JOURNAL OF BACTERIOLOGY, June 1994, p. 3568–3575
0021-9193/94/$04.00+0

Vol. 176, No. 12

The *cobT* Gene of *Salmonella typhimurium* Encodes the NaMN: 5,6-Dimethylbenzimidazole Phosphoribosyltransferase Responsible for the Synthesis of N^1-(5-Phospho-α-D-Ribosyl)-5,6-Dimethylbenzimidazole, an Intermediate in the Synthesis of the Nucleotide Loop of Cobalamin

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Received 2 December 1993/Accepted 6 April 1994

We present in vitro evidence which demonstrates that CobT is the nicotinate nucleotide:5,6-dimethylbenzimidazole (DMB) phosphoribosyltransferase (EC 2.4.2.21) that catalyzes the synthesis of N^1-(5-phospho-α-D-ribosyl)-5,6-dimethylbenzimidazole, a biosynthetic intermediate of the pathway that assembles the nucleotide loop of cobalamin in *Salmonella typhimurium*. Mutants previously isolated as DMB auxotrophs are shown by physical and genetic mapping studies and complementation studies to carry lesions in *cobT*. Explanations for this unexpected phenotype of *cobT* mutants are discussed. The expected nucleotide loop assembly phenotype of *cobT* mutants can be observed only in a specific genetic background, i.e., *cobB* deficient, an observation that is consistent with the existence of an alternative CobT function (G. A. O'Toole, M. R. Rondon, and J. C. Escalante-Semerena, J. Bacteriol. 175:3317–3326, 1993). Computer analysis of CobT homologs showed that at the amino acid level, enteric CobT proteins were 80% identical whereas *Pseudomonas denitrificans* and *Rhizobium meliloti* CobT proteins were 95% identical. Interestingly, the degree of identity between enteric and nonenteric CobT homologs was only 30%. The same pattern of homologies was reported for the *S. typhimurium* CobA, *Escherichia coli* BtuR, and *P. denitrificans* CobO proteins (S.-J. Suh and J. C. Escalante-Semerena, Gene 129:93–97, 1993), suggesting evolutionary divergence between the *cob* genes found in the enteric bacteria *E. coli* and *S. typhimurium* and those found in *P. denitrificans* and *R. meliloti*.

Most of the cobalamin biosynthetic (*cob*) genes of *Salmonella typhimurium* make up a large 17-kb operon located at 41 min of the chromosome (9, 16). The results of nutritional analyses performed with *cob* mutants with lesions in the 41-min region yielded three phenotypically distinct classes of strains. The mutants were classified as being blocked in the synthesis of adenosyl-cobinamide, auxotrophic for 5,6-dimethylbenzimidazole (DMB), or unable to synthesize adenosyl-cobalamin from its precursors adenosyl-cobinamide and DMB; i.e., these mutants cannot assemble the nucleotide loop of cobalamin which joins DMB to the corrin ring (6, 9, 14). Previous reports refer to these mutant classes as *cobI*, *cobII*, and *cobIII*, respectively (9).

Analysis of the DNA sequence of the region encoding the functions necessary for nucleotide loop assembly suggested that it was made up of three open reading frames designated *cobU*, *cobS*, and *cobT* (16). These genes were shown to direct the synthesis of three polypeptides with molecular masses that were in good agreement with those predicted by the DNA sequence (14). On the basis of homology to *Pseudomonas denitrificans cob* genes with assigned biochemical activities, the following functions for the CobU, CobT, and CobS proteins were proposed: (i) CobU, a bifunctional ATP:adenosyl-cobinamide kinase, GTP:adenosyl-cobinamide-phosphate guanylyltransferase whose final product is adenosyl-cobinamide-GDP; (ii) CobT, the nicotinate nucleotide (NaMN):DMB phosphoribosyltransferase whose product is N^1-(5-phospho-α-D-ribosyl)-DMB (also known as α-ribazole-5′-P) (7); and (iii) CobS, the cobalamin synthase that joins adenosyl-cobinamide-GDP and α-ribazole-5′-P to generate adenosyl-cobalamin (16).

Interestingly, previously reported genetic and physical analysis of strains defective in nucleotide loop assembly suggested that no mutations in *cobT* had been isolated. In the same report, genetic evidence was also presented to document that CobT was not required for cobalamin biosynthesis (14). These observations were surprising considering the predicted function for CobT in nucleotide loop assembly. The lack of *cobT* mutants was proposed to be due to the existence of an alternative function for CobT (14, 15). If an alternative function did exist, one would expect that strains carrying mutations only in *cobT* should not have a nucleotide loop assembly phenotype, thus explaining the previous inability to isolate *cobT* mutants with this phenotype.

In this paper we demonstrate that CobT is the NaMN:DMB phosphoribosyltransferase (EC 2.4.2.21) whose product is α-ribazole-5′-P, an intermediate in the assembly of the nucleotide loop of cobalamin in *S. typhimurium*. Additionally, our data show that *cobT* mutants (with an otherwise wild-type background) are DMB auxotrophs. However, *cobT* mutants do display a nucleotide loop assembly phenotype when the *cobB* locus, a putative alternative of *cobT*, is disrupted. These genetic data support the in vivo role of CobT in nucleotide loop assembly.

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MATERIALS AND METHODS

Bacteria, culture media, and growth conditions. All bacterial strains used were derivatives of *S. typhimurium* LT2, and

TABLE 1. Strains and plasmids used in this study

Strain[a] or plasmid	Relevant genotype	Source or reference
Strains		
TR6583	*metE205 ara-9*	K. Sanderson via J. Roth
JE769	*cob-236*::Tn*10d*(Tc)[b] *cobT299*	5
JE772	*cob-236*::Tn*10d*(Tc) *cobT302*	5
JE774	*cob-236*::Tn*10d*(Tc) *cobT304*	5
JE777	*cob-236*::Tn*10d*(Tc) *cob-307*	5
JE1153	*recA1* derivative of JE774	5
JE1171	*recA1* derivative of JE769	5
JE1172	*recA1* derivative of JE772	5
JE1173	*recA1* derivative of JE777	5
JE1856	*cob-111*::Tn*10d*(Tc)	5
JE1857	*cob-109*::Mu dJ(Km^r)[c]	5
JE2423	*recA1* derivative of JE1857	This study
JE2445	*cobB1176*::Tn*10d*(Tc)	This study
JE2461	*cobA367*::Tn*10d*(Tc) pGP1-2/ pJO27	14
JE2462	*cobA367*::Tn*10d*(Tc) pGP1-2/ pJO28	14
JE2501	*cobB1176*::Tn*10d*(Tc) derivative of JE1857	This study
JE2593	*cobT109*::Mu dJ Δ*cobB1177*	This study
JE2612	*cobT1187*::Mu dI1734(Km^r)	This study
Plasmids		
pJO11	Marker rescue clone, Cm^r	This study
pJO26	*cobT*$^+$ Cm^r	14
pJO27	*cobT*$^+$ overexpression vector, Ap^r	14
pJO28	*cobT*$^+$ (wrong orientation to be expressed) Ap^r	14
pJO37	Marker rescue clone, Cm^r	This study
pSU19	Cloning vector, Cm^r	13
pSU20	Cloning vector, Cm^r	13
pSU21	Cloning vector, Cm^r	13
pJE2	Ap^r Cm^r	5

[a] Unless otherwise stated, the strains used were derivatives of *S. typhimurium* LT2 and were available from the laboratory strain collection. All JE strains are derivatives of TR6583. Formerly SA2779.

[b] Tn*10Δ16Δ17* is referred as Tn*10d*(Tc) throughout the text (24).

[c] Mu dJ is used to abbreviate Mu dI1734 insertion elements (3).

their genotypes are listed in Table 1. The composition of rich and chemically defined culture media and the concentrations of nutritional supplements and antibiotics have been reported previously (14, 23). All cultures were grown at 37°C.

In vivo assessment of cobalamin biosynthesis. Cobalamin biosynthesis was assessed indirectly by the ability of a *metE* mutant to synthesize methionine on minimal medium (9). Strains carrying a *metE* mutation methylate homocysteine to methionine in a reaction catalyzed by the cobalamin-dependent MetH enzyme (21). Thus, *metE* mutants that carry mutations in *cob* genes are methionine auxotrophs.

Genetic techniques. All transductional crosses were performed with mutant phage P22 HT *105 int-201* (17, 18), and all phage manipulations were performed as previously described (4). Transductional crosses that selected kanamycin-resistant (Km^r) or chloramphenicol-resistant (Cm^r) transductants were first preincubated nonselectively as described elsewhere (14).

Recombinant DNA techniques. Unless otherwise noted, all protocols for DNA manipulation were those of Maniatis et al. (12). All enzymes were obtained from New England Biolabs, Beverly, Mass., and were used as recommended by the manufacturer. GeneClean (Bio 101, La Jolla, Calif.) was used for the isolation of DNA fragments from agarose gels.

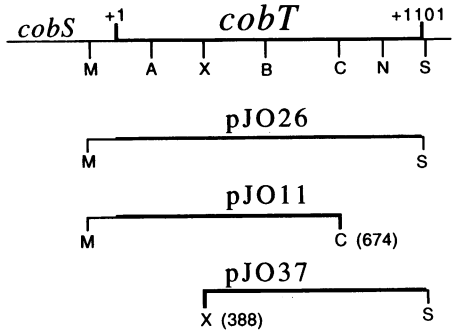

FIG. 1. Plasmids used in complementation and marker rescue studies. Shown at the top of the figure is a simplified restriction map of the *cobT* gene and its flanking regions, and below are the plasmids used in complementation and marker rescue studies. Plasmid pJO26 contains the entire 1,101-bp *cobT* coding sequence as well as 95 bp upstream and 3 bp of downstream flanking sequence. Plasmids pJO11 and pJO37 were used in the marker rescue studies. Plasmid pJO11 contains 674 bp of upstream *cobT* coding sequence and 95 bp of flanking sequence. Plasmid pJO37 contains 713 bp of downstream *cobT* coding sequence and 3 bp of flanking sequence. Numbers in parentheses indicate the position (in base pairs relative to the +1 nucleotide of the *cobT* sequence) of the restriction sites used to generate the marker rescue clones. Thick lines indicate *cobT* coding regions, and thin lines indicate flanking sequences. Restriction enzymes are abbreviated as follows: M, *Msc*I; S, *Sma*I; A, *Alu*I; X, *Xmn*I; B, *Bgl*I; C, *Cla*I; N, *Nde*I.

Plasmids were introduced into appropriate recipients by electroporation with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as previously described (14). All plasmids used in this study are listed and their genotypes are described in Table 1. (i) Plasmid pJO26 (*cobT*$^+$) carries the complete wild-type 1,101-bp *cobT* coding sequence (Fig. 1). Two subclones (pJO11 and pJO37) containing different fragments of the *cobT* gene were constructed for the marker rescue studies (Fig. 1). (ii) Plasmid pJO11 was constructed by cloning the *Msc*I-to-*Cla*I fragment of pJE2 (5) into pSU20 (13) previously digested with *Hinc*II and *Cla*I. Plasmid pJO11 contains the upstream 674 bp of the *cobT* coding sequence as well as approximately 75 bp of upstream flanking sequence. (iii) Plasmid pJO37 contains the *Xmn*I-to-*Sma*I fragment of *cobT* as shown in Fig. 1. This plasmid was constructed by cutting pJO26 with *Xmn*I and *Hin*dIII and cloning this fragment into pSU21 previously digested with *Hinc*II and *Hin*dIII (13). The *Hin*dIII site of pJO26 lies within the multiple cloning site; therefore this *Xmn*I-to-*Hin*dIII fragment contains the *Xmn*I-to-*Sma*I fragment of *cobT*. Plasmid pJO37 contains 713 bp of downstream *cobT* coding sequence and 3 bp of downstream flanking sequence. (iv) Construction of *cobT* under the T7 promoter has been reported previously (14).

Complementation and marker rescue. Complementation and marker rescue were assessed as described elsewhere (14). Mutant strains carrying only the cloning vector were used as a negative control in both complementation and marker rescue experiments.

Preparation of cell extracts. Cell extracts were obtained by sonication of cell suspensions in 100 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.2), half-duty, at 0°C for two 4-min intervals separated by 4 min of incubation at 0°C. Cell debris was discarded after centrifugation at 43,140 × *g* for 1 h (SS34 rotor; Sorvall RC-5B refrigerated centrifuge; Dupont Instruments, Wilmington, Del.). Proteins were visualized by using standard Coomassie blue staining of sodium dodecyl

sulfate-polyacrylamide gel electrophoresis gels (11). Protein concentration of extracts was determined by using the bicinchoninic acid method (19).

In vitro phosphoribosyltransferase activity assays. A modification of the assay described by Cameron et al. for phosphoribosyltransferase activity was used (2). We used [^{14}C]DMB as the substrate in our reaction mixture. The reaction mixture contained 25 nmol of NaMN, 23 nmol of radiolabeled DMB (5.1×10^{-2} μCi/μmol), 2.5 μmol of glycine-NaOH buffer (pH 9.7), and crude cell extract (containing approximately 50 μg of protein) in a final volume of 25 μl. The reaction was initiated by transferring the reaction mixture to a 37°C heating block for 30 min and terminated by heating at 80°C for 10 min. Denatured protein was pelleted at 15,850 × *g* for 5 min in a Marathon 13K/M microcentrifuge (Fisher Scientific, Itasca, Ill.). Reactants and products that remained in the supernatant were resolved by thin-layer chromatography (TLC) (see below); radioactivity was quantitated by scintillation counting (Tri-Carb 4530; United Technologies, Packard Instruments, Inc., Downers Grove, Ill. [counting efficiency, 91% for ^{14}C]) in Poly-Fluor (Packard, Meriden, Conn.) as the scintillation cocktail. One unit of phosphoribosyltransferase activity was defined as the amount of protein that catalyzed the synthesis of 1 μmol of α-ribazole-5′-P per min. Specific activity was expressed as units per milligram of protein.

Initial observations which suggested that the product of the CobT reaction is phosphorylated. The product of the CobT reaction was collected from TLC plates by extraction with 100% methanol. Methanol was evaporated under vacuum, and the pellet was resuspended in 50 μl of distilled H_2O and treated with 1 U of alkaline phosphatase resuspended in 10× alkaline phosphatase buffer (Promega Corp., Madison, Wis.). The digestion mixture was incubated for 1 h at 37°C, and the reaction was terminated by heating the mixture to 80°C for 10 min. Denatured proteins were pelleted at 15,850 × *g* for 5 min as above. Products and reactants were separated by TLC (see below) and quantitated by scintillation counting.

Synthesis and chromatographic analysis of [2-^{14}C]DMB. Radiolabeled DMB was synthesized by the method of Hörig and Renz (8) as follows. A sealed glass ampule containing 220 μmol of 4,5-dimethylphenylenediamine, 0.182 μmol of [^{14}C]formic acid (NEN Dupont, Boston, Mass.; specific radioactivity, 55 mCi/mmol), and 104 μmol of formic acid (Fisher Scientific) in 0.5 ml of 2.0 N HCl was placed in a 130°C oil bath; after 2 h of incubation the ampule was opened, 1 ml of concentrated ammonia was added, and the mixture was dried under vacuum with a SpeedVac concentrator (Savant Instruments, Farmingdale, N.Y.). Radiolabeled DMB was purified by TLC on silica gel with chloroform-methanol (3:2) as the mobile phase. The observed R_f value for DMB in this system was 0.85. The location of DMB in the chromatogram was determined by its blue fluorescence emitted upon irradiation with UV light. The identity of the blue fluorescent spot was confirmed to be DMB since it satisfied the DMB auxotrophy of an indicator strain. The strain used in this bioassay (JE1857) cannot synthesize cobalamin unless DMB is provided in the culture medium (5, 10).

Chromatographic techniques. (i) TLC. TLC was performed on silica gel plates (20 by 20 cm; no. 13179; Kodak) without fluorescent indicator (Eastman Kodak Co., Rochester, N.Y.). Lanes (1 cm wide) were scored on the plates. After application of the sample, the plates were developed (typically for 2 h) with a chloroform-methanol (3:2) mobile phase, each lane was cut into 1-cm pieces, and radioactivity in each piece was quantitated by scintillation counting. R_f values were expressed relative to the solvent front.

(ii) HPLC analysis. The phosphoribosyltransferase reaction mixture was scaled up to a final volume of 1 ml. Incubation at 37°C was increased to 1 h; inactivation at 80°C was extended to 15 min. After removal of pelleted denatured proteins, the supernatant was concentrated under vacuum and extracted with 100% methanol. The precipitate was pelleted and discarded, and the supernatant was concentrated under vacuum and resuspended in 300 μl of distilled water. The sample was filtered with a 0.45-μm-pore-size cellulose-acetate Spin-X filter (Costar, Cambrige, Mass.) and purified by high-pressure liquid chromatography (HPLC) on a Glyco-Pak DEAE column (Waters, Milford, Mass.) previously equilibrated with the mobile phase at a flow rate of 0.6 ml/min. The HPLC system used was a Waters model 990 Plus computer-controlled chromatograph equipped with a photodiode array detector and a Waters model 600 multisolvent delivery system. Spectral data were collected from 240 to 400 nm, and elution was monitored at 280 nm. Two different gradient systems were used: gradient 1 was a 40-min linear gradient (curve 6) from 0 to 1 M NaCl, and gradient 2 was a 30-min convex gradient (curve 3) from 0 to 1 M NaCl. The flow rate was 0.6 ml/min. Eluted fractions were tested for biological activity by using the bioassay described below.

Biological activity assay. Cells from an overnight culture grown in rich medium were pelleted and washed once with 0.085% sterile NaCl, and ca. 10^8 cells were resuspended in 3 ml of molten 0.7% agar and overlaid on E minimal medium supplemented with 20 nM dicyanocobinamide. A 5- or 10-μl sample was applied directly on the overlay and incubated overnight at 37°C. A cobalamin solution (5 μl of 0.15 μM stock) was applied elsewhere on each plate to serve as a positive control; a DMB solution (5 μl of 30 mM DMB) served as a negative control.

Mass spectrometry. Fast atom bombardment mass spectra were obtained with an MS-50TC ultra-high-resolution mass spectrometer (Katos, Ltd., Manchester, England). The spectrometer was equipped with a xenon fast atom bombardment gun (Ion Tech, Ltd., Teddington, United Kingdom). The acceleration potential was 7 kV, the source temperature was 25°C, and 3-nitrobenzyl alcohol (3-NBA) was used as the matrix. Data were collected and processed by using a Katos DS-55 data system.

Amino acid sequence analysis. All analyses of amino acid sequences were performed by using the programs of the Genetics Computer Group, Madison, Wis. Amino acid sequence comparisons were constructed by using the program Pileup.

RESULTS

***cobT* encodes the NaMN:DMB phosphoribosyltransferase of *S. typhimurium*.** Evidence for the CobT-dependent synthesis of a product from DMB and NaMN is shown in Fig. 2. Extracts containing chromosomally encoded CobT (strain JE2462) did not alter the mobility of the radioactive spot on the TLC plate (R_f = 0.86, which corresponds to DMB). Incubation of the substrate with extracts containing overexpressed levels of CobT (strain JE2461) resulted in a drastic change in the location of the radioactivity on the TLC plate. Under these conditions a radiolabeled compound with an R_f of 0.0 (origin) accumulated. Accumulation of this compound was dependent on the presence of active CobT and NaMN in the reaction mixture (data not shown).

The specific activity for the synthesis of the product by extracts containing overexpressed levels of CobT was 4.6

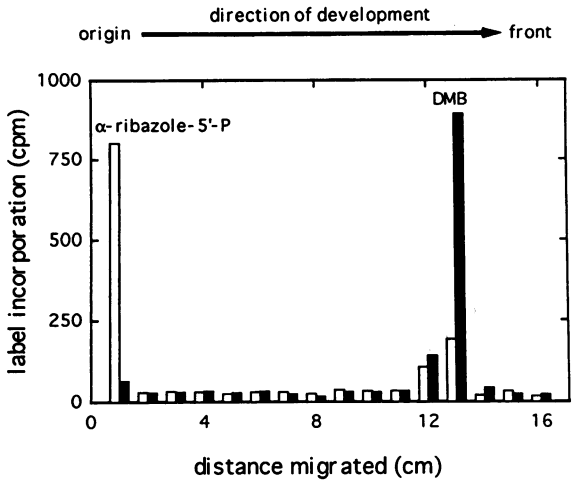

FIG. 2. Separation of reactants and products of the CobT reaction by TLC. Solid bars show the results of a representative assay performed with cell extract containing chromosomally encoded levels of CobT. Open bars correspond to results from assays performed with cell extracts containing overexpressed levels of CobT. The positions of α-ribazole-5'-P and DMB are labeled above the corresponding bars. Counts per minute (ordinate) are plotted versus distance migrated from the origin on the TLC plate (abcissa). The counting efficiency of the scintillation counter used was 91% for ^{14}C. Bars represent the relative radioactivity of each sample counted.

μU/mg of protein, in contrast to 0.25 μU/mg of protein obtained with extracts containing nonoverexpressed CobT levels. The 0.25 μU/mg of protein was interpreted to represent the contribution of the chromosomal CobT and potentially alternative CobT function(s). Dependence on NaMN and the lack of partitioning into the relatively nonpolar mobile phase used to develop the TLC strongly suggested that the product of the reaction was α-ribazole-5'-P and not α-ribazole.

Identification of α-ribazole-5'-P as the product of the reaction catalyzed by CobT. To investigate the possibility that the product of the CobT reaction was α-ribazole-5'-P, the labeled product of the in vitro reaction which remained at the origin of the TLC plate was extracted with methanol, evaporated to dryness under vacuum, resuspended in phosphatase buffer, and treated with alkaline phosphatase. After digestion, the reaction mixture was separated by TLC by using the same mobile phase as above. The radiolabeled compound partitioned into the mobile phase, resulting in an R_f of 0.61 (data not shown), indicating that the product of the CobT reaction could serve as a substrate for alkaline phosphatase; i.e., the product of the CobT reaction was phosphorylated, supporting the idea that the product of the CobT reaction was α-ribazole-5'-P.

The product of the CobT reaction was identified as α-ribazole-5'-P on the basis of UV-visible and fast atom bombardment mass spectra of HPLC-purified material. Figure 3A shows the chromatographic behavior of the major peak (bracketed); Fig. 3B shows chromatographically homogeneous product whose UV-visible spectrum is shown in Fig. 3C. The UV-visible spectrum resembles α-ribazole, as expected (1), but is not identical; e.g., the double peak at 280 nm of the α-ribazole spectrum is much more highly resolved than the peak at 280 nm shown here for α-ribazole-5'-P.

Mass measurements performed on HPLC-purified material are shown in Fig. 4. Signals elicited by the matrix were detected with *m/z* ratios of 482 (three 3-NBA molecules + Na) and 329 (two 3-NBA molecules + Na). Signals with *m/z* ratios of 403 (M + 2Na), 381 (M + Na), and 359 (M + H) were elicited by

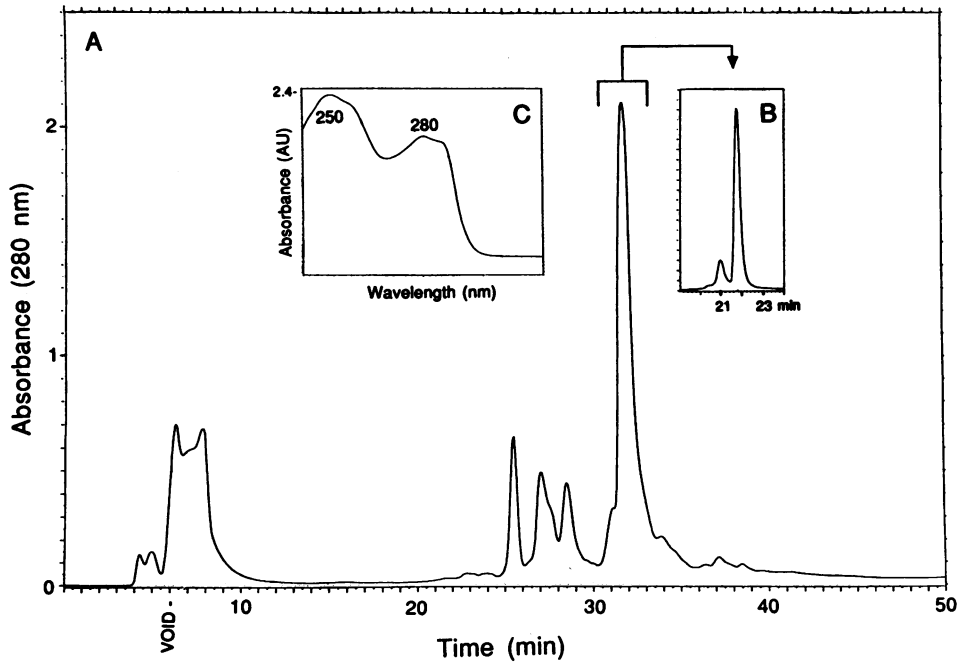

FIG. 3. HPLC analysis of the CobT-catalyzed reaction product. (A) Resolution of α-ribazole-5'-P on Glyco-Pak DEAE developed with gradient 1 (retention time, 31.9 min). (B) The bracketed peak was further resolved on the same column by using gradient 2, which separated α-ribazole-5'-P (retention time, 21.8 min) from a contaminant. (C) UV-visible spectrum of chromatographically homogeneous α-ribazole-5'-P. The spectrum was obtained with a photodiode array detector during development of the column. Conditions for the development of the column are described in detail in Materials and Methods.

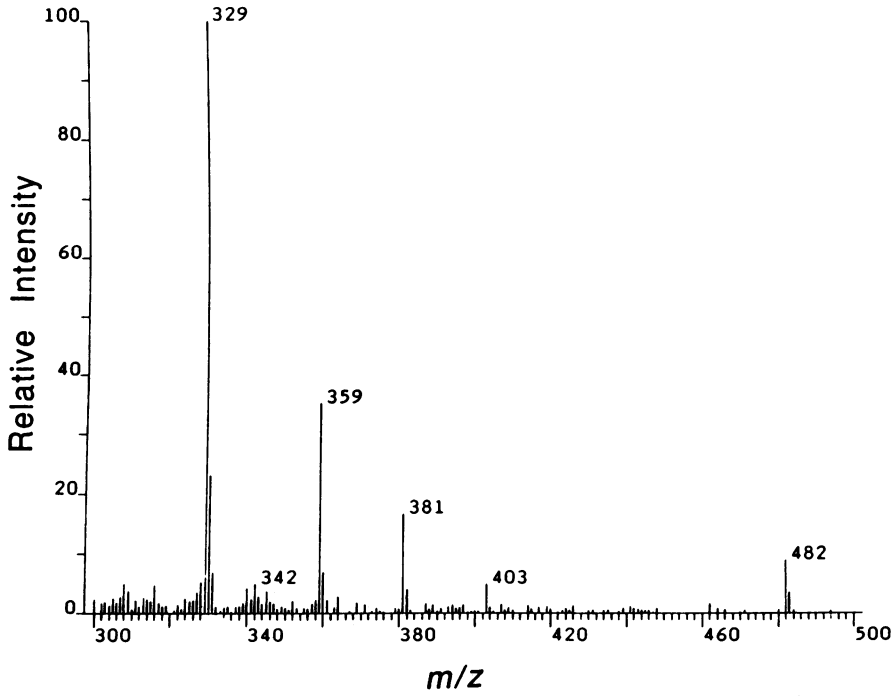

FIG. 4. Mass spectrum of the CobT-catalyzed reaction product. Shown here is the positive-ion, fast atom bombardment mass spectrum of HPLC-purified α-ribazole-5′-P. The signal at *m/z* 359 was identified as the molecular ion plus one proton (M + H); gain of Na yielded *m/z* 381; and gain of 2Na yielded *m/z* 403. The spectrum was obtained by using a 3-NBA matrix. The signal at *m/z* 329 was identified as two molecules of 3-NBA + Na; the signal at *m/z* 482 was identified as three molecules of 3-NBA + Na.

the product of the CobT reaction. These signals are consistent with the molecular weight of α-ribazole-5′-P.

Taken together, these in vitro data provide strong evidence that CobT is the NaMN:DMB phosphoribosyltransferase of *S. typhimurium* and that the reaction catalyzed by this enzyme can be written as shown in Fig. 5.

***cobT* mutants are DMB auxotrophs.** It was observed that mutants previously isolated as DMB auxotrophs (referred to as *cobII* mutants [5]) could be complemented by a *cobT*$^+$ clone. Plasmid pJO26 (*cobT*$^+$ [Fig. 1]) corrected the DMB auxotrophy of the following representative, recombination-deficient mutants: *cob-302* (JE1172), *cob-307* (JE1173), *cob-299* (JE1171), *cob-304* (JE1153), and *cob-109*::Mu dJ (JE2423). That is, in the presence of pJO26, these strains were able to grow on minimal medium containing only cobinamide. The phenotype of all other DMB auxotrophs (recombination proficient) tested was also rescued by plasmid pJO26 (data not shown). Plasmid pSU19, into which *cobT*$^+$ was cloned, served as a negative control for the complementation tests. Strains receiving plasmid pSU19 remained auxotrophic for DMB, implying that the observed complementation by pJO26 was due to CobT and not to the vector. These data strongly suggested that DMB auxotrophs have mutations in the *cobT* gene.

Marker rescue studies confirmed that previously isolated DMB auxotrophs carry mutations which are alleles of *cobT*. Plasmids pJO11 and pJO37 (Fig. 1) contain upstream and downstream portions of the *cobT* gene, respectively. Neither plasmid alone complemented any of the recombination-deficient DMB auxotrophs tested.

Alleles *cob-302* (JE772) and *cob-111*::Tn*10d*(Tc) (JE1856) were recombinationally repaired by pJO11; alleles *cob-304* (JE777) and *cob-109*::Mu dJ (JE1857) were recombinationally repaired by pJO37; alleles *cob-299* (JE769) and *cob-307* (JE777) were recombinationally repaired by both pJO11 and pJO37. The relative positions of these alleles within *cobT* as determined by the marker rescue studies were in agreement with their location on the genetic map (5). These marker rescue data further support the conclusion that mutations causing DMB auxotrophy are alleles of the *cobT* gene.

The DMB auxotrophic phenotype of *cobT* mutants was surprising considering the role of CobT in nucleotide loop assembly (see above). Given the enzymatic function of CobT, we would expect a *cobT* mutant to be unable to assemble the

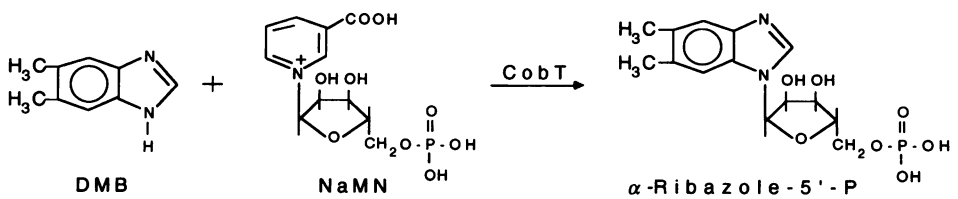

FIG. 5. CobT-dependent synthesis of α-ribazole-5′-P. CobT, NaMN:D MB phosphoribosyltransferase.

TABLE 2. Supplementation of *cobT*, *cobB*, and *cobT cobB* double mutants

Strain	Relevant genotype[a]	Growth on minimal medium with added[b]:		
		CBI	CBI + DMB	CBL
TR6583	*cobT*⁺ *cobB*⁺	+	+	+
JE2445	*cobT*⁺ *cobB*	+	+	+
JE2612	*cobT cobB*⁺	−	+	+
JE2501	*cobT cobB*	−	−	+

[a] All strains carry the *metE205* mutation.

[b] E minimal agar (23) supplemented with glucose (11 mM) as carbon and energy source. Plates were grown at 37°C for 16 to 24 h. Cobinamide (CBI) and cobalamin (CBL) were provided as their cyano derivatives at 15 nM and at 300 μM, respectively.

nucleotide loop of cobalamin. To explain this paradox, we hypothesized the existence of an alternative CobT function which would mask the nucleotide loop assembly phenotype of *cobT* mutants (14). Possible explanations for the DMB auxotrophy of *cobT* mutants are addressed in Discussion.

The nucleotide loop assembly phenotype of *cobT* mutants is background dependent. If the idea of an alternative CobT function is correct, inactivation of the gene encoding such a function should result in the expected nucleotide loop assembly phenotype of a *cobT* mutant. We isolated derivatives of a *cobT* mutant which were unable to assemble the nucleotide loop of cobalamin (JE2501). The mutation in this strain defined a new locus designated *cobB* (22). Importantly, *cobB* mutants did not have a detectable cobalamin auxotrophy in an otherwise wild-type background, suggesting that *cobB* does not encode a function necessary for nucleotide loop assembly (Table 2). Since the nucleotide loop assembly phenotype of a *cobT* mutant can be observed if *cobB* is inactivated, we suspect that *cobB* may encode the previously proposed alternative CobT function (14). Further characterization of *cobB* is in progress.

Biological activity of the product of the CobT reaction. If a strain is blocked in the synthesis of an intermediate in cobalamin biosynthesis (e.g., α-ribazole-5′-P), one should be able to provide that intermediate exogenously and restore cobalamin biosynthesis. As predicted, enzymatically synthesized, HPLC-purified α-ribazole-5′-P restored the ability of a *cobT cobB* mutant (JE2593) to synthesize cobalamin, suggesting that this double mutant was blocked in the synthesis of α-ribazole-5′-P. These in vivo results suggested that α-ribazole-5′-P is an intermediate of the cobalamin biosynthetic pathway in *S. typhimurium*.

Amino acid sequence comparisons among CobT homologs. An alignment of proteins homologous to CobT identified in *P. denitrificans*, *Escherichia coli*, and *Rhizobium meliloti* is shown in Fig. 6A. Only the complete DNA sequences of *cobU* of *P. denitrificans* (2) and *cobT* of *S. typhimurium* (16) have been determined. The biochemical activities of the CobU protein of *P. denitrificans* (2) and the CobT protein of *S. typhimurium* (see above) have been demonstrated to be identical. Despite the fact that the amino acid sequences of the *E. coli* and *R. meliloti* CobT homologs are only partially known, the degree of identity to the *S. typhimurium* and *P. denitrificans* CobT homologs, respectively, is striking (see below).

Figure 6B summarizes the relatedness among the four polypeptides. The CobT protein of *S. typhimurium* and its homolog in *E. coli* show 85% identity at the amino acid level, while the CobT homologs of *P. denitrificans* and *R. meliloti* are greater than 95% identical to each other. However, there is

A.

```
     1                  **     *    *    *                  *   **  *  **  **    50
Pd     ....SASGLP FDDFRELLRN LPGPDAAALV AARERDAQLT KPPGALGRLE
Rm     .......... .......... .......... .......... ..........
Ec     .......... MQILADLLNT IPAIDSTAMS RAQRHIDGLL KPVGSLGKLE
St     MSRLLRTRHL MQTLHALLRD IPAPDAEAMA RTQQHIDGLL KPPGSLGRLE

    51  *  **     *     * *      *         * **    **    *   ***    100
       EIAFWLAAWT G..KAPVVNR PLVAIFAGNH GVTRQGVTPF PSSVTAQMVE
       .......... .......... .......... .......... ..........
       VLAIQLAGMP GLNGIPHVGK KAVLVMCADH GVWEEGVAIS PKEVTAIQAE
       TLAVQLAGMP GLNGTPQVGE KAVLVMCADH GVWDEGVAVS PKIVTAIQAA

   101 *    *                  * *                            * *   150
       NFAAGGAAIN QICVSHDLGL KVFDLALEYP .......... ....TGDITE
       .......... .......... .......... .......... ..........
       NMTRGTTGVC VLAEQAGANV HVIDVGIDTA EPIPGLINMR VARGSGNIAS
       NMTRGTTGVC VLAAQAGAKV HVIDVGID.A EPIPGVVNMR VARGCGNIAV

   151  * *                      *   *    ** *   * ** ***          200
       EAALSERDCA ATM....AFG MEAIAGGTDL LCIGEMGIGN TTIAAAINLG
       .......... .......... .......... .......... ..........
       APAMSRRQAE KLLLDVICYT QELAKNGVTL FGVGELGMAN TTPAAAIVX.
       GPAMSRLQAE ALLLEVSRCA CDLAQRGVTL FGVGELGMAN TTPAAAMVSV

   201                        *    *    * *          *         *  250
       LYGGTAEEWV GPGTGSEGEV LKRKIAAVEK AVALHRDHLS DPLELMRRLG
       .......... .......... .MRKIAAVEK AVALHRDHLS DPLEVMRRLG
       .......... .......... .......... .......... ..........
       FTGSDAKEVV GIGANLPPSR IDNKVDVVRR AIAINQPNPR DGIDVLSKVG

   251 *     * *    * *      * *  **       **        *          *  300
       GREIAAMAGA ILAARVQKVP VIIDGYVATA AASILKAANP SALDHCLIGH
       GREIAAMAGA ILAARMQKVP VIIDGYVATA AAAILKAANP AALDHCLIGH
       .......... .......... .......... .......... ..........
       GFDLVGMTGV MLGAARCGLP VLLDGFLSYS AALAACQIAP AVRPYLIPSH

   301  * * *   *    *   * *    ******  *****  **   ** *    *    350
       VSGEPGHLRA IEKLGKTPLL ALGMRLGEGT GAALAAGIVK AAAACHSGMA
       VSSEPGHMRA IEKLGKTPLL ALGMRLGEGT GAALAAGIVK AAAACHSGMA
       .......... .......... .......... .......... ..........
       FSAEKGARIA LAHLSMEPYL HMAMRLGEGS GAALAMPIVE AACAMFHNMG

   351  *         *                                             368
       TFAQAGVSNK E.......
       TFAQAGVSNK E.......
       .......... ........
       ELAASNIVLP EGNANATZ
```

B.

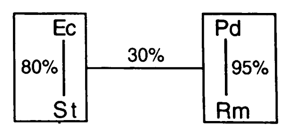

FIG. 6. Amino acid sequence comparison among CobT homologs. (A) Abbreviations: Pd, *P. denitrificans*; Rm, *R. meliloti*; Ec, *E. coli*; St, *S. typhimurium*. Asterisks indicate a three-of-three match of amino acids. Numbers indicate amino acids relative to the beginning of the *S. typhimurium* CobT protein. The complete *S. typhimurium* (CobT) and *P. denitrificans* (CobU) protein sequences are shown. Only the N-terminal half of the *E. coli* (CobT) protein and the C-terminal half of the *R. meliloti* homolog have been reported. The percent identity to the *E. coli* and *R. meliloti* proteins was calculated by using only the portion of these proteins which have been determined. Nucleotide sequence accession numbers: Pd, P29935; Rm, S27658; Ec, L25054; St, L12006. (B) Shown is a representation of the relatedness of CobT and its homologs. Numbers represent average percent identity (at the amino acid level) among the respective proteins.

only 30% identity between the enteric CobT proteins and their homologs in *P. denitrificans* and *R. meliloti*. The regions of identity, however, span the entire lengths of the proteins. This relatedness suggests a great deal of evolutionary divergence of the cobalamin biosynthetic genes between enteric and nonenteric organisms.

DISCUSSION

The data presented in this paper support three conclusions: (i) CobT catalyzes the transfer of the phosphoribosyl moiety of NaMN to DMB, yielding α-ribazole-5′-P; (ii) α-ribazole-5′-P is an intermediate of the pathway that assembles the nucleotide

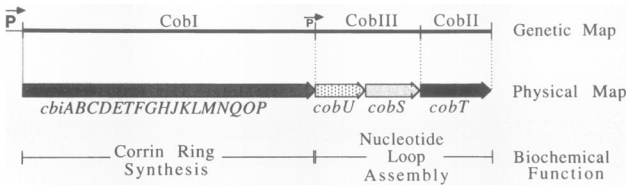

FIG. 7. Correlation of the genetic and physical maps of the *cob* region with biochemical functions required for the synthesis of cobalamin. The top of the figure represents a simplified version of the reported genetic map of the *cob* region. Promoters predicted from genetic studies are also indicated (5). The upstream-most promoter is regulated, whereas the downstream promoter is believed to be a weak constitutive promoter. Below the genetic map are the physical map of the *cob* operon (16) and the assigned roles of the genes in the biosynthesis of cobalamin. The *cbi* genes were originally defined by mutations which could be rescued by providing the preformed corrin macrocycle and are required for the synthesis of the corrin ring. Mutations in the *cobU* and *cobS* genes map to the CobIII region. Mutations in the *cobT* gene map to the CobII region of the genetic map. The *cobUST* genes are involved in the assembly of the nucleotide loop of cobalamin. The physical map is not to scale and represents only the relative order of the *cob* genes.

loop of cobalamin in *S. typhimurium*; and (iii) mutations in *cobT* in an otherwise wild-type genetic background result in DMB auxotrophy.

The last conclusion raises an important question relating to a possible role for CobT in DMB biosynthesis. Two models can help us explain the observed DMB auxotrophy of *cobT* mutants. It should be kept in mind that both models require the existence of an alternative phosphoribosyltransferase activity.

In the first model, CobT is a multifunctional enzyme that catalyzes the synthesis of α-ribazole-5′-P from NaMN and DMB and also participates in the synthesis of DMB. If this were correct, we would predict that null alleles of *cobT* would result in the absence of both CobT activities. The presence of the alternative phosphoribosyltransferase function in this background would mask a deficiency in nucleotide loop assembly but not a deficiency in DMB biosynthesis. This explains the DMB auxotrophy of *cobT* mutants and suggests that the alternative phosphoribosyltransferase enzyme does not substitute for CobT in DMB biosynthesis. Although we have demonstrated the phosphoribosyltransferase activity associated with CobT, at present no in vitro evidence supporting the involvement of CobT in DMB biosynthesis is available.

An alternative model suggests that the DMB auxotrophy of *cobT* mutants reflects intrinsic biochemical properties of CobT and its proposed alternative function, rather than indicating a block in the DMB biosynthetic pathway. One can propose that the alternative CobT function has a higher K_m for DMB than does CobT; hence, in a *cobT* mutant the endogenous level of DMB would have to be supplemented in order for the alternative phosphoribosyltransferase to drive the synthesis of α-ribazole-5′-P. This explains why phenotypically a *cobT* mutant appears to be blocked in DMB biosynthesis even though genotypically the strain is DMB proficient. At present no in vitro evidence supporting this model is available.

Figure 7 shows the correlation between the genetic and physical maps of the *cob* region and assigned biochemical functions. Mutations originally defined by their cobinamide auxotrophy (CobI) define the *cbi* genes and are required for corrin ring synthesis. Mutations in the CobIII region are alleles of *cobU* and *cobS*, and mutations in the CobII region are alleles of *cobT*. The *cobU*, *cobS*, and *cobT* genes are required for the assembly of the nucleotide loop of cobalamin. For the sake of simplicity and clarity, we suggest that hereafter the use of the terms CobI, CobII, and CobIII be disregarded in favor of gene designations and/or functions.

The analysis of the amino acid sequences of three homologs of CobT has also been presented. Interestingly, the low level of relatedness of the enteric CobT homologs (*E. coli* and *S. typhimurium*) to their homologs in *P. denitrificans* and *R. meliloti* suggests a relatively large degree of evolutionary divergence between these two groups of proteins. A similar divergence was observed for CobA homologs. *E. coli* BtuR and *S. typhimurium* CobA are 89% identical at the amino acid level but only 42% identical to CobO from *P. denitrificans* (20). This pattern of relatedness suggests that there are at least two lines of descent among *cob* genes in bacteria.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM40313 from the National Institute of General Medical Sciences to J.C.E.-S., by the College of Agricultural and Life Sciences of the University of Wisconsin—Madison, and by University of Wisconsin—Madison Graduate School project 930684.

We thank R. Randall from the Mass Spectrometry Laboratory of the Biochemistry Department at the University of Wisconsin—Madison for obtaining the mass spectra.

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