenzimidazole, as well as the genes needed to form cobalamin from these precursors. We isolated strains carrying mutations (*cobD*) which are unlinked to any of the previously described B_{12} biosynthetic genes. Mutations in *cobD* are recessive and map at minute 14 of the linkage map, far from the major cluster of B_{12} genes at minute 41. The *cobD* mutants appear to be defective in the synthesis of 1-amino-2-propanol, because they can synthesize B_{12} when this compound is provided exogenously. Labeling studies in other organisms have shown that aminopropanol, derived from threonine, is the precursor of the chain linking dimethylbenzimidazole to the corrinoid ring of B_{12}. Previously, a three-step pathway has been proposed for the synthesis of aminopropanol from threonine, including two enzymatic steps and a spontaneous nonenzymatic decarboxylation. We assayed the two enzymatic steps of the hypothetical pathway; *cobD* mutants are not defective in either. Furthermore, mutants blocked in one step of the proposed pathway continue to make B_{12}. We conclude that the aminopropanol for B_{12} synthesis is not made by this pathway. Expression of a *lac* operon fused to the *cobD* promoter is unaffected by vitamin B_{12} or oxygen, both of which are known to repress the main *cob* operon, suggesting that the *cobD* gene is not regulated.**

Cobalamin (B_{12}) is a large coenzyme consisting of a complex tetrapyrrole ring with a central cobalt atom to which the base dimethylbenzimidazole (DMB) is coordinated as a lower ligand (Fig. 1). The DMB base is present as a nucleotide, connected to one of the pyrrole rings via an aminopropanol moiety. The biosynthetic pathway for cobalamin is incompletely understood, but much progress has been made in the past few years, particularly with respect to the synthesis of the corrin ring (8, 9, 37, 39). The bacterium *Salmonella typhimurium* is able to synthesize B_{12} de novo under anaerobic conditions (24). The possibility of genetic manipulation of *S. typhimurium* makes it an ideal organism for studying the biosynthetic pathway of coenzyme B_{12}.

In *S. typhimurium*, cobalamin is required as a cofactor in four known enzymatic reactions. One of these enzymes (MetH) is a methyltransferase that catalyzes the last step in methionine biosynthesis (36). *S. typhimurium* has another enzyme (MetE) capable of catalyzing the same reaction in the absence of B_{12}; thus, the ability of *S. typhimurium* to synthesize cobalamin can be scored by testing methionine production in a strain carrying a *metE* mutation. Anaerobically, *S. typhimurium* is able to synthesize B_{12}, and therefore *metE* mutants can grow on minimal medium. Under aerobic conditions, a *metE* mutant requires either B_{12} (or a corrin ring precursor to B_{12}) or methionine in the medium, since it cannot make its own B_{12} in the presence of oxygen (12). We have isolated mutants blocked in B_{12} synthesis (*cob*) by using a *metE* strain and screening for mutants that cannot grow anaerobically unless either methionine or B_{12} is provided (24). *S. typhimurium* has approximately 25 genes involved in B_{12} synthesis. All the genes studied to date, except one, are located close together in a large gene cluster at minute 41 on the chromosome near the *his* locus (24). Recent data suggest that this cluster is transcribed as a single operon (10a). Genes in this cluster are grouped by function in the pathway. Mutants with mutations involved in the synthesis of the corrin ring (CobI) are identified because their growth is restored by exogenous cobinamide; these genes are located near the promoter. The promoter-distal group of genes (CobII) is required for the synthesis of DMB, and the central group (CobIII) is needed for combining DMB with cobinamide to form cobalamin. In addition to the main B_{12} operon, another B_{12} gene has been identified (*cobA*) which is unlinked to the main operon and is involved in the adenosylation of corrinoids (13).

We report here the isolation and genetic characterization of a new class of mutants (*cobD*) which appear to be unable to synthesize one component of cobalamin, 1-amino-2-propanol. The new gene maps at minute 14 on the chromosome, far from the main B_{12} operon, but very close to the pyridine synthetic gene (*nadD*), encoding nicotinic acid mononucleotide adenylyltransferase.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, media, and growth conditions. All strains used are derivatives of *S. typhimurium* LT2 and are described in Table 1. Two transposition-defective derivatives of the specialized transducing phage Mu dl(Amp^r *lac* cts) of Casadaban and Cohen (4) were used to form operon fusions: Mu dl-1734 Kan^r (5) (called Mu dJ) and Mu dl-8 Amp^r (21) (called Mu dA). Two transposition-defective derivatives of Tn*10* were used: Tn*10del16del17*Tet^r (40) (called Tn*10d*Tet), and Tn*10d*Cam (11). Complex medium was Difco nutrient broth (8 g/liter), containing 5 g of NaCl per liter. Medium E with glucose (0.2%) as a carbon source was used as a minimal medium (38). When glycerol (0.2%) was used as the carbon source, the citrate of medium E was omitted. Solid medium contained 1.5% Bacto-Agar

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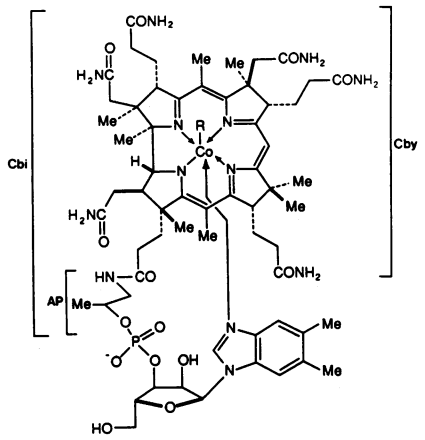

FIG. 1. Structure of cobalamin. R is (-CN) in synthetic compounds and adenosine in the coenzyme form of cobalamin. The cobyric acid (Cby), cobinamide (Cbi), and aminopropanol (AP) moieties are indicated. Me, methyl.

(Difco Laboratories). Anaerobic growth was monitored on medium E-glucose plates containing 1 μM $CoCl_2$, incubated in an anaerobic chamber (model 1024; Forma Scientific). For anaerobic liquid medium, either glucose served as the carbon source or glycerol using KNO_3 (20 mM) as the electron acceptor. The media were made anoxic as previously described (12). The tubes were incubated at 37°C with gentle shaking.

When added, the concentration of antibiotic was (micrograms per milliliter): tetracycline, 20; kanamycin, 40; ampicillin, 30. Other additions included methionine (45 μg/ml); threonine (0.3 mM); $MgSO_4$ (1 mM); cyanocobalamin (B_{12}), dicyanocobinamide, and dicyanocobyric acid (10 nM); DL-1-amino-2-propanol (0.01%); 5′,6′-DMB (16 μg/ml); 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), (20 mg/liter); and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), (10 mM).

Genetic techniques. (i) Transductions. A high-frequency, generalized transducing phage P22 mutant (HT 105/1 *int*-201) was used for transductional crosses at a multiplicity of infection of approximately 1 (10, 35). Inheritance of the kanamycin resistance (Kan^r) marker of Mu dJ required incubation of the transduction mix on nonselective medium (nutrient broth) prior to exposure to the antibiotic. Phage-free, nonlysogenic transductants were isolated as described previously (6).

TABLE 1. List of strains

Strain	Genotype	Source[a]
TR6583	*metE205 ara-9*	Strain collection
TT8782	*nadA216*::Mu dA	Strain collection
TT8785	*nadA219*::Mu dA	Strain collection
TT10288	*hisD9953*::Mu dJ *his-9944*::Mu dl	Strain collection
TT15660	*metE205 ara-9 cobD498*::Mu dJ	
TT15661	*metE205 ara-9 cobD498*::Mu dJ *zbe-3687*::Tn*10*	
TT15662	*metE205 ara-9 cobD499*::Mu dJ	
TT15663	*metE205 ara-9 cobD500*::Mu dJ	
TT15692	*metE205 ara-9* DUP1087 [(*nadA219*)*Mu dA* (*proC693*)]	
TT16529	*metE205 ara-9* DEL902(Δ*cobA-trp*) DEL299(Δ*hisG-cob*)	
TT15709	*metE205 ara-9 nadD157 zbe-1028*::Tn*10*	

[a] Unless otherwise indicated, all strains were constructed during this work.

(ii) Mutant isolation. Random Mu dJ insertions were isolated as described by Hughes and Roth (23). To avoid isolating mutants for previously studied B_{12} genes, we constructed a strain (TT16529) which contained deletions of the known B_{12}-related genes (Δ*his-cob* Δ*cobA*). Approximately 26,000 colonies containing independent Mu dJ insertions were isolated and pooled; P22 transducing phage was grown on the pool. Strain TR6583 (*metE*) was transduced to Kan^r with the lysate, and approximately 12,000 colonies were replica plated to anaerobic minimal medium with or without B_{12} supplementation to assess their ability to make B_{12}. Three new *cob* mutants were isolated from two independent searches; all three were unable to grow without B_{12} (Cob$^-$).

Localized mutagenesis was performed by the method of Hong and Ames (10, 18).

(iii) Determination of the direction of transcription of *cobD*. The orientation of *cobD*::Mu dJ insertions was determined as described by Hughes and Roth (22); insertions (Lac$^+$ and Lac$^-$) in the previously characterized *nadA* gene (located at 17 min) were used as a reference (42). The three Mu dJ insertions in *cobD* were converted to Mu dA insertions by the method of Castilho et al. (5) to increase the frequency of recombination between the two Mu d elements used in this cross (i.e., *cobD* and *nadA*). Crosses were performed to demonstrate whether a duplication could form by recombination between a *cobD*::Mu d insertion and each of the reference *nadA*::Mu d insertions. By comparing orientation and phenotype (Lac$^+$ or Lac$^-$), the orientation of transcription of each *cobD* element was determined. A Lac$^+$ colony (scored as a blue colony on X-Gal plates) results from a Mu d insertion being oriented in a gene such that the transcript of the *lac* operon (carried on the Mu d element) is fused to the transcript of the target gene into which the Mu d is inserted.

Labeling of cobalt-containing compounds. Cells were grown anaerobically in minimal glucose medium containing 1.5 μCi of $^{57}CoCl_2$ per ml and 45 μg of methionine per ml. ^{57}Co-labeled compounds were extracted from cells, spotted on silica gel plates, and visualized as previously described (13, 31).

Enzyme assays. (i) β-Galactosidase. Activity was measured in cultures (5 ml) grown to the early log phase (approximately 70 Klett units) in minimal medium supplemented with methionine; cells were pelleted by centrifugation and resuspended in 0.1 M NaCl. β-Galactosidase activity was assayed by the method of Miller (29) and expressed as nanomoles of *o*-nitrophenyl-β-galactoside per minute per unit of optical density at 650 nm.

(ii) L-Threonine dehydrogenase and D-1-amino-2-propanol: NAD$^+$ oxidoreductase. Protocols for the preparation of cell extracts and assays of threonine dehydrogenase activity have been described previously (2, 26). In one assay, the amount of aminoacetone formed from threonine was determined colorimetrically (17); in the second assay, NADH formation was monitored (at 340 nm). Activity of 1-amino-2-propanol:NAD$^+$ oxidoreductase was assayed by similar assays described by Kelley and Dekker (26).

Chemicals. Dicyanocobyric acid was a gift from Rhône-Poulenc (Vitry sur Seine, France); (*R*)-1-Amino-2-propanol was purchased from Aldrich; all other chemicals were from Sigma Chemical Co.

TABLE 2. Phenotype of *cobD* strains grown on cobyric acid and aminopropanol[a]

Strain	Growth on minimal medium supplemented with:									
	None		AP		Cby		Cby + AP		Cbi	
	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$
metE	−	+	−	+	+	+	+	+	+	+
metE cobD	−	−	−	+	−	−	±	+	+	+

[a] Cells were grown in minimal medium with 0.2% glucose and 1 μM $CoCl_2$. Abbreviations: AP, DL-1-amino-2-propanol; Cby, dicyanocobyric acid; Cbi, dicyanocobinamide.

RESULTS

Isolation of *cobD* mutants. Mu dJ insertional mutagenesis of the entire *Salmonella* chromosome was performed so as to avoid recovery of mutants for previously described genes (Materials and Methods). Two searches yielded three mutants which were unable to grow without B_{12} supplementation. P22 phage was grown on all three new *cob* mutants, and the Mu dJ insertions were transduced into a new *metE* strain (TR6583) by selection for Kan^r recombinants. The resulting strains (TT15660, TT15662, and TT15663) had identical phenotypes and represented one new class of mutants (see below). The genetic locus defined by these mutations was called *cobD*. Strain TT15660 was Lac^+ (formed blue colonies on plates containing X-Gal), indicating the formation of a fusion of the *lac* operon to the *cobD* promoter; colonies of strains TT15662 and TT15663 were Lac^- (white) and proved to have fusions in the opposite orientation (see below). Strain TT15660 (*cobD498*::Mu dJ) was used in subsequent experiments.

Localized chemical (hydroxylamine) mutagenesis of the *cobD* region by the method of Hong and Ames (18) resulted in the isolation of point mutants with the same phenotypes as the insertion mutants.

Phenotype of *cobD* mutants. The CobD phenotype was scored in a *metE* strain in which methionine synthesis is dependent on the presence of B_{12}. Addition of B_{12} allowed growth of *cobD* mutants and therefore indicated a defect in B_{12} biosynthesis. To examine what aspect of B_{12} synthesis was perturbed by *cobD* mutations, we fed two B_{12} precursors, cobinamide and DMB, to a *cobD* strain under anaerobic growth conditions. The addition of cobinamide was sufficient to allow growth of a *cobD* strain, indicating that the defect of *cobD* strains was prior to the intermediate cobinamide and likely to be in corrin ring synthesis. DMB had no effect on the growth of *cobD* strains.

The structure of cobinamide (Fig. 1) includes a 1-amino-2-propanol moiety attached to a propionyl substituent of the completed corrin ring (cobyric acid). The addition of the precursor cobyric acid alone did not affect *cobD* strains. B_{12}-dependent growth was restored to *cobD* mutants under anaerobic conditions if 1-amino-2-propanol was provided, suggesting that *cobD* mutants are defective in aminopropanol synthesis (Table 2). Aminopropanol also corrected *cobD* mutants under aerobic conditions; however, in the presence of oxygen, a corrinoid compound (cobyric acid) must also be provided since even wild-type cells cannot form the corrin ring aerobically. Cobyric acid permitted growth of *cobD* mutants only if aminopropanol was also provided; however, the *cobD* mutant grew slower (approximately 50%) than the wild-type strain under these conditions.

In addition to DL-1-amino-2-propanol, the *R*-1-amino-2-propanol stereoisomer also corrected *cobD* strains. The *S* stereoisomer was inactive, in agreement with previous reports that the *R* form of 1-amino-2-propanol is present in vitamin B_{12} (41).

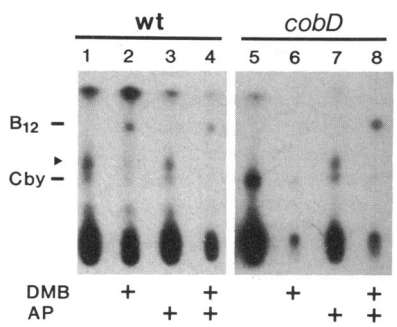

FIG. 2. Autoradiograph of a thin-layer chromatogram of ^{57}Co-labeled compounds produced in anaerobically grown wild-type (wt) and *cobD* strains (for experimental details, see the Materials and Methods section). The migration positions of unlabeled vitamin B_{12} (B_{12}) and dicyanocobyric acid (Cby) used as standards are shown at left. The concentration of DMB was 40 μg/ml and that of (*R*)-1-amino-2-propanol (AP) was 0.02%.

Effect of *cobD* on the expression of the *cob* operon. The B_{12}^- phenotype of *cobD* mutants could be explained by a regulatory effect on the expression of the other B_{12} biosynthetic genes. Thus, we studied the effect of *cobD* mutations on the expression of *lacZ* transcriptional fusions to the three regions of the main *cob* operon. Strains were constructed that carried both a *cobD* point mutation and an operon fusion to the CobI, -II, or -III region. These strains were grown under conditions that normally cause expression (anaerobic respiration) or repression (aerobic growth) of the operon. The *cobD* mutation had no effect on the expression of the *cob*::*lac* fusions under these conditions in either the absence or presence of B_{12} (data not shown).

Cobalt labeling experiments. To characterize the effect of *cobD* mutations on B_{12} synthesis, we used $^{57}CoCl_2$ to specifically label corrin compounds in anaerobically grown cells. Figure 2 shows the accumulations of labeled compounds visualized by thin-layer chromatography and autoradiography. DMB was required in the growth medium to permit a detectable accumulation of B_{12} in both wild-type and mutant strains (Fig. 2, lanes 2, 4, and 8); in the absence of DMB, an unidentified intermediate accumulated (arrowhead, Fig. 2, lanes 1, 3, and 7). Wild-type cells synthesized B_{12} de novo and produced a labeled compound which showed the same mobility as the B_{12} standard (lanes 2 and 4). Mutant (*cobD*) strains did not synthesize B_{12} but accumulated a new compound (lanes 5 and 6). This compound migrated with the same R_f as the cobyric acid standard. When aminopropanol (in addition to DMB) was included in the growth medium of a *cobD* mutant, a compound accumulated which migrated with the same R_f as the compound labeled in wild-type cells, presumably B_{12} (Fig. 2, lane 8). Wild-type cells were unaffected by the presence of aminopropanol.

Possible pathway for aminopropanol formation. Since addition of aminopropanol restored B_{12} synthesis to *cobD* mutants, we inferred that the mutants were defective in aminopropanol synthesis and that preformed aminopropanol can be attached to cobyric acid to form cobinamide. Decarboxylation of threonine seems the most likely source of aminopropanol, and labeling studies in other organisms

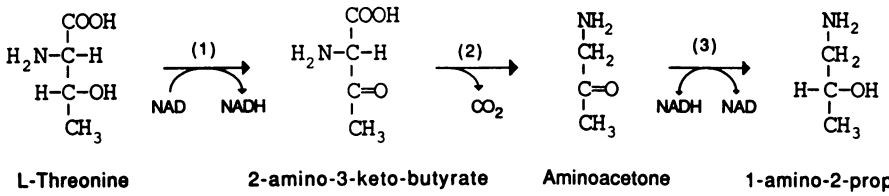

FIG. 3. Proposed pathway for (*R*)-1-amino-2-propanol synthesis from L-threonine (30). 1, threonine dehydrogenase; 2, spontaneous decarboxylation; 3, 1-amino-2-propanol:NAD^+ oxidoreductase.

support conversion of threonine to aminopropanol (27). However, the direct decarboxylation reaction has not been demonstrated despite repeated attempts (16). Instead, an alternative three-step pathway has been proposed (30) that could account for the production of aminopropanol from threonine (shown in Fig. 3). We tested whether our *cobD* mutants were defective in this pathway.

The suggested pathway, whose enzymatic activities have been reported previously (2, 26, 30), involves the enzymatic oxidation of threonine to 2-amino-3-ketobutyrate, which can spontaneously decarboxylate to form aminoacetone, which in turn is enzymatically reduced to form aminopropanol (Fig. 3). The first step is catalyzed by threonine dehydrogenase, which oxidizes threonine to form aminoketobutyrate. The second step is proposed to occur nonenzymatically since aminoketobutyrate is unstable and has been shown to have half-life of less than 1 min at neutral pH (28). The third step involves the reduction of aminoacetone to form aminopropanol and is catalyzed by D-1-amino-2-propanol:NAD^+ oxidoreductase (26).

Since the gene for threonine dehydrogenase (*tdh*) maps at minute 81 in *Escherichia coli* (33), it was unlikely that *cobD* (which maps at minute 14 in *S. typhimurium*, see below) would affect the *tdh* gene. Nevertheless, threonine dehydrogenase activity was assayed in cell extracts, and no difference was observed between wild-type and *cobD* strains. However, no mutants in the oxidoreductase (step 3) have been reported, leaving open the possibility that *cobD* strains are deficient in this enzyme activity. To test this, we prepared cell extracts of wild type and *cobD* and determined the oxidoreductase activity (monitoring the oxidation of aminopropanol to aminoacetone) by two different methods (see Materials and Methods). Both assay methods indicated comparable levels of the oxidase activity in wild-type and *cobD* strains (data not shown).

Isolation of mutants lacking threonine dehydrogenase activity. The above results indicated that the *cobD* mutation did not affect the enzyme activities of the proposed three-step pathway. To test further whether this pathway was contributing to B_{12} synthesis in *S. typhimurium*, we isolated mutants lacking threonine dehydrogenase activity (the first enzyme in the pathway). To do so, we first isolated a mutant strain which was able to use threonine as a carbon source. This type of mutant has been studied in *E. coli* and has been shown to be due to the overproduction of threonine dehydrogenase (3), the first step of a pathway which produces acetyl coenzyme A and glycine via aminoketobutyrate (32, 34). Our strain showed 23-fold-higher threonine dehydrogenase activity than wild-type cells. By insertional mutagenesis, we then isolated mutants unable to grow on threonine as a carbon source (but able to grow on acetate). Several mutants contained no threonine dehydrogenase activity, and their mutations mapped near minute 80 (where *tdh* maps in *E. coli* [33]) (unpublished data). All mutants were unaffected for B_{12} synthesis.

Chromosomal map location of *cobD*. A strain (TT15661) was isolated which had a Tn*10* transposon 75% linked to the *cobD498*::Mu dJ (10). The Tn*10* was located by Hfr mapping (7) between the *purE* and *pyrC* loci (minutes 12 and 23) on the chromosome. Transduction linkage tests with various Tn*10* insertions between 12 and 23 minutes showed that *cobD498*::Mu dJ was approximately 90% linked to the *nadD* gene at minute 14 between the *lip* and *leuS* loci. The other two *cobD*::Mu dJ insertions were found to share the same map location. The location of *cobD* with respect to markers in the area is shown in Fig. 4. Results from three-factor crosses are shown in Table 3. We infer that the *nadD* and *cobD* genes are close together and lie in the order presented in Fig. 4.

Direction of transcription of *cobD*. As discussed above, two types of *cobD lac* fusion mutants were isolated—those that were Lac^+ (scored here as having the ability to form blue colonies on plates containing X-Gal) and those that were Lac^- (formed white colonies on X-Gal plates). The two types of insertions were shown to coincide with different orientations in the *cobD* gene. These two insertion strains and two strains containing insertions in the nearby *nadA* gene were used to determine the orientation of transcription of the *cobD* gene. The *cobD* gene was shown to be transcribed in a clockwise direction. Transducing fragments carrying insertion *cobD498*::Mu dA (Amp^r Lac^+) can recombine with other transducing fragments carrying a *nadA216*::Mu dA (Amp^r Lac^+) insertion to generate prototrophic transductants that are Amp^r and carry a duplication of the *cobD-nadA* region; the inherited hybrid Mu d

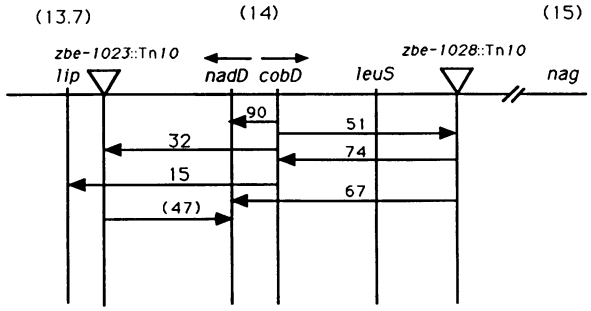

FIG. 4. P22-mediated cotransduction frequencies of the *cobD* region of the *S. typhimurium* genetic map. A portion of the map from *lip* to *leuS* (minute 14) is shown here. Arrows start at the selected gene in transductional crosses and point toward the unselected gene. Linkage data in parentheses are taken from Hughes et al. (20). The direction of transcription of the *nadD* gene has been shown to be counterclockwise (19a).

TABLE 3. Three-factor crosses: position of *cobD* with respect to *lip*, *nadD*, and linked *zbe-1028*::Tn*10*

Cross	Strain tested	Genotypes and phenotype				No. with indicated genotype[a]	Relative frequency (% of total)
		lip	*nadD*	*cobD*	Tet		
1[b]	Donor		−	+	r		
	Recipient		+	−	s		
	Tet^r recombinants (238)		−	+	r	157 (DT)	66
			+	+	r	19	8
			+	−	r	60	25
			−	−	r	2 (DCO)	1
2[c]	Donor	+	+	r			
	Recipient	−	−	s			
	Kan^r recombinants (333)	−	+	r		269	81
		+	+	r		42 (DT)	13
		−	−	r		18	5
		+	−	r		4 (DCO)	1

[a] DT, donor type; DCO, double-crossover class.
[b] Donor was strain TT15709 [*nadD157*(Ts) *cobD*$^+$ *zbe-1028*::Tn*10*] and recipient was strain TT15660 (*nadD*$^+$ *cobD498*::Mu dJ Tet^s). r, resistant; s, sensitive.
[c] Donor was strain TT15660 (*lip*$^+$ *nadD*$^+$ *cobD498*::Mu dJ) and recipient was strain TT7261 [*lip nadD157*(Ts) *cobD*$^+$]. The *cobD* gene was scored as Kan^r.

element is at the duplication join point. Duplication formation requires recombination between two insertions in the same orientation in the chromosome. Since duplications were formed by two Lac$^+$ insertions in the *cobD* and *nadA* genes, these genes must be transcribed in the same direction. This was confirmed by the observation that *cobD*::Mu dA (Amp^r Lac$^-$) insertions were unable to form duplications with the *nadA*::Mu dA (Lac$^+$) insertion but could recombine with a *nadA*::Mu dA (Lac$^-$) insertion (whose Mu dA element is in the opposite orientation). The *nadA* gene has been shown previously to be transcribed in a clockwise direction (42), and we therefore conclude that the direction of *cobD* transcription is also clockwise. This is indicated in Fig. 4. A similar method was used by Hughes (19a) to demonstrate that the nearby *nadD* gene is transcribed in a counterclockwise direction.

Regulation of *cobD* transcription. Expression of the *cobD498*::*lacZ* (Lac$^+$) fusion in TT15660 was assayed in cells grown under various conditions. Table 4 shows that expression of this fusion was constitutively low (2 to 11 β-galactosidase units/A_{650}) and was unaffected by carbon source or by the addition of cobyric acid, cobinamide, aminopropanol, DMB, threonine, or B_{12}. A slight but reproducible increase (two- to threefold) was measured under anaerobic growth conditions.

TABLE 4. Expression of *cobD*::*lac* fusions under various conditions[a]

Addition[b]	β-Galactosidase activity during growth on:			
	Glucose		Glycerol	
	$+O_2$	$-O_2$	$+O_2$	$-O_2$
None	3	9	4	11
Cby	4	11	3	10
DMB	3	—	5	10
AP	—	—	3	9
Thr	3	—	—	11
Cbi	3	7	4	10
B_{12}	3	9	4	9

[a] Strain TT15660 was grown on medium E with concentrations of additions as described in Materials and Methods. Values represent β-galactosidase activity measured in early-log-phase cultures. Dash (—) indicates that assays were not performed.
[b] Cby, dicyanocobyric acid; AP, aminopropanol; Cbi, dicyanocobinamide.

Dominance studies. A strain (TT15692) containing a tandem duplication of the chromosomal region containing *cobD* (between minutes 8.5 and 17) was constructed by the method of Hughes and Roth (22). Insertion *cobD498*::Mu dJ was introduced into one copy of the duplicated region, resulting in a merodiploid with tandem duplications containing both the *cobD*$^+$ and *cobD*$^-$ mutant alleles. The merodiploid retained a CobD$^+$ phenotype (grew on minimal medium anaerobically with no B_{12} added), indicating that the wild-type gene is dominant to the *cobD* allele.

DISCUSSION

We identified a new gene in *S. typhimurium* that is required for vitamin B_{12} biosynthesis. This gene, called *cobD*, is involved in the synthesis of 1-amino-2-propanol, indicated by the fact that addition of aminopropanol restores B_{12}-dependent growth. Aminopropanol was also shown in $^{57}CoCl_2$-labeling experiments to restore the ability of *cobD* strains to synthesize B_{12}. The accumulation of a cobalt-labeled compound in the mutant is evidence that the *cobD* mutation is involved only in aminopropanol synthesis and does not prevent production of the corrin ring portion of B_{12}. Indeed, *cobD* mutations have no effect on expression of *lac* operon fusions to the main *cob* operon (located at minute 41 on the chromosome). The B_{12} intermediate produced in *cobD* strains migrates in thin-layer chromatography with cobyric acid (the compound expected to accumulate if no aminopropanol were available); however, the structure of the accumulated compound has not been identified by other means.

The *cobD* gene maps at minute 14 on the chromosome, very near *nadD*. This linkage is interesting and may be significant since the substrate of the *nadD* gene product (nicotinic acid mononucleotide) is the source of the ribose of vitamin B_{12} as well as a precursor of NAD (20). The functional or regulatory relationship (if any) between these two genes remains to be elucidated. Current evidence supports divergent transcription of the two genes.

The *cobD* gene is one of two known genes which map outside of the main *cob* operon in *S. typhimurium*. The other, *cobA*, is involved in adenosylation of both exogenous corrinoids and an endogenous intermediate in de novo cobinamide synthesis (13). The *cobD* gene could similarly be used for both de novo B_{12} synthesis and assimilation of

exogenous cobyric acid. Cobyric acid has been reported to be widely distributed in nature (16), and our results (Table 2) show that *S. typhimurium* can convert it to B_{12} aerobically. Both *cobD* and *cobA* genes are expressed at a low constitutive level, while the main *cob* operon is repressed aerobically and by corrinoids (12) and is subject to redox control (1, 12). We speculate that the position and expression of these genes may reflect a role in the assimilation of useful corrinoids under aerobic growth conditions. If B_{12} were useful aerobically but could not be synthesized (perhaps because of the inability of *S. typhimurium* to protect oxygen-sensitive intermediates), it would seem logical that genes involved in assimilation of exogenous corrinoids might be regulated differently from the main biosynthetic gene cluster.

In the structure of vitamin B_{12}, aminopropanol forms the link between the corrin ring and the lower nucleotide. In other bacteria, the aminopropanol moiety has been shown to be derived from threonine (27). However, there has been much speculation as to the biosynthetic pathway involved. A direct decarboxylation of threonine would form aminopropanol in one step; however, no decarboxylating activity has ever been reported despite serious efforts (16). Therefore, an alternative three-step pathway was proposed (30) but has never been shown to be important for cobalamin synthesis. The alternative route (Fig. 3) involves the (i) oxidation of L-threonine (by threonine dehydrogenase) yielding 2-amino-3-ketobutyrate, (ii) spontaneous decarboxylation of this unstable compound to form aminoacetone, and (iii) the sterospecific reduction of aminoacetone to yield (*R*)-1-amino-2-propanol (16, 30). This alternative pathway seems unlikely for B_{12} synthesis in *S. typhimurium* since the *cobD* mutants described here require (*R*)-1-amino-2-propanol and are not defective in either of the two enzymatic steps of this proposed pathway (steps 1 and 3 described above). The *cobD* mutants were the only class of mutants found that are corrected by aminopropanol, whereas this pathway would predict the existence of at least two separate genes involved. In addition, as observed before (16, 19), the fact that the enzyme activities are present in other bacteria unable to make B_{12} and are absent in some bacteria that do make B_{12} also indicates that this pathway is unrelated to B_{12} synthesis. Furthermore, we have evidence that mutants lacking threonine dehydrogenase activity (the first step of the pathway) retain the ability to make B_{12}.

A third means of decarboxylating threonine has been suggested which involves first the attachment of threonine as a ligand to the corrinoid cobalt, after which the decarboxylated product is shifted to the ring of cobyric acid (14, 15). The *cobD* mutants described here may affect such a mechanism. If this pathway was operating, one might expect the involvement of several enzymes in the attachment of threonine, decarboxylation of the corrinoid adduct, and rearrangement to place aminopropanol at its proper position. If this pathway operated, *cobD* mutants could be defective in attaching threonine, and another enzyme could accept exogenous aminopropanol in lieu of the decarboxylated adduct to form cobinamide. We have evidence that at least two more genes (in addition to *cobD*) may play a role in the conversion of cobyric acid to cobinamide in *S. typhimurium*. One gene is encoded within the main Cob operon, and the other is located near *cobD* (unpublished data). Strains with mutations in the second gene have been recently isolated as mutants corrected by cobyric acid; however, when combined with a *cobD* mutation, the addition of cobyric acid and aminopropanol fail to allow growth as would be expected if these two substituents were synthesized independently. Although these mutants have not yet been well studied, they do support the involvement of a more complex scheme for aminopropanol synthesis than an independent pathway for its formation and then attachment to cobyric acid to form cobinamide.

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