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Salmonella typhimurium Synthesizes Cobalamin (Vitamin B_{12}) De Novo Under Anaerobic Growth Conditions

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In this paper, we report that the enteric bacterium *Salmonella typhimurium* synthesized cobalamin de novo under anaerobic culture conditions. Aerobically, *metE* mutants of *S. typhimurium* need either methionine or cobalamin as a nutritional supplement for growth. The growth response to cobalamin depends upon a cobalamin-requiring enzyme, encoded by the gene *metH*, that catalyzes the same reaction as the *metE* enzyme. Anaerobically, *metE* mutants grew without any nutritional supplements; the *metH* enzyme functioned under these conditions due to the endogenous biosynthesis of cobalamin. This conclusion was confirmed by using a radiochemical assay to measure cobalamin production. Insertion mutants defective in cobalamin biosynthesis (designated *cob*) were isolated in the three major branches of the cobalamin biosynthetic pathway. Type I mutations blocked the synthesis of cobinamide, type II mutations blocked the synthesis of 5,6-dimethylbenzimidazole, and type III mutations blocked the synthesis of cobalamin from cobinamide and 5,6-dimethylbanzimidazole. Mutants that did not synthesize siroheme (*cysG*) were blocked in cobalamin synthesis. Genetic mapping experiments showed that the *cob* mutations are clustered in the region of the *S. typhimurium* chromosome between *supD* (40 map units) and *his* (42 map units). The discovery that *S. typhimurium* synthesizes cobalamin de novo only under anaerobic conditions raises the possibility that anaerobically grown cells possess a variety of enzymes which are dependent upon cobalamin as a cofactor.

The enteric bacteria *Salmonella typhimurium* and *Escherichia coli* possess two enzymes that are known to use cobalamin (vitamin B_{12}) as a cofactor. These enzymes are tetrahydropteroylglutamate methyltransferase (EC 2.1.1.13) and ethanolamine ammonia lyase (EC 4.3.1.7) (5, 6, 14). The in vivo activities of these enzymes depend upon the cells being provided with an exogenous source of the vitamin, either cobalamin itself or its biosynthetic precursor cobinamide. This nutritional requirement for a preformed corrinoid ring has led to the generally held belief that the enteric bacteria are unable to synthesize cobalamin de novo.

We were concerned by several questions which these observations provoked. Why should these bacteria produce enzymes whose cofactor they cannot provide? In the intestinal tract, cobalamin can be obtained only by competition with a very efficient host uptake system (21). If the enteric bacteria cannot synthesize cobalamin de novo, why is a partial pathway that converts cobinamide to cobalamin present? It seems unlikely that this biosynthetic intermediate is available in the normal environments of the cells.

We considered the possibility that cobalamin is synthesized de novo by a conditional pathway, the expression of which is triggered by a particular set of environmental conditions. Several of the known cobalamin-dependent enzymes have been detected only in strictly anaerobic bacteria (12), and most of the research on cobalamin biosynthesis and cofactor function has been done with anaerobes (e.g., the pioneering work done by H. A. Barker and his colleagues with *Clostridium tetanomorphum* [3, 27]). In addition, it has been shown that cells of *Propionibacterium shermanii* grown aerobically produce less cobalamin than when those cells are grown anaerobically (19). Thus, we tested *S. typhimurium* under anaerobic conditions and found it capable of the complete synthesis of cobalamin. This observation opens the possibility that the enteric bacteria possess a set of novel metabolic capabilities which are expressed only in the absence of oxygen. This shadow metabolism may consist of a number of previously unrecognized enzymes that require cobalamin as a cofactor.

MATERIALS AND METHODS

Bacterial strains. The designation, genotype, and source of each bacterial strain used in this study are given in Table 1. All strains were derived from *S. typhimurium* LT2.

Culture media and growth conditions. The E medium of Vogel and Bonner (26) with 0.2% glucose as the carbon source was the general-purpose minimal medium. Final concentrations of nutritional supplements, added as needed, were: amino acids, purines, and pyrimidines, 0.1 to 0.5 mM; cobinamide dicyanide, 20 μg/liter; cyanocobalamin, 20 μg/liter; and 5,6-dimethylbenzimidazole (DMBI), 50 mg/liter. The complex medium was nutrient broth (8 g/liter, Difco Laboratories) with added NaCl (5 g/liter). Solid medium contained agar (15 g/liter; Difco). Final concentrations of antibiotics were: tetracycline hydrochloride, 10 or 20 mg/liter; kanamycin sulfate, 125 or 50 mg/liter; and streptomycin sulfate, 1,000 or 100 mg/liter in minimal medium and in complex medium, respectively. Biochemicals were supplied by Sigma Chemical Co. and ICN Nutritional Biochemicals. Inorganic chemicals were supplied by Mallinckrodt, Inc.

Cobalamin-supplemented NCE minimal medium (11) with 0.2% lactose as the sole carbon source was used to select Hfr donor strains for the conjugation experiments (see below).

Unless otherwise specified, all cultures were grown at 37°C. Aerobic liquid cultures were grown in an incubator rotary shaker (New Brunswick Scientific Co.) at 200 rpm. Anaerobic liquid cultures were grown in 60-ml Wheaton serum bottles with rubber stopper seals. Petri dishes were incubated anaerobically in GasPak jars (BBL Microbiology Systems) under an H_2-CO_2 atmosphere.

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TABLE 1. *Salmonella typhimurium* strains used in this study.

Strain	Genotype	Reference or source[a]
TR1984	*metE859 pro-636*	RCC
TR2380	*cysG382 malB119*	RCC
TR5654	*thrA9 rpsL1*	11
TR5655	*leu-485 rpsL1*	11
TR5656	*proA36 rpsL1*	11
TR5657	*purE8 rpsL1*	11
TR5658	*pyrC7 rpsL1*	11
TR5660	*pyrF146 rpsL1*	11
TR5662	*hisO1242 his-2236 rpsL1*	11
TR5663	*purF145 rpsL1*	11
TR5664	*cysA533 rpsL1*	11
TR5665	*cysC519 rpsL1*	11
TR5666	*serA13 rpsL1*	11
TR5667	*cysG439 rpsL1*	11
TR5668	*cysE396 rpsL1*	11
TR5669	*ilv-508 rpsL1*	11
TR5670	*metA53 rpsL1*	11
TR5671	*pyrB64 rpsL1*	11
TR5686	*aroD140 rpsL1*	RCC
TR5688	*purA155 rpsL1*	11
TR5890	Δ*hisOG203* CRR289[b]	RCC
TR5900	Δ*hisOG203* CRR299[b]	RCC
TR6431	*metE205 metP760 ara-9*	RCC
TR6510	*metE205 metH465 ara-9*	K. E. Sanderson
TR6526	*metH463*	D. A. Smith
TR6527	*metE404 metH464*	D. A. Smith
TR6528	*metE230 metH465*	D. A. Smith
TR6529	*metE205 metH776*	D. A. Smith
TR6530	*metH465 ara-9*	This study
TR6573	*metP760 ara-9*	This study
TT172	*cysG1510*::Tn*10*	RCC
TT219	*metE863*::Tn*10*	RCC
TT220	*metE864*::Tn*10*	RCC
TT230	*metE874*::Tn*10*	RCC
TT256	*metA900*::Tn*10*	RCC
TT628	*pyrC7 rpsL1*(F'ts114 *lac*$^+$ *zzf-21*::Tn*10*)	8
TT629	*pyrC7 rpsL1*(F'ts114 *lac*$^+$*zzf-22*::Tn*10*)	8
TT1518	*tre-57*::Tn*10*	RCC
TT7570	*metE205 metP760 ara-9 cob-1*::Tn*10*	This study
TT7571	*metE205 metP760 ara-9 cob-2*::Tn*10*	This study
TT7572	*metE205 metP760 ara-9 cob-3*::Tn*10*	This study
TT7573	*metE205 metP760 ara-9 cob-4*::Tn*10*	This study
TT7610	*supD10 zea-609*::Tn*10*	RCC
TT7744	*metE205 metP760 ara-9 cob-5*::Tn*5*	This study
TT7745	*metE205 metP760 ara-9 cob-6*::Tn*5*	This study
TT7746	*metE205 metP760 ara-9 cob-7*::Tn*5*	This study
TT7747	*metE205 metP760 ara-9 cob-8*::Tn*5*	This study
TT8100	*metE205 metP760 ara-9 cob-9*::Tn*10*	This study
TT8651	*cob-3*::Tn*10*	This study
TT8652	*metE205 metP760 ara-9 cob-9*::Tn*10*(F'ts114 *lac*$^+$ *zzf-21*::Tn*10*)	This study
TT8653	*metE205 metP760 ara-9 cob-9*::Tn*10*(F'ts114 *lac*$^+$ *zzf-22*::Tn*10*)	This study
TT8654	*supD10 zea-609*::Tn*10*(F'ts114 *lac*$^+$ *zzf-21*::Tn*10*)	This study
TT8655	*supD10 zea-609*::Tn*10*(F'ts114 *lac*$^+$ *zzf-22*::Tn*10*)	This study
TT8656	*metE205 metP760 ara-9 cob-7*::Tn*5 rpsL1*	This study
TT8720	*cysG382 malB119 metE864*::Tn*10*	This study
TT8721	*metE205 metP760 ara-9 cysG1510*::Tn*10*	This study
TT8722	*metE205 metP760 ara-9 cob-10*::Tn*10*	This study
TT8723	*metE205 metP760 ara-9 cob-11*::Tn*10*	This study
TT8724	*metE205 metP760 ara-9 cob-12*::Tn*10*	This study
TT8725	*metE205 metP760 ara-9 cob-13*::Tn*10*	This study
TT8726	*metE205 metP760 ara-9 cob-14*::Tn*10*	This study
TT8727	*metE205 metP760 ara-9 cob-15*::Tn*10*	This study
TT8728	*metE205 metP760 ara-9 cob-16*::Tn*10*	This study
TT8729	*metE205 metP760 ara-9 cob-17*::Tn*10*	This study
TT8730	*metE205 metP760 ara-9 cob-18*::Tn*10*	This study
TT8731	*metE205 metP760 ara-9 cob-19*::Tn*10*	This study
TT8732	*metE205 metP760 ara-9 cob-20*::Tn*10*	This study
TT9656	*metP760 ara-9 cob-4*::Tn*10*	This study
TT9657	*metP760 ara-9 cob-10*::Tn*10*	This study
TT9658	*metP760 ara-9 cob-11*::Tn*10*	This study

[a] RCC, Roth culture collection. K. E. Sanderson and D. A. Smith, University of Calgary, Alberta, Canada, and University of Birmingham, England.
[b] Spontaneous histidinol-utilizing (Hol$^+$) revertant of parent strain Δ*hisOG203*. CRR, Chromosomal rearrangement.

Transductional methods. The high-frequency generalized transducing mutant bacteriophage P22 HT 105/1 *int*201 (1) was used for all transductional crosses. Maximum stationary-phase cell cultures (ca. 4×10^9 CFU/ml) grown in complex medium were mixed with phage at a multiplicity of infection of 0.1 for 30 min. Samples (0.1 ml) were then plated onto selective medium. Transductant colonies growing on the selective plates were restreaked onto the same medium and then streaked nonselectively onto green indicator plates. Putative phage-free clones were checked for phage sensitivity by cross-streaking the P22 H5 (clear-plaque mutant) phage strains and onto green plates.

Mutant selection. Full-grown liquid cultures of strain TR6431 were transduced with bacteriophage grown on independent pools of over 10,000 *S. typhimurium* LT2-derived clones containing random insertions of the transposons Tn*5* (encoding kanamycin resistance) or Tn*10* (encoding tetracycline resistance) into the chromosome (11, 18). Antibiotic-resistant transductants were selected on complex medium and then replica printed to minimal plates containing the same antibiotic. Replica plates supplemented with either cobinamide or cobalamin were incubated aerobically to identify mutants which were unable to synthesize cobalamin from cobinamide. Clones which grew on cobalamin-supplemented plates but not on cobinamide-supplemented plates were isolated and purified, and their phenotypes were double-checked. A similar procedure was used to identify mutants blocked before cobinamide in the biosynthetic pathway, except that the replica plates were either unsupplemented or supplemented with cobalamin and then incubated anaerobically. Phage lysates were grown on each of these mutants and were used to transduce the parent strain TR6431 to antibiotic resistance to demonstrate cotransfer of the Cob$^-$ phenotype with the antibiotic resistance marker.

Conjugational methods. Hfr donor strains were constructed by Tn*10*-directed integration of an F′ plasmid into the desired site on the *S. typhimurium* chromosome (8). F′ts114 *lac*$^+$ plasmids carrying Tn*10* insertions in opposite orientations were mated from donor strains TT628 and TT629 into each appropriate recipient strain containing a chromosomal Tn*10* insertion. Matings were performed at 30°C by cross-streaking donor and recipient strains onto lactose selective medium and purifying Lac$^+$ transconjugants by restreaking them onto the same medium. Liquid cultures of several

TABLE 2. Phenotypes of cobalamin-related mutants of *S. typhimurium*.

Strain	Aerobic and anaerobic growth on E minimal medium with indicated supplements[a]:							
	None		Cobinamide		Cobalamin		+ L-Methionine	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
LT2	+	+	+	+	+	+	+	+
metE TR1984, TR6431, TT219, TT220, TT230	−	+	+	+	+	+	+	+
metE cob (types II and III) TT7571, TT7573, TT7744, TT7746, TT7747, TT8100	−	−	−	−	+	+	+	+
cob (type III) TT8651	+	+	+	+	+	+	+	+
metE metH TR6510, TR6527, TR6528 TR6529	−	−	−	−	−	−	+	+
metH TR6526, TR6530	+	+	+	+	+	+	+	+
metA TT256	−	−	−	−	−	−	+	+

[a] Medium contain 0.2% glucose as the carbon source, and no electron acceptor was provided.

transconjugant clones were grown at 30°C in lactose medium, diluted 1:100 into fresh medium, and grown to full density at 42°C. These 42°C cultures were plated for single colonies at 42°C onto the same medium. Several large, healthy colonies from each selection were subcultured as putative Hfr donor strains, in which integration of the F′ plasmid into the chromosome by Tn*10* homology had occurred.

Hfr strains were grown overnight at 42°C in lactose medium, diluted 1:10 with nutrient broth, and incubated for 2 h at 37°C. For conjugation, a donor culture (0.1 ml) and a nutrient broth-grown recipient culture (0.1 ml) were spread directly onto an E minimal medium-streptomycin plate. Prototrophic recombinants were counted after a 2-day incubation at 37°C.

Radiochemical assay for cobalamin production. Total cobalamin in acid extracts of cells grown under various culture conditions was measured by a radioisotope dilution assay.

Cells were grown aerobically or anaerobically to the maximum stationary phase in E minimal medium supplemented with $CoCl_2 \cdot 6\ H_2O$ (1.2 mg/liter); some cultures were also supplemented with L-methionine (0.3 mM). The medium did not contain nitrate or any other alternative electron acceptor. For aerobic cultures, this medium was diluted to one-half strength to limit the population size at the stationary phase. This was necessary to prevent the cultures from reaching a density which was sufficiently oxygen limited, even with vigorous aeration, that cobalamin biosynthesis would be induced. Growth was measured as absorbance at 540 nm; at the time of harvesting, the culture density was adjusted by dilution to an absorbance of 1.0. Each culture was harvested (3,000 × *g*, 15 min, 0°C), washed once with 0.06 M potassium phosphate buffer (pH 7.2), resuspended in 0.3 M HCl at a 75-fold concentration, and incubated overnight at 4°C. The acid extract was centrifuged in a microcentrifuge (Eppendorf; Brinkmann Instruments Co.) to remove cell debris, and the supernatant was decanted and neutralized with 1 M Tris buffer (pH 8.6) (1 volume of Tris to 2 volumes of supernatant).

The neutralized extracts were assayed for cobalamin content by using a commercial radioassay (Quantaphase; Bio-Rad Laboratories). In general, the instructions of the manufacturer were followed. The extracts were diluted appropriately in 0.05 M sodium borate buffer (pH 9.2). Samples (0.2 ml), to which bovine serum albumin (4 mg) was added, were boiled in a cyanide-containing solution to convert all forms of cobalamin to cyanocobalamin. The vitamin content of each sample was then quantitated by the degree of competition between unlabeled cyanocobalamin (in the sample) and a fixed amount of ^{57}Co-labeled cyanocobalamin tracer for binding sites on immobilized porcine intrinsic factor. Bound cobalamin was recovered, and radioactivity was measured in a gamma spectrophotometer (Biogamma II; Beckman Instruments). Counts were then converted to picograms of cyanocobalamin by using a standard curve.

To determine the specificity of the radioassay for cobalamin, samples of a stock solution of cobinamide dicyanide (230 pg/ml) were assayed; the cross-reactivity with the labeled cyanocobalamin tracer was less than 0.2%.

RESULTS

Physiological assay for de novo cobalamin biosynthesis. In *S. typhimurium* and *E. coli*, the final step in methionine biosynthesis, transfer of a methyl group to the sulfur atom of homocysteine, is catalyzed by either of two distinct enzymes (5, 14). Tetrahydropteroyltriglutamate methyltransferase (EC 2.1.1.14), encoded by the gene *metE*, carries out the reaction in cells growing on minimal medium. Tetrahydropteroylglutamate methyltransferase (EC 2.1.1.13), encoded by the gene *metH*, catalyzes the same reaction, but the cells require an exogenous supply of cobalamin to activate the *metH* apoprotein. Thus, aerobically grown *metE* mutants are methionine auxotrophs, but the nutritional requirement for methionine can also be satisfied by cobalamin (10, 22). Mutants defective in *metH* alone are prototrophs, since the functional *metE* enzyme can supply the required methionine (7). These growth requirements are shown in Table 2. We used growth of *S. typhimurium metE* mutants in the absence of any nutritional supplement as a preliminary indicator of cobalamin biosynthesis.

***S. typhimurium* synthesizes cobalamin when grown anaerobically.** When incubated anaerobically, *metE* mutants grew on minimal medium. Thus, they had regained the ability to synthesize methionine. This suppression of the auxotrophic phenotype of the cells depended upon possession of a functional *metH* gene (Table 2). The simplest interpretation of this observation is that *S. typhimurium* was able to

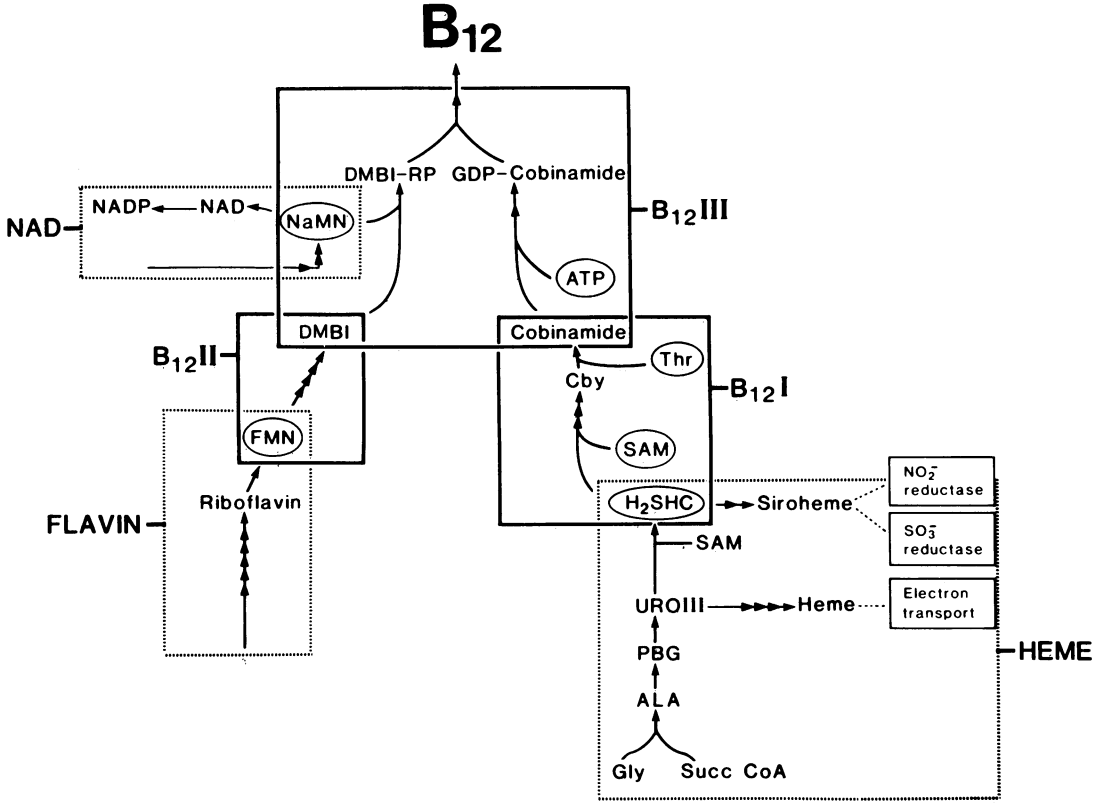

FIG. 1. An outline of cobalamin biosynthesis as it is currently known. Branch I (B_{12} I) of the pathway represents synthesis of the corrinoid ring, branch II (B_{12} II) represents synthesis of DMBI and branch III (B_{12}III) represents assembly of the several parts to form the mature cobalamin molecule. Encircled compounds are those that make a direct contribution to the final structure. Abbreviations: ALA, 5-aminolevulinic acid, Cby, cobyric acid; DMBI-RP, 1-α-D-ribofuranosido-DMBI; FMN, flavin mononucleotide; GDP-cobinamide, guanosine diphosphocobinamide; Gly, glycine; H_2SHC, dihydrosirohydrochlorin; NaMN, nicotinic acid mononucleotide; PBG, porphobilinogen; SAM, *S*-adenosylmethionine; Succ CoA, succinyl coenzyme A; Thr, L-threonine; and URO III, uroporphyrinogen III.

synthesize cobalamin under anaerobic conditions. Several lines of evidence, presented below, support this interpretation.

Mutants unable to convert cobinamide to cobalamin. Under aerobic conditions, *metE* mutants are able to grow if they are supplied with either cobalamin or its precursor cobinamide (13). That is, the cells express a partial pathway that converts cobinamide to cobalamin and thereby activates the cobalamin-dependent *metH* enzyme. In a *metE* parent strain (TR6431), we isolated insertion mutants blocked in converting cobinamide to cobalamin. We designated these mutations *cob* (for defects in cobalamin biosynthesis). All of the *metE cob* double mutants were able to grow aerobically when supplied with methionine or cobalamin, but could not use cobinamide to correct the methionine auxotrophy. In contrast to the parent strain, these mutants retained the auxotrophic phenotype even under anaerobic growth conditions (Table 2). This demonstrates that anaerobic suppression of the *metE* phenotype depended upon possession of a complete pathway of cobalamin biosynthesis and strongly supports the contention that *S. typhimurium* synthesizes cobalamin anaerobically.

Mutants blocked in the synthesis of DMBI. Work in other organisms (4, 15–17) has led to a general outline of the biosynthetic pathway for cobalamin (Fig. 1). The mutants described above have a phenotype consistent with blocks in either of two major branches of the pathway: branch III, the synthesis of cobalamin from cobinamide or branch II, the synthesis of DMBI. We tested these *cob* mutants aerobically to see whether growth was restored when DMBI was added as a nutritional supplement. Of the nine mutants tested, one strain (TT7747; *cob-8*::Tn*5*) converted exogenously supplied cobinamide to cobalamin when DMBI was also supplied. Anaerobically, the addition of DMBI alone was sufficient to restore the growth of this strain. Presumably, the *cob-8* mutation blocked DMBI synthesis. Thus, correction of the Cob^- phenotype by exogenous DMBI constitutes an additional phenotype by which mutations that block either branch II or branch III of the cobalamin biosynthetic pathway are distinguishable.

Mutants blocked in the synthesis of cobinamide. We performed an anaerobic selection and isolated additional *cob* mutants unable to correct the *metE* auxotrophy. One of these mutants (TT8722; *cob-10*::Tn*10*) possessed the DMBI-correctable phenotype described in the preceding section. The rest of the mutants retained the ability to synthesize cobalamin from cobinamide and so must have been blocked in the pathway before cobinamide (branch I in Fig. 1). A total of 10 such mutants have been isolated so far (*cob-11* through *cob-20*).

No cobalamin synthesis by *cysG* mutants. Mutations at the *cysG* locus result in simultaneous loss of sulfite reductase and nitrite reductase activities (9). The most obvious feature these two enzymes have in common is the prosthetic group

TABLE 3. Production of cobalamin by *S. typhimurium* strains which were grown under various culture conditions.

Strain	Cobalamin production (ng/ml of acid extract) under the following conditions[a]:			
	Aerobic		Anaerobic	
	Without L-methionine	With L-methionine	Without L-methionine	With L-methionine
metE$^+$				
LT2	<0.02	<0.02	1.80	1.45
TR6573	<0.02	<0.02	1.60	1.25
metE				
TR6431	NG	<0.02	1.90	1.25
metE cob				
TT7573 (type III), TT8722 (type II), TT8723 (type I)	NG	<0.02	NG	<0.20[b]
cob				
TT9656 (type III), TT9657 (type II), TT9658 (type I)	<0.02	<0.02	<0.20[b]	<0.20[b]

[a] A competitive binding assay with radiolabeled cobalamin was used to determine the amounts of unlabeled cobalamin in acid extracts of cells (see text). The presence of cobalamin in an extract of LT2 and its absence in the extract of a *cob* mutant have been confirmed by chromatographic analysis (R. Jeter, unpublished data). Assay results in a dilution series of the acid extracts of cobalamin-producing cultures did not precisely parallel the assay results in a dilution series of the cobalamin standard. This made estimates of the amount of cobalamin in an extract dependent upon the dilution used. We chose to standardize our measurements to the dilution (or concentration) of each extract that would give a signal equivalent to 0.20 ng of cobalamin per ml on the standard curve. NG, No growth.

[b] Values ranged from 0.08 to 0.20 in the assays.

siroheme, an iron-containing isobacteriochlorin. Thus, *cysG* mutants are thought to be blocked in siroheme biosynthesis. Recent biochemical studies have strongly suggested that cobalamin and siroheme have several biosynthetic steps in common (4). We sought to determine whether *cysG* mutants of *S. typhimurium* were also blocked in cobalamin biosynthesis. We constructed *metE cysG* double mutants (TT8720 and TT8721) and tested their anaerobic phenotypes. Although *metE* mutants grew anaerobically on minimal medium and *cysG* mutants only required cysteine as a nutritional supplement, *metE cysG* mutants did not grow anaerobically unless both methionine and cysteine were supplied. Methionine could be replaced by exogenous cobalamin or cobinamide. Thus, *cysG* mutations conferred a phenotype consistent with a block in branch I of the cobalamin biosynthetic pathway.

Overview of mutant phenotypes. To summarize, strains that carry the *metE* mutation possessed a cobalamin-dependent growth phenotype in the absence of exogenous methionine. In one such *metE* strain of *S. typhimurium*, we isolated three types of mutants that represented blocks in three major branches of the cobalamin biosynthetic pathway as outlined in Fig. 1.

(i) Type I: mutants blocked before cobinamide. These mutants could synthesize cobalamin from cobinamide but were not corrected by DMBI alone (*cob-11* through *cob-20*).

(ii) Type II: mutants blocked in DMBI synthesis. These mutants could synthesize cobalamin when supplied with DMBI plus cobinamide (aerobically) or with DMBI alone (anaerobically) but were not corrected by cobinamide alone (*cob-8*, *cob-10*).

(iii) Type III: mutants unable to convert cobinamide to cobalamin. These mutants were not corrected by either cobinamide or DMBI (*cob-1* through *cob-7*, *cob-9*).

All of the above mutant strains, which carry a *metE* allele, grew aerobically and anaerobically when supplied with exogenous cobalamin or methionine.

Radiochemical assay of cobalamin biosynthesis. Cobalamin production was measured in acid extracts of aerobically and anaerobically grown cultures of *S. typhimurium* by using a competitive binding radioassay (see above). The results of these experiments are presented in Table 3. Two major conclusions can be drawn from this data. First, the results fell into three classes: aerobically grown cells make no cobalamin detectable by the assay; anaerobically grown cells that do not carry a *cob* mutation make cobalamin in readily detectable amounts; and anaerobically grown cells that carry a *cob* mutation give a barely detectable signal when high levels of extract were assayed. In fact, the small signal from these latter extracts might represent the accumulation of biosynthetic precursors of cobalamin in the cells as a consequence of the mutational blocks or a nonspecific interference with the assay by an unidentified component of the extract.

Second, the cultures that were grown anaerobically in the presence of exogenous L-methionine made only slightly reduced amounts of cobalamin. The lack of a major reduction makes it unlikely that methionine exerts a direct regulatory effect on the biosynthesis of cobalamin.

Cotransduction of *cob* mutations. All of the *cob* mutations are genetically linked in transductional crosses. We demonstrated the linkage of *cob* mutations that block branches II and III of the cobalamin biosynthetic pathway by transducing the *cob*::Tn*5* insertion mutants with P22 lysates grown on each *cob*::Tn*10* strain and scoring tetracycline-resistant recombinants for the frequency at which Tn*5* was retained. In every cross, the frequency of double antibiotic resistance (kanamycin and tetracycline) among the individual transductant clones was less than 8% (Table 4). We then performed a similar set of transductions with one of the type III mutants (TT7746; *cob-7*::Tn*5*) as the recipient and P22 lysates grown on each type I *cob*::Tn*10* strain as donors. The average frequency of double antibiotic resistance among the individual transductant clones in these crosses was 78% (Table 4).

Thus, all of the *cob* mutations we selected were divided into two groups by the P22 cotransduction results. Mutants blocked in branch I of the cobalamin biosynthetic pathway appeared to form one cluster; mutants blocked in branches II and III of the pathway formed a second tightly linked cluster. However, these two clusters of *cob* mutations were cotransducible at a detectable frequency, and so they lie

TABLE 4. Transductional linkage of *cob* mutations[a]

Donor bacteriophage	% Tc^r transductants retaining Km^r in strains:		
	TT7744 (*cob-5*::Tn*5*; type III)	TT7746 (*cob-7*::Tn*5*; type III)	TT7747 (*cob-8*::Tn*5*; type II)
φTT7571 (*cob-2*::Tn*10*; type III)	<1.0 (0/52)	3.8 (2/52)	1.9 (1/52)
φTT7573 (*cob-4*::Tn*10*; type III)	1.9 (1/52)	1.9 (1/52)	<1.0 (0/52)
φTT8100 (*cob-9*::Tn*10*; type III)	1.9 (1/52)	<1.0 (0/52)	<1.0 (0/52)
φTT8722 (*cob-10*::Tn*10*; type II)	1.9 (1/52)	<1.0 (0/52)	7.7 (4/52)
φTT8723 (*cob-11*::Tn*10*; type I)		85 (44/52)	
φTT8724 (*cob-12*::Tn*10*; type I)		71 (37/52)	
φTT8725 (*cob-13*::Tn*10*; type I)		81 (42/52)	
φTT8726 (*cob-14*::Tn*10*; type I)		69 (36/52)	
φTT8727 (*cob-15*::Tn*10*; type I)		83 (43/52)	
φTT8728 (*cob-16*::Tn*10*; type I)		87 (45/52)	
φTT8729 (*cob-17*:: Tn*10*; type I)		71 (37/52)	
φTT8730 (*cob-18*::Tn*10*; type I)		56 (29/52)	
φTT8731 (*cob-19*::Tn*10*; type I)		87 (45/52)	
φTT8732 (*cob-20*::Tn*10*; type I)		87 (45/52)	
φTT1518 (*tre-57*::Tn*10*)[b]	100 (52/52)	100 (52/52)	100 (52/52)

[a] Expressed as percent tetracycline-resistant (Tc^r) transductants that retained the kanamycin-resistant (Km^r) phenotype. The numbers of Tc^r Km^r transductants per Tc^r transductants scored are given as ratios in parentheses.
[b] Positive control (no cotransduction of markers possible).

very close to each other at a single locus on the *S. typhimurium* chromosome.

Genetic mapping by conjugation. We determined the chromosomal location of the *cob* locus that had been identified in the cotransduction studies. Hfr mapping experiments were performed by the method of Tn*10*-directed insertion of an F′ plasmid into the chromosome (8) to generate the appropriate donor strains for conjugation. In the initial experiments, donors constructed with origin of transfer in *cob-9*::Tn*10* (strain TT8100; type III) were mated with a standard set of auxotrophic recipients representing markers at various locations around the entire chromosome. A clear discontinuity in the numbers of prototrophic recombinants occurred between *aroD* and *his* (Table 5, first and second columns). These data place the *cob* locus between 36 and 42 map units, respectively (locations of marker positions in map units on the *S. typhimurium* chromosome are as specified in reference 20).

We mapped the position of the *cob* locus more precisely in a second round of conjugation experiments. Donors were constructed from strains carrying Tn*10* insertions into known genetic markers in this region of the *S. typhimurium* chromosome. We used a streptomycin-resistant derivative of strain TT7746 (*cob-7*::Tn*5*; type III) as a recipient and selected Cob$^+$ recombinants. Data from matings with a set of donor strains derived from TT7610 (*supD10 zea-609*::Tn*10*) are presented in Table 5 (third and fourth columns). These data place the *cob* locus between *supD* and *his* at 40 and 42 map units, respectively.

Several *cob* markers, representing blocks in the three branches of the biosynthetic pathway, were tested for cotransduction with *supD*, *phs*, or *hisG*; none cotransduced at a detectable frequency (greater than 1%) with P22 (data not shown). However, cobalamin biosynthesis was eliminated in two independently isolated strains (TR5890 and TR5900) with spontaneous deletions extending upstream out of the *his* operon. The sizes of these deletions have not been determined, but neither deletion was repaired by P22 transduction. A rough genetic map of the region is presented in Fig. 2.

DISCUSSION

Evolutionary considerations led us to the observation that *S. typhimurium* synthesizes cobalamin de novo under anaerobic conditions. This sort of reasoning now poses new questions. Why do these cells synthesize cobalamin at all? Neither of the two known cobalamin-dependent enzymes plays an essential role in cellular metabolism. Why is cobalamin made de novo only in the absence of oxygen? Both cobalamin-dependent enzymes function aerobically when supplied with the vitamin exogenously, and at first glance, the need of the cell for the products supplied by these enzymes would not appear to be greater anaerobically.

Mechanistically, the inability of *S. typhimurium* to make cobalamin aerobically could be due either to some active form of regulation (transcriptional or posttranscriptional) of the cobalamin biosynthetic enzymes or to an intermediate or an enzyme in cobinamide synthesis being sensitive to oxygen. We are currently conducting experiments to distinguish between these alternatives. In any case, *S. typhimurium* has made a large investment in cobalamin synthesis: we estimate that at least twenty enzymes are directly involved in the de novo pathway. Thus, we suspect that the vitamin plays a more important role in anaerobic metabolism than has been realized before. It is not yet clear what this role is. We have checked the ability of single *cob* mutants (without the *metE* mutation) to grow anaerobically on standard glucose minimal medium and such mutants are not growth impaired. Thus, cobalamin is not absolutely necessary for anaerobic biosynthesis of any of the essential cellular components.

One possibility is that, although a demand for methionine exists in growing cells both aerobically and anaerobically, the efficiency of methionine formation becomes more critical anaerobically. The cobalamin-independent *metE* methyltransferase is much less efficient than the cobalamin-dependent *metH* enzyme; in vitro, the turnover numbers of purified holoenzyme preparations from *E. coli* have been measured to be 14 and 780, respectively (25, 28). The cells compensate for the extreme inefficiency of the *metE* enzyme by synthesizing it in large amounts: two independent studies have found that, under aerobic culture conditions, 3 to 5% of cellular protein in *E. coli* consists of this enzyme (23, 28). We assume that the same situation exists in *S. typhimurium*, although this has not been experimentally demonstrated. The cost imposed by the *metE* enzyme on protein synthesis is enormous relative to that of other biosynthetic enzymes. Anaerobically, this cost may be energetically prohibitive, making it selectively advantageous for the cells to synthesize cobalamin to more efficiently produce methionine.

Another possibility is that cobalamin-dependent enzymes hitherto unrecognized in *S. typhimurium* are required for the anaerobic dissimilation of carbon compounds other than glucose. The majority of known cobalamin-containing enzymes catalyze a variety of molecular rearrangement reac-

TABLE 5. Conjugational mapping of the *cob* region on the *S. typhimurium* chromosome

Recipient strain	No. of prototrophic recombinants for which Hfr donor was derived from strain:				
	TT8652 (*cob-9*::Tn*10*)	TT8653 (*cob-9*::Tn*10*)	TT8654 (*zea-609*::Tn*10*)[a]	TT8655 (*zea-609*::Tn*10*)[a]	None
TR5654 (*thrA*)	15	18			0
TR5655 (*leu*)	23	32			0
TR5656 (*proA*)	18	24			0
TR5657 (*purE*)	11	1	28	9	0
TR5658 (*pyrC*)	28	13	68	5	0
TR5660 (*pyrF*)	211	16	505	19	0
TR5686 (*aroD*)	190	4	346	7	0
TT8656 (*cob-7*::Tn*5*)			2	161	1
TR5662 (*his*)	12	750	24	1,470	0
TR5663 (*purF*)	5	540	9	516	0
TR5664 (*cysA*)	2	128	1	357	0
TR5665 (*cysC*)	3	270	6	398	0
TR5666 (*serA*)	2	71			0
TR5667 (*cysG*)	0	25			0
TR5668 (*cysE*)	0	15			0
TR5669 (*ilv*)	2	147			0
TR5670 (*metA*)	4	3			0
TR5688 (*purA*)	0	0			0
TR5671 (*pyrB*)	11	44			0
None	8	5	0	0	

[a] 90% linked to *supD*.

tions that serve just such a purpose in the fermentative metabolism of strict anaerobes (12). To our knowledge, differences in the metabolic capabilities of *S. typhimurium* in the presence and absence of oxygen have not been rigorously studied.

The fact that the cobalamin-dependent *metH* enzyme carries out the methylation of homocysteine in *S. typhimurium* raises the intriguing possibility that cobalamin participates in other methyl transfer reactions in the cell. Under anaerobic growth conditions, the methylation of chemotaxis proteins or the modification reactions of DNA and tRNA might easily involve cobalamin. In this connection, Antoshkina et al. (2) have recently reported that DNA methylation in cobalamin-deficient cells of *P. shermanii* is impaired. Cobalamin is also known to be involved in the methylation of several metal cations, including mercury (24).

The data we present in this paper clarify two previous and somewhat puzzling observations. A study of in vitro methionine biosynthesis by crude extracts of *S. typhimurium* and *E. coli* was made by Cauthen et al. (5). They concluded that *S. typhimurium* can "synthesize cobalamin to a limited, though significant, extent," although the amount of "the vitamin synthesized is patently insufficient to satisfy the growth requirement." More recently, Smith and Neidhardt (23) have published their observation that "certain *metE* mutants [of *E. coli*] do not require methionine for anaerobic growth." In the same paper, they also reported a nearly threefold induction of tetrahydropteroyltriglutamate methyltransferase (*metE* enzyme) when *E. coli* cells were shifted from anaerobic to aerobic growth conditions. This fits with the economic arguments for methionine biosynthesis presented above and makes it probable that the same pattern of cobalamin biosynthesis exists both in *E. coli* and in *S. typhimurium*.

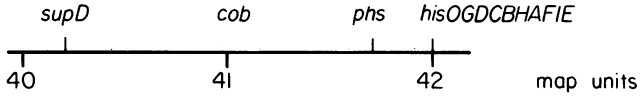

FIG. 2. A genetic map of the region around *cob* on the chromosome of *S. typhimurium*.

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