JOURNAL OF BACTERIOLOGY, Feb. 2008, p. 1160–1171 Vol. 190, No. 4
0021-9193/08/$08.00+0 doi:10.1128/JB.01386-07
Copyright © 2008, American Society for Microbiology. All Rights Reserved.

One Pathway Can Incorporate either Adenine or Dimethylbenzimidazole as an α-Axial Ligand of B_{12} Cofactors in *Salmonella enterica*▽

Peter J. Anderson,[1]* Jozsef Lango,[2] Colleen Carkeet,[3] Audrey Britten,[1] Bernhard Kräutler,[4] Bruce D. Hammock,[5] and John R. Roth[1]

Section of Microbiology, University of California, Davis, California 95616[1]; Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California 95616[2]; Department of Nutrition, University of California, Davis, California 95616-5270[3]; Institute of Organic Chemistry and Centre of Molecular Biosciences, University of Innsbrück, A-6020 Innsbrück, Austria[4]; and Department of Entomology and Cancer Research Center, University of California, Davis, California 95616[5]

Received 26 August 2007/Accepted 23 October 2007

Corrinoid (vitamin B_{12}-like) cofactors contain various α-axial ligands, including 5,6-dimethylbenzimidazole (DMB) or adenine. The bacterium *Salmonella enterica* produces the corrin ring only under anaerobic conditions, but it can form "complete" corrinoids aerobically by importing an "incomplete" corrinoid, such as cobinamide (Cbi), and adding appropriate α- and β-axial ligands. Under aerobic conditions, *S. enterica* performs the corrinoid-dependent degradation of ethanolamine if given vitamin B_{12}, but it can make B_{12} from exogenous Cbi only if DMB is also provided. Mutants isolated for their ability to degrade ethanolamine without added DMB converted Cbi to pseudo-B_{12} cofactors (having adenine as an α-axial ligand). The mutations cause an increase in the level of free adenine and install adenine (instead of DMB) as an α-ligand. When DMB is provided to these mutants, synthesis of pseudo-B_{12} cofactors ceases and B_{12} cofactors are produced, suggesting that DMB regulates production or incorporation of free adenine as an α-ligand. Wild-type cells make pseudo-B_{12} cofactors during aerobic growth on propanediol plus Cbi and can use pseudo-vitamin B_{12} for all of their corrinoid-dependent enzymes. Synthesis of coenzyme pseudo-B_{12} cofactors requires the same enzymes (CobT, CobU, CobS, and CobC) that install DMB in the formation of coenzyme B_{12}. Models are described for the mechanism and control of α-axial ligand installation.

Coenzyme B_{12} (Ado-Cbl) and vitamin B_{12} (CN-B_{12}) (Fig. 1) include the base 5,6-dimethylbenzimidazole (DMB) as an α-axial ligand. Prokaryotes, the only producers of vitamin B_{12}, make "complete" corrinoid cofactors with a variety of alternative α-axial ligands, including benzimidazoles, phenols, and purines (16, 52). These alternative corrinoids may be functional equivalents of coenzyme B_{12} for many bacterial enzymes, but functional differences are suggested by the selectivity of the human assimilatory protein, intrinsic factor for cobalamin, the DMB-containing vitamin B_{12} (53).

Under strictly anaerobic growth conditions, *Salmonella enterica* synthesizes corrinoids de novo (44) and installs either adenine (to form pseudo-coenzyme B_{12} [Ado-pseudo-B_{12}]) or 2-methyl-adenine (to form adenosyl-factor A) as an α-axial ligand (22). If even trace amounts of oxygen are present, DMB-containing B_{12} (cobalamin [Cbl]) is also made (39). When grown aerobically on glucose, *S. enterica* cannot synthesize the corrin moiety, which must be supplied as an "incomplete" corrinoid, such as cobinamide (Cbi), with its corrin ring and aminopropanol side chain. Under these conditions, coenzyme B_{12} is made, with DMB as the α-axial ligand (20). Figure 2 diagrams the de novo (anaerobic) synthetic pathway and assimilation of exogenous Cbi or CN-B_{12} (42). Notice that the β-ligand adenosyl can be added (by CobA) to three different substrates and that the $(CN)_2$Cbi supplied as the corrin ring source is not a normal CobA substrate.

Here, the origins and installation of α-axial ligands are approached genetically using an unexpected feature of *S. enterica*. While *S. enterica* can use exogenous Cbi to produce B_{12} cofactors (cobalamins) during aerobic growth on glucose (20), it makes only about 100 molecules per cell (2). This is apparently insufficient B_{12} coenzyme to support growth on ethanolamine (5) unless the DMB base is also supplied. That is, under these conditions, cells neither make sufficient DMB nor install an alternative ligand to allow corrinoid-dependent aerobic growth on ethanolamine (5).

This situation allowed positive selection of mutants that can grow on ethanolamine plus Cbi without added DMB. These mutants were expected to show either increased endogenous DMB production or to install an alternative base as an α-axial ligand. All of the mutants made pseudo-B_{12} cofactors, which have adenine as an α-axial ligand, and most of these mutations affected purine metabolism so as to increase the intracellular level of free adenine base. The same set of enzymes (CobUSTC) installs either adenine base (to form pseudo-B_{12}) or DMB (to form vitamin B_{12}). A model suggests how the choice is made.

MATERIALS AND METHODS

Bacterial strains and transposons. Strains were derived from *S. enterica* (serovar Typhimurium) LT2 (Table 1).

Media and chemicals. Pseudo-vitamin B_{12} (CN-pseudo-B_{12}) was isolated as described previously (16). Regular agar was from EMD Inc., Gibbstown, NJ. Noble agar was from U.S. Biological, Swampscott, MA. Rich medium was nu-

* Corresponding author. Present address: Department of Microbiology, Monash University, Clayton, Victoria 3800, Australia. Phone: 61 3 9905 1545. Fax: 61 3 9905 8241. E-mail: Peter.Anderson@med.monash.edu.au.

▽ Published ahead of print on 2 November 2007.

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries

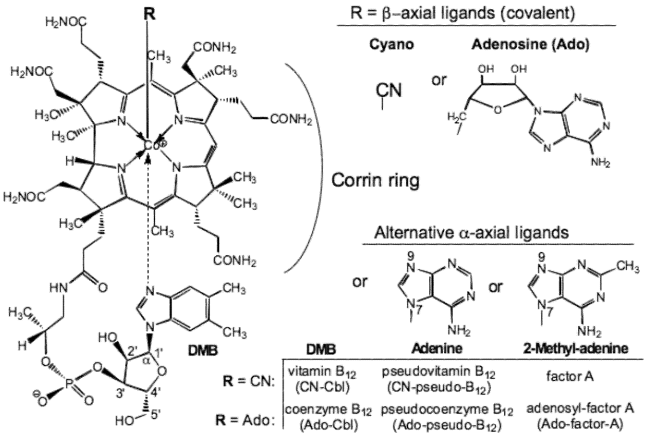

FIG. 1. Alternative "complete" corrinoids in *S. enterica*. The CN(β) derivatives are formed in the process of extracting corrinoids from cells. A methyl group (not shown) can also serve as a β-axial ligand and is involved in methyl transfer reactions. The corrin moiety is provided to cells as dicyanocobinamide $(CN)_2$Cbi, which has only the aminopropanol side chain and no ligand nucleotide; in the presence of CN^- ions, it has CN as both the β- and α-axial ligand.

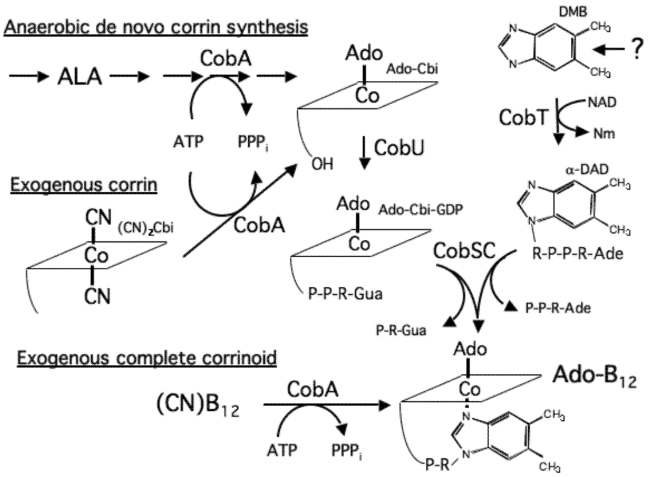

FIG. 2. Pathways for formation of complete corrinoids. In the anaerobic de novo pathway, the β-axial ligand adenosine is added to cobyrinic acid diamide (CobA), followed by four amidations and addition of the aminopropanol side chain to produce Ado-Cbi. Under aerobic conditions, the corrin ring is supplied as $(CN)_2$Cbi, which is adenosylated by CobA to produce Ado-Cbi. The aminopropanol side chain of this corrin is activated by the addition of GDP (CobU), and the α-axial ligand loop is completed by replacing GMP with a nucleoside of either DMB or adenine (CobS and CobC), thereby generating a complete corrinoid. CobT produces the dinucleotide that donates the nucleoside group. Note that the corrin ring compound $(CN)_2$Cbi is not the natural substrate for the CobA enzyme. Ade, adenine; ALA, aminolevulinic acid; Co, corrin; Gua, guanine; Nm, nicotinamide.

trient broth (NB) (0.8%; Difco Laboratories) supplemented with NaCl (5 g/liter). Minimal media were variants of E medium, which contains citrate. NCE medium lacks citrate (25), and NCN medium contains neither citrate nor a nitrogen source (4). Ethanolamine hydrochloride (Aldrich) was used as a carbon source in NCE medium (40 mM), as a nitrogen source in NCN medium (10 mM) with glycerol (27 mM). CN-Cbl (100 nM; Sigma Chemical Co.) was the usual exogenous vitamin B_{12} source. Amino acids and purines were added to minimal medium at the concentrations previously recommended (9). NB agar medium contained antibiotics at the following concentrations: ampicillin, 30 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 20 μg/ml. Solid media were prepared by adding agar (1.5%; EMD) to E, NCN, NCE, or NB medium.

Testing growth in liquid medium using ethanolamine as a nitrogen source. The growth medium was NCN medium with glycerol (0.2%) and ethanolamine (10 mM). To test for growth of strain TR10000 in this medium, duplicate Erlenmeyer flasks containing 25 ml of growth medium were set up containing (i) 0 nM cobinamide, (ii) 20 nM cobinamide, (iii) 20 nM cobinamide and 20 nM DMB, and (iv) 20 nM CN-B_{12}. Inoculation was by 1,000-fold dilution of a culture of TR10000 grown in NCE medium with glycerol. Growth was assessed by measuring optical density at 600 nm (OD_{600}) after 24 h.

Selection of nitrosoguanidine-induced mutants. Cells were spread onto NB plates, and a small crystal of 1-methyl-3-nitro-1-nitrosoguanidine (NG) (Aldrich) was placed in the center of the plate. Plates were incubated for 16 h at 37°C and then replica plated to minimal NCE plates containing 2% Noble agar (U.S. Biological), 40 mM ethanolamine HCl (Sigma), and 100 nM cobinamide dicyanide [$(CN)_2$-Cbi] (Sigma) and incubated at 37°C. After 48 to 72 h, 27 mutant colonies appeared in a circle around the killing zone of the NG.

Preparation and extraction of corrinoids. Cultures (200 ml) of the *apt-18* deletion mutant (TT25778) were grown aerobically on minimal NCE medium containing 40 mM ethanolamine and 1 μM cobinamide. Culture A contained no DMB; culture B contained 1 μM DMB. Both cultures were grown aerobically for 72 h at 30°C with shaking at 200 rpm. Strains TT25752 through TT25751 were grown aerobically for 24 h in minimal NCE medium containing 27 mM glycerol, 40 mM ethanolamine, and 1 mM dicyanocobinamide. In all cases, corrinoids were extracted and prepared for high-performance liquid chromatography/mass spectrometry (HPLC/MS) analysis by the method previously described (5), in which extracted cyanidated corrinoids were washed with water on a Sep-Pac column and eluted with methanol prior to drying and resuspension in water.

HPLC methods. An HPLC system from Waters Alliance 2795 (Bedford, MA) was used to deliver solvent at a flow rate of 250 μl/min. The solvents used in the gradients in the mobile phase were solvent A (94.5% water, 5.0% methanol, and 0.5% formic acid [by volume]) and solvent B (99.5% methanol and 0.5% formic acid [by volume]). The times and gradients were as follows: 0 to 5 min, 0% solvent B; 45 min, 100% solvent B; 45 to 58 min, 100% solvent B; and 60 min, 100% solvent A. The column was an XTerra MS C_{18} column (3.5-μm particle size) (3.0- by 100-mm column) (catalog no. 186000418; Waters, Bedford, MA), coupled to an XTerra C_{18} column (3.5-μm particle size) (3.0- by 20.0-mm column; (catalog no. 186000640; Waters, Bedford, MA) guard column was used. The column temperature was kept constant at 30°C. For UV-visible light signal detection, the Waters 996 PDA detector was used as a UV-visible light detector with a wavelength range of 210 to 620 nm, resolution of 1.2 nm, and sampling rate of 1 spectrum/s.

MS. All the exact mass measurement experiments were performed in positive mode on a Waters/Micromass LCT using orthogonal acceleration–time-of-flight mass spectrometer (Waters/Micromass, Manchester, United Kingdom). Sample source conditions were as follows: capillary voltage of 3,150 V, sample cone voltage of 30 V, extraction cone voltage of 3 V, source temperature of 110°C, and desolvation temperature of 300°C. Transfer optics settings were follows: rf (radio frequency) lens, 300 V; rf dc (direct current) offset-1 4-OV; rf dc offset-2 6-OV; aperture, 2.0 V; acceleration, 200.0 V; focus, 2.0 V; and steering, −0.5 V. Analyzer settings were as follows: multichannel plate detector, 2,600 V; ion energy, 31.0 V; tube lens, 9.0 V; grid, 2 31.0 V; time-of-flight tube, 4,599 V; and reflectron, 1,813 V. The pusher cycle time was 75 μs, data files were acquired in continuum mode, and spectra were stored from *m/z* 100 to 2,100 with a 2.1-second scanning cycle consisting of a 2.0-second scan and a 0.1-second interscan time. The cone gas and desolvation gas were set at 50 and 675 liter/hour, respectively. Lock spray parameters were identical to sample setting parameters. Lock spray sampling frequency mode was set at 5, i.e., every fifth spectrum generated was the signal from the lock spray inlet. Analyzer pressure measured 7.75×10^{-7} mbar. MassLynx 4.0 software (Waters/Micromass, Manchester, United Kingdom) was used for instrument control, data acquisition, and data evaluation.

Mapping of mutations. Strains were isolated with Tn*10* inserted near new DMB-independent mutations as described previously (23). A collection of about 100,000 random Tn*10* (tetracycline-resistant) insertion mutants were pooled and used to prepare transducing phage. This lysate served as the donor in a cross with the mutant to be characterized. Tetracycline-resistant transductants were selected and screened to identify those that lost the recipient mutant phenotype when they acquired tetracycline resistance. This is expected to occur when the Tn*10* insertion is coinherited with a nearby wild-type allele of the recipient mutant gene. Each of several Tn*10* insertions was sequenced and tested for linkage to the whole set of DMB-independent mutations. This gave the general

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries

TABLE 1. Strains used in this study

Strain	Genotype[a]	Source or reference
TR10000	Wild-type *S. enterica* (serovar Typhimurium) LT2	Lab collection
TT24729	*cobT110*::MudJ	Lab collection
TT25112	STM2008-1::Tn*10*dTc *amnA52*(*G935A*)	This report
TT25454	*cobU2620*::Frt(sw)	This report
TT25522	*cobS2621*::Frt(sw)	This report
TT25575	*cobT111*::Tn*10*	Lab collection
TT25587	*ydjA391*::Cm^r(sw)	This report
TT25588	*metE205 ara-9 ydjA*::Cm^r(sw)	This report
TT25720	*metE2119*::MudJ	This report
TT25721	*metE2119*::MudJ *cobT111*::Tn*10*	This report
TT25722	*metE2119*::MudJ *cobS2621*::Frt(sw)	This report
TT25723	*metE2119*::MudJ *cobU2620*::Frt(sw)	This report
TT25777	STM2008-1::Tn*10*dTc *amnA52*(*G935A*) *cobT110*::MudJ	This report
TT25778	*apt-18*::Cm^r(sw)	This report
TT25779	*apt-18*::Cm^r(sw) *cobT110*::MudJ	This report
TT25780	*nfnB121*::Kn^r(sw)	This report
TT25781	*mdaA121*::Zeo^r(sw)	This report
TT25782	*metE205 ara-9 nfnB121*::Kn^r(sw)	This report
TT25783	*metE205 ara-9 mdaA121*::Zeo^r(sw)	This report
TT25784	*amnA57*::(*lacZ*$^+$ *lacA*::Cm^r) (*lacZ* fused to *amn* transcript)	This report
TT25785	*amnA58*::Kn^r(sw deletion of *amn* promoter and gene) *amn-57*::(*lacZ*$^+$ *lacA*::Cm^r) (*lacZ* fused to *amn* transcript)	This report
TT25786	*apt-18*::Cm^r(sw) *cobC1175*::Tn*10*dTc	This report
TT25787	*apt-18*::Cm^r(sw) *cobU2620*::Frt(sw)	This report
TT25788	*apt-18*::Cm^r(sw) *cobS2621*::Frt(sw)	This report
TT25789	*amnA53*::Kn^r(sw)	This report
TT25793	STM2503-1::Tn*10*dTc *dmb-1*	This report
TT25797	*hisG203*(del) *trp-2475*::Tn5/F′$_{128}$ *pro*$^+$ *lacY4654*::Cm^r(sw)	This report
TT25810	*cobC1175*::Tn*10*dTc	Lab collection

[a] (sw) indicates a constructed (swap) mutation in which the indicated sequence element replaces the reading frame of the affected gene.

map positions of several mutations and indicated the region in which sequence determination might identify them.

Sequencing of Tn*10* insertion sites. Single-primer PCR was used to amplify join points between Tn*10* insertions and chromosomal DNA. The single primer used for PCR was TP93 (ACCTTTGGTCACCAACGCTTTTCC), which is homologous to the right arm of the Tn*10*dTet. The resulting PCR fragment was sequenced using the nested primer TP91 (ATCATTAGGGGATTCATCAG).

Construction of mutations by linear transformation. (i) Deletion of the *apt* gene. The chloramphenicol resistance (Cm^r) cassette was PCR amplified from strain TT24696 using primers TP1864 (ATGACCGCGACTGCACAGCAGCT TGAGTTTCTCAAAAACACACCAAACACCCCCCAAAACC) and TP1865 (TCAATGCCCTGGAAACGGCACCAGGCTATAACAGGTAATCCACA CAACCACACCACACCAC), whose 5′ ends are homologous to the ends of the *apt* sequence. The amplified fragment was electroporated into strain TT22971, which carries a plasmid with the Red genes of phage lambda (8), selecting Cm^r on NB plates with 20 μg/ml chloramphenicol. Successful deletion was confirmed by PCR characterization of the region from the final strain (TT25778).

(ii) Deletion of the *amn* gene. Primers TP1712 (GATCGCCTGGAGGAGC TATACGAGCAGTCGGTTAACGCGCCACCAAACACCCCCCAAAACC) and TP1713 (TGAGTGCAAACGATCACCTTCTGCGCGTAGTAAATCAA TAGCACACAACCACACCACACCAC) were used to amplify a kanamycin resistance (Kn^r) cassette. The amplified fragment was electroporated into strain TT22971, and recombinants were selected on NB plates containing kanamycin (50 μg/ml). The 5′ ends of this fragment support recombination to integrate the Kn^r determinant in place of the *amn* coding sequence. After recombination, the *amnA59*::Kn^r(sw) deletion [(sw) indicates a constructed (swap) mutation in which the indicated sequence element replaces the reading frame of the affected gene] was moved by transduction into the wild-type *S. enterica* strain TR10000.

(iii) Construction of an *amn*::*lacZ* transcriptional fusion. Primers TP1981 (AGCTGTCGCCTGCTGCTGGACAGGAGCGGAAGAGGCGTATCACA CAGGAAACAGCTATGACC) and TP1982 (AGAACCGCCTCCCGCTAAG GCTTCATTCAGCCACTTTCCACACACAACCACACCACACCAC) were used to amplify a fragment including *lacZ*$^+$ and a Cm^r determinant (in strain TT25797). The amplified fragment was electroporated into strain TT22971, which carries a plasmid with the Red recombination genes of phage lambda (8). The sequences at the 5′ ends of this fragment allow recombination to generate an *amn*::*lacZ*$^+$ fusion allele that expresses LacZ from the *amn* promoter; this allele was transduced into the wild-type strain TR10000 to form TT25784.

(iv) Deletion of the *amn* promoter and coding sequence. Primers TP1990 (CCAGTACCCTTCTTTTTGAGCGATGATGGTGGCGCTAAGACACACA ACCACACCACACCAC) and TP1991 (TGAGTGCAAACGATCACCTTCTG CGCGTAGTAAATCAATAGCACCACCAAACACCCCCCAAAACC) were used to amplify a Kn^r determinant and generate a fragment whose 5′ ends supported recombination events that allow the Kn^r determinant to replace the promoter and coding sequence of the *amn* gene of strain TT25784 (see above). The resultant DNA was electroporated into strain TT25784 containing the λ-Red recombinase plasmid pKD46 (8), and 50 μg/ml kanamycin was used to select inheritance of the incoming cassette. The resulting strain, TT25785, is isogenic with TT25784 but has no promoter for the *lacZ* sequence.

In-frame deletions of *cobU* and *cobS* genes. Chromosomal in-frame deletion mutations were constructed for the *cobU* and *cobS* genes using linear transformation. Each deleted region was replaced by an inserted chloramphenicol resistance (Cm^r) cassette flanked by FLP recombination target (Frt) sites, which did not disrupt the reading frame. Primers used to amplify the Cm^r cassette prior to recombination were TP1778 (GCACGTAGTGGTAAAAGCCGTCATGCTGA AGCCTTAATTGTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCC ACCAAACACCCCCCAAAACC) and TP1779 (ACCTGAGACTACCAGCC AGACCTCATCCGCCGCTGCCGCCGAAGTTCCTATTCTCTAGAAAGT ATAGGAACTTCACACACAACCACACCACACCAC) for *cobU* and primers TP1780 (ATGCTCGCTTTTATTAGCCGCTTGCCCGTACCGTCACGCCTG AAGTTCCTATACTTTCTAGAGAATAGGAACTTCCACCAAACACCCCC CAAAAC) and TP1781 (GATCAATTCACCAAGTTCGATCGCCGCGCCCA GCGTATCGGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCACAC ACAACCACACCACACCAC) for *cobS*. After the Frt-Cm^r-Frt sequence was recombined in place of the normal gene sequence, the Cm^r cassette was removed by recombination of the Frt sites induced by transducing in the temperature-sensitive pCP20 plasmid which encodes Frt recombinase. The plasmid was then removed by overnight growth of cells at 37°C, leaving a single in-frame Frt sequence at the site of the deletion. The constructed deletions were each transduced by a P22-mediated cross into a strain with a wild-type genetic background. The crosses were verified by PCR amplification of the mutant region and DNA sequencing of the deletion endpoints.

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries

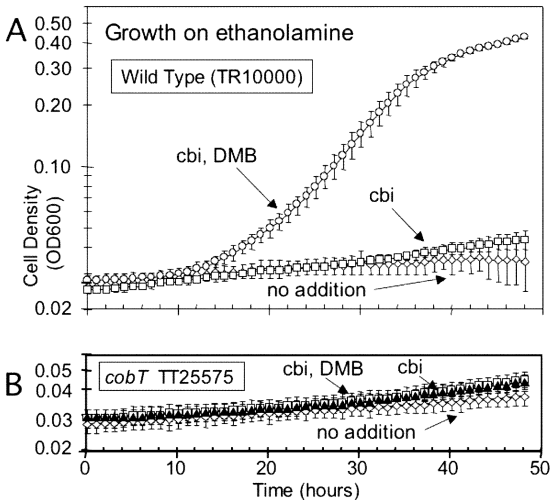

FIG. 3. DMB-dependent aerobic growth on ethanolamine. (A) Aerobic growth of wild-type *S. enterica* (TR10000) on minimal NCE medium with additions as shown. (B) A *cobT* mutant (TT25575) cannot grow even when both Cbi and DMB are provided. Culture OD_{600} was monitored using a BioTek Synergy HT plate reader and KC4 software; it is presented on a logarithmic scale.

RESULTS

Conditions that prevent synthesis or installation of any α-axial cobalt ligand. Wild-type *S. enterica* can use ethanolamine as an aerobic source of carbon and energy only when both Cbi and DMB are provided (Fig. 3A). The Cbi requirement reflects the inability to synthesize the corrinoid precursor in the presence of oxygen. The requirement for DMB suggests that cells make insufficient DMB and can insert no alternative α-axial ligand into "complete" corrinoids under these conditions.

Use of exogenous DMB requires CobT enzyme (Fig. 3B), which is thought to exchange DMB with the nicotinamide moiety of NAD^+ to form α-DAD (α-5,6-dimethylbenzimidazole-5-β-[9H-adenine]-dinucleotide = DMB-R-P-P-R-Ade) (30).

Selection of mutants able to use ethanolamine aerobically without added DMB. Mutants were sought that could grow on ethanolamine plus Cbi without exogenous DMB. Wild-type *S. enterica* (strain TR10000) was plated on rich medium with a crystal of the mutagen NG. The mutagenized bacterial lawn was replica plated to minimal medium containing ethanolamine and Cbi (100 nM), but no DMB. While these conditions do not support growth of the population as a whole, a ring of colonies grew at the position corresponding to the edges of NG killing zone on the plate with rich medium. The 27 independent mutants isolated in this way are listed in Table 1 and described below.

Figure 4A and C show the growth of two representative mutants (described below); all others exhibited similar growth phenotypes. DMB-independent growth required the CobT enzyme (Fig. 4B and D), which is known to be necessary for the addition of DMB as an α-axial ligand (30, 55).

All DMB-independent mutants make pseudo-B_{12} coenzymes (α-axial adenine). Mutants were grown on NCE medium with ethanolamine and Cbi and with glycerol added to enhance growth; the same results were obtained when ethanolamine was the sole carbon source. Corrinoids were extracted in the presence of excess cyanide and fractionated on a C_{18} HPLC column without CN^- ions in the buffer as described in Materials and Methods. All of the mutants tested produced pseudo-B_{12} cofactors, isolated as CN-pseudo-B_{12} (= pseudo-vitamin B_{12}), which has adenine as the α-ligand. No cyanocobalamine CN-B_{12} (DMB base) was detected.

Figure 5A shows the CN-Cbl and β-CN-pseudo-B_{12} standards, and Fig. 5B shows the corrinoids extracted from cells of the *apt-18* mutant. On the basis of accurate MS measurements (Fig. 5D), peaks 1 and 3 are both mono-CN-pseudo-B_{12}

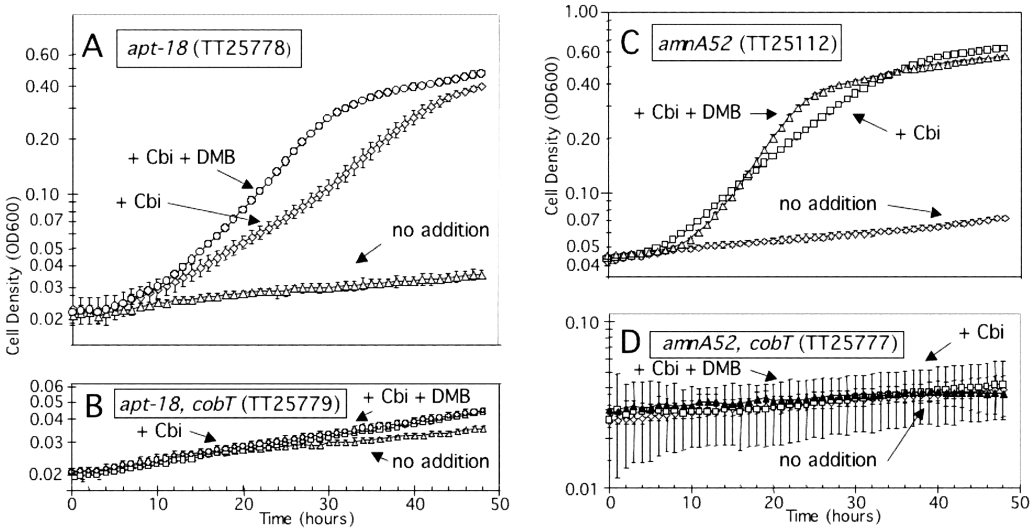

FIG. 4. Growth of DMB-independent *apt* and *amn* mutants. Growth was tested aerobically on minimal NCE medium with ethanolamine as the sole carbon source, and growth was monitored at OD_{600}. (A) Strain TT25778 carries the constructed deletion mutation *apt-18*. (B) Strain TT25779 carries the *apt-18* deletion and a *cobT* insertion mutation. (C) Strain TT25112 carries the *amnA52* point mutation (G935A). (D) Strain TT25777 carries both the *amnA52* point mutation and a *cobT* insertion.

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries

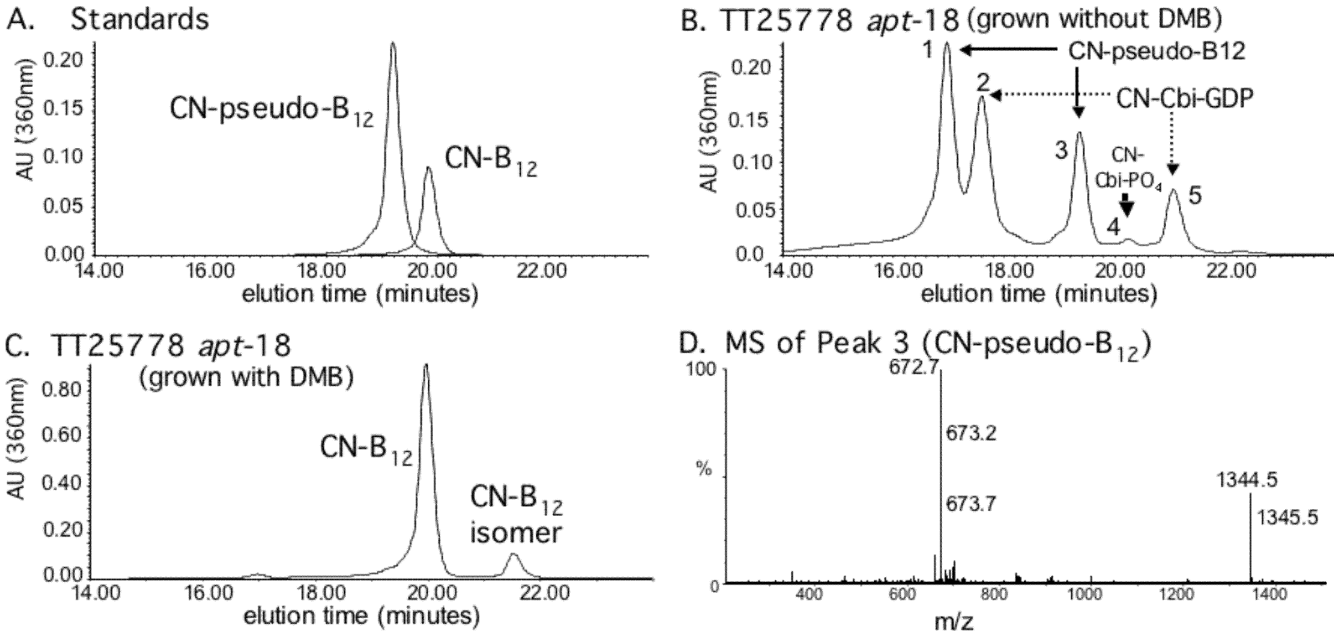

FIG. 5. HPLC/MS of cyanidated extracts from the *apt-18* deletion mutant (TT25778). (A) Separation of standards. CN-pseudo-B_{12} elutes at 19.39 min, and vitamin B_{12} (CN-Cbl) elutes at 20.03 min under the chromatographic conditions used for all separations. (B) Cyanidated extracts from cells grown on ethanolamine and cobinamide. Peak 1 (16.97 min), α-CN-pseudo-B_{12} isomer; peak 2 (17.60 min), CN-cobinamide-GDP isomer; peak 3 (19.38 min), CN-pseudo-B_{12}; peak 4 (20.03 min), CN-cobinamide phosphate; peak 5 (21.07 min), CN-cobinamide-GDP. (C) Cyanidated extracts from cells grown on ethanolamine and cobinamide in the presence of DMB. The peaks are vitamin B_{12} (20.00 min) and B_{12} isomer (21.55 min). AU, absorbance units. (D) Electrospray ionization mass spectrum of peak 3 having a molecular ion $(M+H)^+$ of 1344.5453, the exact mass of CN-pseudo-B12 (change of 0.5 ppm). Peaks with standard β-CN-corrinoid forms are indicated by solid black arrow; alternative α-CN isomers are indicated with dashed lines.

(=pseudo-vitamin B_{12}), which appears in two forms due to the process used to extract pseudo-B_{12} cofactors. The extraction in the presence of CN^- ions converts corrinoids to dicyano forms. When CN^- is removed, a mixture of monocyano forms (α or β) is generated. This mixture was subjected to HPLC fractionation before it had been converted to the β-CN isomer (pseudo-vitamin B_{12}), which is the equilibrium form in the presence of traces of CN ion. Only the β-CN isomer (peak 3) allows the adenine attached to the nucleotide loop to act as the α-ligand; this form runs with the standard (19.38 min). According to the MS data and UV-visible light spectral evidence, peak 1 is suggested to be the monocyano (α-CN) form, which runs differently, because α-CN prevents adenine from coordinating with cobalt. The chemical background for the above interpretation has been described previously (23a). All of the mutants yielded these two CN-pseudo-B_{12} forms following growth on ethanolamine plus $(CN)_2$Cbi.

Peaks 2 and 5 in Fig. 5B are two monocyano (α and β) isolation forms of the corrin precursor Ado-Cbi-GDP, a cyanidated derivative of the activated corrin compound [Ado(β)-Cbi-GDP], a known intermediate formed when CobU enzyme acts on Ado-Cbi (Fig. 2). Peak 4 is one of the monocyano (α or β) derivative of Cbi-PO_4, the preceding intermediate in the pathway; the second monocyano isomer of this intermediate is present but only in very small amounts. These monocyanidated precursors of pseudo-B_{12} coenzymes were found for all of the mutants described below following growth on $(CN)_2$Cbi.

When the same mutants were grown with both DMB and $(CN)_2$Cbi, the extracted corrinoids included two isolation forms of CN-B_{12} (= CN-Cbl = vitamin B_{12} with DMB as the lower ligand), rather than CN-pseudo-B_{12} (pseudo-vitamin B_{12}). Data for the *apt-18* deletion mutant are shown in Fig. 5C. Mass measurements and UV-visible light spectral evidence demonstrated that both forms were mono-CN-Cbl, i.e., complete B_{12} corrinoids with DMB attached to the nucleotide loop, and are assumed to form as described above for pseudo-B_{12}. The first (more polar) form is inferred to be the β-CN-B_{12} (i.e., vitamin B_{12}), and it elutes at the same time as the CN-B_{12} standard. This result demonstrates that although mutants are able to make pseudo-B_{12} cofactors, they make vitamin B_{12} only when DMB is provided.

Genetic characterization of the mutations that cause pseudo-B_{12} synthesis during aerobic growth on ethanolamine. Insertions of transposon Tn*10* were isolated near several of the mutations (see Materials and Methods). Four distinct Tn*10* insertions were identified, each closely linked (in transduction crosses) to a different subset of the mutations. Each mutation with a linked insertion was moved into an unmutagenized wild-type strain (TR10000) using Tn*10* as the selective marker, demonstrating that the DMB-independent growth phenotype was due to a single, heritable mutation in the region. The junction sequence between each Tn*10* element and the adjacent chromosomal region (see Materials and Methods) revealed the genomic position of the insertion. Most of the point mutations were identified by determining the sequence of the chromosomal region surrounding the linked Tn*10* insertion (Table 2).

Mutations that cause loss of adenine phosphoribosyltransferase (Apt). Seventeen of the 27 mutations affect the *apt* gene

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries

TABLE 2. Mutants able to grow aerobically on ethanolamine plus Cbi

Affected enzyme (gene)	Strain	Gene(s) linked to Tn*10*	Map position	Allele	Base change	Amino acid change
Adenine phosphoribosyltransferase (*apt*)	TT25725	*acr, fsr*	11.5	*apt-1*	C401T	T134I
	TT25726	*acr, fsr*	11.5	*apt-2*	G212A	G71D
	TT25727	*acr, fsr*	11.5	*apt-3*	G439A	G147R
	TT25728	*acr, fsr*	11.5	*apt-4*	G212A	G71D
	TT25729	*acr, fsr*	11.5	*apt-5*	G307A	E103K
	TT25730	*acr, fsr*	11.5	*apt-6*	G388A	A130T
	TT25733	*acr, fsr*	11.5	*apt-7*	G212A	G71D
	TT25734	*acr, fsr*	11.5	*apt-8*	G409A	A137T
	TT25735	*acr, fsr*	11.5	*apt-9*	C224T	A75V
	TT25737	*acr, fsr*	11.5	*apt-10*	G212A	G71D
	TT25738	*acr, fsr*	11.5	*apt-11*	G-10A	(rbs)[a]
	TT25740	*acr, fsr*	11.5	*apt-12*	C224T	A75V
	TT25742	*acr, fsr*	11.5	*apt-13*	C64T	P22S
	TT25743	*acr, fsr*	11.5	*apt-14*	C196T	R66C
	TT25744	*acr, fsr*	11.5	*apt-15*	C275T	P92L
	TT25747	*acr, fsr*	11.5	*apt-16*	G185A	G62D
	TT25750	*acr, fsr*	11.5	*apt-17*	G242A	G81D
AMP nucleosidase (*amn*)	TT25731	STM2008	43.4	*amnA51*	G1096A	V366M
	TT25732	STM2008	43.4	*amnA52*	G935A	S312N
	TT25739	STM2008	43.4	*amnA53*	G1096A	V366M
	TT25741	STM2008	43.4	*amnA54*	C1064T	T355I
	TT25746	STM2008	43.4	*amnA55*	G1096A	V366M
	TT25745	STM2008	43.4	*amnA56*	Not done	
Unknown	TT25736	None	Unknown	*amnX61*	Not done	
	TT25748	None	Unknown	*amnX62*	Not done	
	TT25724	STM2503	54.2	*dmb-1*	Not done	
	TT25749	None	Unknown	*dmb-2*	Not done	

[a] (rbs), ribosome binding site.

(Table 2). The Apt enzyme catalyzes reaction of adenine with phosphoribosyl pyrophosphate to form AMP and pyrophosphate (PP). This salvages free adenine and is likely to be driven by hydrolysis of PP. Two lines of evidence suggest that the *apt* mutations owe their DMB independence phenotype to loss of Apt activity. First, the *apt-11* mutation is a G-A transition outside the *apt* coding region (position −10) that removes one of the three matches to the ribosome binding (Shine-Dalgarno) consensus sequence and alters one of the two adjacent G residues shared by most such elements. This change is predicted to reduce *apt* expression. Second, a constructed in-frame deletion mutation *aptA18*::Cm^r(sw) caused the same growth phenotype as the several *apt* point mutations (DMB-independent growth on ethanolamine). The *apt* mutations thus impair the adenine salvage pathway and are expected to cause an increase in the intracellular concentration of free adenine.

Mutations that increase AMP nucleosidase activity (Amn). Six of the 27 mutations allowing synthesis of pseudo-B_{12} coenzymes were linked to a Tn*10* insertion (STM2008), adjacent to the distal end of operon that encodes the B_{12} biosynthetic enzymes (*cob*). Five mutations linked to this Tn*10* insertion were sequenced (Table 2), and each had a base substitution inferred to cause an amino acid substitution in the Amn protein. This enzyme removes adenine from the ribose of AMP, leaving ribose-5′-PO_4 (26, 28, 51), and while considerable biochemical work has been done on the enzyme, no mutant phenotype has been reported, and no physiological role has been demonstrated.

The *amnA* mutations that allow DMB-independent growth appear to increase the level of nucleosidase activity (AMP → adenine + ribose-5-PO_4). They might do this by increasing the half-life of the enzyme or by causing resistance to feedback inhibition. Consistent with this idea, these mutations were rarer than those in *apt* (6 compared to 17 of 27), and three of the five independent *amn* mutations sequenced have the same base change. In addition, a constructed *amnA* deletion mutation, *amnA59*::Kn^r(sw), did not cause a DMB-independent growth phenotype. Furthermore, the *amnA59* deletion mutation eliminated the activity of several DMB-independent mutations unlinked to *amnA*, suggesting that their DMB-independent phenotype depends on Amn activity, which they may increase or deregulate.

Mutations in Amn all affect the Amn catalytic domain. The Amn protein has been studied at both a functional (17, 27, 46–48) and structural (14, 58) level. The enzyme is feedback inhibited by inorganic phosphate (P_i) (47, 48) and activated by MgATP (48, 49). Structural studies have identified the binding sites for the substrate, AMP, and for P_i, a feedback inhibitor of the *Escherichia coli* enzyme (58).

The primary protein sequence of Amn from *S. enterica* serovar Typhimurium is 93% identical with that of the *E. coli* enzyme with most of the sequence divergence in the 180-residue N-terminal domain. All of the *S. enterica* serovar Typhimurium Amn mutations described above are clustered in the conserved C-terminal catalytic domain near residues (K367 and T372) shown previously to bind P_i (58). Three mutations

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries

(*amnA51*, *amnA53*, and *amnA55*) are identical (V366M) and alter the amino acid adjacent to one of the P_i binding residues K367. Mutation *amnA54* alters a residue (T355I) in the same region. We predict that these *amn* mutations may interfere with P_i binding, thereby releasing the enzyme from feedback inhibition. Such activation of the enzyme would be expected to increase the level of free adenine and could account for the ability of these mutants to incorporate adenine into pseudo-B_{12}. The third mutation [*amnA52*(S312N)] affects the AMP binding site, but it is not clear how this substitution might affect Amn function.

A heritability test was done for mutation *amnA52*(*G935A*) to demonstrate that it is responsible for the DMB-independent growth phenotype. A donor strain carrying *amnA52* and the nearby Tn*10* insertion (TT25112) was crossed with a wild-type recipient selecting for growth on tetracycline. Of 40 transductants analyzed, 37 inherited the donor ability to grow without DMB and all of the 5 randomly chosen for sequencing acquired the donor *amnA52* base change (G935A). Conversely, the three rare recombinants whose growth remained dependent on DMB all showed the wild-type *amn* sequence.

Model for how *apt* and *amn* mutations stimulate pseudo-B_{12} synthesis. Features of the model are listed below and diagrammed later (see Fig. 7). (i) In the synthesis of pseudo-B_{12} cofactors, free adenine base is the precursor of the α-axial ligand. (ii) The *apt* mutations increase the pool of free adenine by impairing the adenine salvage pathway. (iii) The *amnA* mutations increase the pool of free adenine by increasing the activity of Amn, a glycohydrolase that releases adenine from AMP. (iv) Adenine base (like DMB base) is a substrate of the CobT enzyme, which catalyzes the exchange of DMB base with the nicotinamide moiety of NAD to form the DMB-adenine dinucleotide, α-DAD (30). By attaching the N-7 of adenine to C-1 of ribose, CobT forms adenine(7)-adenine dinucleotide (α-Ade7-1R5-P-P-5R1-9Ade). The CobT product (α-A^7A^9D) thus has one adenine attached at N-7 and the other at N-9. (v) Either of the two dinucleotides (α-DAD or α-A^7A^9D) can serve as a substrate for CobS, which is proposed to attach the entire dinucleotide to AdoCbiGDP by an attack of a free 3′OH of a dinucleotide ribose on the pyrophosphate bond of the activated corrin compound, Ado-Cbi-PP-R-guanine. This releases GMP from the corrin precursor and produces a corrinoid whose nucleotide loop ribose has three substitutions (see Fig. 7). (vi) The CobC enzyme catalyzes hydrolytic release of an ADP moiety from the 5′ position of ribose to leave the final complete corrinoid.

Evidence that free adenine stimulates pseudo-B_{12} synthesis. The model described above predicts that adenine is a substrate for CobT and the precursor of the α-axial ligand of pseudo-B_{12} cofactors. Exogenous adenine was tested for its effect on the ability of *S. enterica* to use ethanolamine aerobically without added DMB. As seen in Fig. 6, adenine was found to improve growth and substitute in part for added DMB. While stimulation was less than that seen for DMB, it increased with adenine concentration and required a functional CobT enzyme. It is not surprising that the stimulation by adenine is weak, since import of adenine is driven by internal formation of AMP (by the Apt enzyme), so only slight increases in internal adenine are expected.

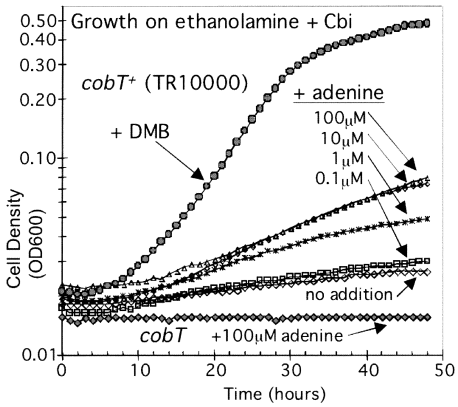

FIG. 6. Effect of adenine base on the ability to use ethanolamine. Wild-type cells were tested for the ability to grow aerobically on minimal NCE medium with ethanolamine as the sole carbon source and the indicated supplementation by adenine. Culture OD_{600} was monitored using a BioTek Synergy HT plate reader and KC4 software and is presented on a logarithmic scale.

Two mutations unlinked to *amn* require Amn activity for their phenotype. The constructed *amn* deletion mutation *amnA59*::Kn^r(sw) was crossed into all 27 of the DMB-independent mutants. All six of the *amn* point mutants lost DMB independence when they received the deletion, as expected, since the gain-of-function *amn* point mutations would be removed. None of the 17 *apt* mutants lost DMB independence, demonstrating that other sources of free adenine are sufficient to provide the growth phenotype when the salvage pathway is blocked. Except for the two mutations described below, all other DMB-independent mutants maintained their DMB-independent growth phenotype after introduction of an *amn* deletion.

Two additional mutations (*amnX61* and *amnX62*) have not been mapped but are not linked to the *amn*, *apt*, or *guaAB* loci (see below). Both of these mutants lost their DMB-independent growth phenotype when the *amn* deletion mutation was introduced. This suggests that the mode of action of these mutations involves increasing the activity of Amn.

Tests of Amn regulation. To test the possibility that *amn* is transcriptionally controlled, an operon fusion was constructed that places a *lacZ* gene under transcriptional control of the *amn* promoter. Evidence that expression of *lacZ* was actually under control of the *amn* promoter was the finding that deletion of the *amn* promoter essentially eliminated transcription of the *amn-lac* fusion.

Under all conditions tested, *amn* was constitutively transcribed at a low level, producing about 40 Miller units of β-galactosidase regardless of the carbon source or the presence of vitamin B_{12}, DMB, or Cbi. This contrasts with evidence in *E. coli* that *amn* transcription increased in response to added cyclic AMP (26). Since in vitro studies showed that AMP nucleosidase of *E. coli* is subject to feedback inhibition by phosphate, the concentration of phosphate in the medium was varied from 73 mM (E medium) to 1.3 mM (morpholinepropanesulfonic acid [MOPS] medium) (34) but had no effect on the DMB requirement for growth on ethanolamine.

A mutation that maps near *ppk* and *guaAB* stimulates pseudo-B_{12} synthesis independent of Amn. One of the DMB-

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries

independent mutants (*dmb-1*) was 85% linked to a Tn*10* insertion in the STM2503 gene located close to the guanine biosynthetic genes *guaA* and *guaB*. The mutation has not yet been identified.

Synthesis of pseudo-B_{12} (like that of B_{12}) requires CobT, CobS, CobU, and CobU. CobT is required for synthesis of both B_{12} cofactors (Fig. 2) and for the production of pseudo-B_{12} cofactors. The role of CobT in B_{12} production has previously been demonstrated (30, 56). The role of CobT in pseudo-B_{12} production was shown by moving a *cobT* mutation (TT24729) into all of the 27 mutants shown in Table 2, all of which grow without DMB because they synthesize Ado-pseudo-B_{12}. In all mutants, the DMB-independent growth phenotype (ability to make pseudo-B_{12} cofactors) was lost after the addition of the *cobT* mutation (Fig. 4). The role of CobT in adding DMB to form B_{12} cofactors was confirmed by showing that these same mutants failed to make active corrinoid cofactors even when DMB was added.

By similar genetic tests, it was shown that the CobU, CobS, and CobC proteins are required to make pseudo-B_{12} cofactors. In-frame deletions of *cobU* and *cobS* were constructed and moved into strains with the deletion mutation *apt-18*, which causes strains to synthesize only pseudo-B_{12} cofactors. The added *cob* deletions allow normal expression of genes located distal to the promoter in the *cob* operon. For testing the requirement for CobC, a single *cobC*::Tn*10* insertion mutation was transduced into a strain carrying the *apt-18*::Cm^r deletion; polarity is not an issue, since *cobC* is a single gene outside the operon (37). The *cobU*, *cobS*, and *cobC* mutations all eliminated the ability of the *apt-18* deletion mutant to grow on Cbi; since the mutant normally made pseudo-B_{12} (see above), the added deletions are concluded to block incorporation of adenine. As expected, these double mutants failed to make active corrinoid cofactors even if DMB was provided. This confirms the previous finding that CobU, CobS, and CobC are needed for cobalamin production (31, 36). It should be noted that the *cobC* mutation blocked pseudo-B_{12} production, while the same mutation caused only a partial block in synthesis of corrinoids in the presence of DMB. We conclude that the CobT, CobU, CobS, and CobC enzymes are all required for synthesis of both Ado-Cbl (α ligand DMB) and Ado-pseudo-B_{12} (α ligand adenine). The leakiness of the block in the presence of DMB may result from accumulation of the final intermediate when excess DMB was provided, allowing some other enzyme to replace CobC in the last reaction (see Discussion). Since adenine availability seems to limit growth in the absence of DMB (see section on precursor accumulation above), lower accumulation of the penultimate intermediate is expected.

Wild-type cells produce Ado-pseudo-B_{12} during aerobic growth on propanediol. The above evidence for synthesis of pseudo-B_{12} cofactor and its dependence on the CobT, CobU, CobS, and CobC proteins was based on a set of DMB-independent mutants. It seemed important to determine whether this conclusion is true for wild-type *S. enterica* as well.

Several earlier reports suggested the possibility that wild-type *S. enterica* can make corrinoids other than B_{12}. It was reported that wild-type *S. enterica* synthesizes pseudo-B_{12} during growth under strictly anaerobic conditions and standard B_{12} under microaerobic conditions (21, 22). Nutritional experiments suggested that wild-type *S. enterica* uses a ligand other than DMB during growth on propanediol. While DMB was required for aerobic growth on ethanolamine (Cbi), no DMB was required for growth on propanediol under the same conditions (38).

Corrinoids were extracted from wild-type *S. enterica* cells grown aerobically on propanediol (plus Cbi) and examined by HPLC/MS. The corrinoid extracted from these cells was CN-pseudo-B_{12} (data not shown). The ability of wild-type *S. enterica* to make corrinoids and grow on propanediol under these conditions was blocked by a *cobT* mutation. These results provide additional evidence that wild-type cells can make pseudo-B_{12} cofactors and do so using the same pathway as the mutants described above. In addition, providing DMB to these wild-type cell cultures caused a shift from pseudo-B_{12} to B_{12} cofactor production. The question of why DMB is required for aerobic growth on ethanolamine but not on propanediol is addressed below.

Pseudo-B_{12} (or derived forms) can serve as cofactor for MetH and EutBC and PduCDE enzymes. Three known enzymes of *S. enterica* depend on corrinoid cofactors (MetH, EutBC, and PduCDE). The synthesis of two different cofactors raises the question of whether all three enzymes can use both pseudo-B_{12} and pseudo-B_{12} cofactors. Use of pseudo-B_{12} by propanediol dehydratase (PduCDE) is implicit in the finding that only pseudo-B_{12} cofactors were found during growth on propanediol (above). The other enzymes were tested with CobT mutant cells that cannot assemble a corrinoid internally. Pseudo-B_{12} was provided as CN-pseudo-B_{12}. For this added corrinoid to provide a cofactor for ethanolamine ammonia lyase (EutABC) or methonine synthetase (MetH), the vitamin must be transported and used without the loss of its α-ligand adenine base, and it must accept a β-ligand adenosyl group. Since CN-pseudo-B_{12} is a precious commodity, these tests were done by qualitative spot tests on agar plates.

Cells of a *cobT* mutant (TT25575) can grow aerobically on ethanolamine as the sole carbon and energy source only when a "complete" corrinoid with an α-axial ligand (e.g., CN-Cbl) is provided. These cells cannot synthesize the corrin ring aerobically and (because of their CobT defect) cannot add a new α-axial ligand to any provided complete corrinoid. Therefore, they cannot use Cbi as a source of corrinoids. When provided with CN-pseudo-B_{12} (16), this strain grew using ethanolamine as the sole carbon source. Pseudo-B_{12} must therefore serve as an alternative to cobalamin and allow activity of ethanolamine ammonia lyase. This growth is known to require a corrinoid with a β-axial adenosine ligand, suggesting that *S. enterica* can transport CN-pseudo-B_{12} and replace its β-axial CN group with adenosine, using one if its adenosyl transferases, CobA or EutT (11, 50).

Similarly, cells with both a *metE* mutation and a *cobT* mutation (TT25721) were tested for their ability to produce methionine and grow on minimal medium containing glucose using their corrinoid-dependent MetH enzyme. This strain grows if either methionine or B_{12} is provided. Cbi is not sufficient because the CobT defect prevents the addition of an α-axial ligand, even when DMB is given. However, exogenous CN-pseudo-B_{12} allowed strain TT25721 to grow on minimal medium containing glucose but not methionine and thus must serve as precursor for a methyl(β)-corrinoid cofactor used by the MetH enzyme. These results demonstrate that the CobA

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries

enzyme of *S. enterica* can transfer an adenosyl group to CN-pseudo-B_{12} and that the MetH enzyme can replace the β-axial ligand of Ado-pseudo-B_{12} with a methyl group to form the methyl-corrinoid used by MetH (32).

DISCUSSION

Evidence that *Salmonella enterica* synthesizes pseudo-B_{12} coenzymes from provided Cbi using the same enzymes (CobT, CobU, CobS, and CobC) known to be required for production of vitamin B_{12} cofactors is presented. The remarkable feature of the pathway to pseudo-B_{12} is that it appears to use free adenine base as the precursor but does so only when no DMB is available. Use of free adenine in the CobT-catalyzed exchange with nicotinamide of NAD may serve as the means of forming the unusual adenine (N-7)-ribose (C-1) linkage that characterizes pseudo-B_{12} coenzymes (16). Pseudo-B_{12} cofactors can be used by all the corrinoid-dependent enzymes of *S. enterica* and may be the only corrinoid types made under periods of high demand unless DMB is provided exogenously. We suggest that pseudo-B_{12} is the main cofactor used by *S. enterica*, which makes very little DMB but uses it (when provided) in preference to adenine.

Control of α-ligand synthesis. The results presented raise the question, "Why does the ability to assemble complete corrinoids from provided $(CN)_2$Cbi change with the growth conditions?" Only aerobic conditions are discussed, since oxygen is required for synthesis of DMB (15, 29, 54); under these conditions, corrin ring must be provided (as CN_2Cbi). (i) When ethanolamine is the sole carbon source, no complete corrinoids are made unless DMB is provided in addition to Cbi. Selection of the mutants described here depended on this phenomenon. (ii) With glucose as the carbon source, about 100 molecules of B_{12} cofactors (with DMB) are made per cell, presumably using the small amount of DMB synthesized endogenously (2, 20). (iii) When propanediol is the carbon source, pseudo-B_{12} cofactors are made. We suggest a model that explains this behavior using the data described here in combination with previously published results from other labs. The background for this model is discussed below.

Transcription of the divergent operons (*cob* and *pdu*) for cobalamin synthesis and propanediol catabolism is induced by propanediol, but not by ethanolamine (1, 3, 6, 43), and the *cob* operon is repressed by Ado-Cbl (33, 40, 41, 57). Whenever cells induce their ethanolamine or propanediol enzymes, about 10,000 molecules of corrinoids per cell are made; this occurs because the relevant B_{12}-dependent degradative enzymes bind B_{12}, creating a demand for corrinoids that releases *cob* from repression. This in turn creates a demand for lower ligand bases DMB or adenine.

Variation of corrinoid assembly in response to carbon sources. The variation of corrinoid assembly in response to carbon sources can be explained in terms of the above background information.

(i) Growth on ethanolamine plus Cbi. On ethanolamine plus Cbi, the *eut* operon and ethanolamine ammonia lyase are induced (creating demand), but the *cob* and *pdu* operons are tightly repressed. Induction of *eut* requires ethanolamine plus a corrinoid, and even the incomplete corrinoid Ado-Cbi can be used (A. Britten and P. J. Anderson, unpublished); *cob* is repressed by B_{12}, and perhaps also by Cbi. We suggest that DMB can be made only in very small amounts and that some enzyme encoded in the *cob* or *pdu* operons contributes to production of free adenine. This proposal would explain why there is insufficient lower ligand base to produce the corrinoids needed for ethanolamine metabolism when *cob* and *pdu* operons are off (on ethanolamine)—little free adenine is made, and corrinoid demand exceeds the minimal supply of DMB. Therefore, aerobic growth on ethanolamine requires added DMB or enhanced adenine production (by the mutations described here).

(ii) Aerobic growth on glucose plus Cbi. During aerobic growth on glucose plus Cbi, neither major degradative pathway is induced, and corrinoid demand is low. In this situation, extremely low levels of corrinoid are made, and the tiny amount of available DMB is sufficient. That is, very little complete corrinoid is made, and all of it is B_{12}.

(iii) Growth on propanediol. During growth on propanediol, the *cob* and *pdu* operons are induced. There is high demand for corrinoid, and free adenine can be generated by some enzyme encoded within these operons. Endogenous DMB may be present in small amounts but in amounts insufficient to contribute heavily or to inhibit adenine production. This explains the synthesis of pseudo-B_{12} cofactors during growth on propanediol.

The mutants described here increased the level of free adenine and allowed synthesis of considerable corrinoid without induction of the *cob* and *pdu* operons. The addition of exogenous DMB to the mutants or to wild-type cells growing on propanediol (with high levels of the CobS, CobT, and CobC enzymes) causes a shift from pseudo-B_{12} to cobalamins, suggesting that DMB is used preferentially, at least at the high concentrations provided here.

New view of the CobT, CobS, and CobC reactions. There are several uncertainties about this pathway. We have assumed (Fig. 2) that NAD^+ is the CobT substrate that accepts the ligand base (adenine or DMB) in exchange for nicotinamide and that the product of this reaction (α-A^7A^9D or α-DAD) donates the ligand nucleoside to the activated corrin precursor (Ado-Cbi-GDP) by reactions catalyzed by CobS and CobC. However, it was initially suggested that the CobT substrate is the NAD biosynthetic precursor nicotinate mononucleotide (NaMN), which has substantially more favorable K_m and K_{cat} than those of NAD^+(12, 13, 56). If NaMN is the relevant substrate, then the CobT product is a simple nucleotide and all that is required is to remove the phosphate and attach the nucleoside to the activated corrin (using CobS and CobC in either order). Despite its unfavorable kinetic parameters, NAD^+ seemed more relevant because of its higher intracellular concentration and the ability of the NAD^+ exchange product to donate DMB nucleoside in the formation of B_{12} (30). It was therefore proposed (30) that NAD^+ is the substrate and the CobT product is a dinucleotide whose pyrophosphate must be split before one half is attached to the activated corrin. The process is then completed by removal of phosphate. This pyrophosphatase required by the pathway was not revealed by the genetic approaches.

We suggest an alternative pathway (Fig. 7) that accounts for all known mutants and allows either NaMN or NAD^+ to be the substrate of CobT. In this pathway, when NAD^+ is used, the

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries

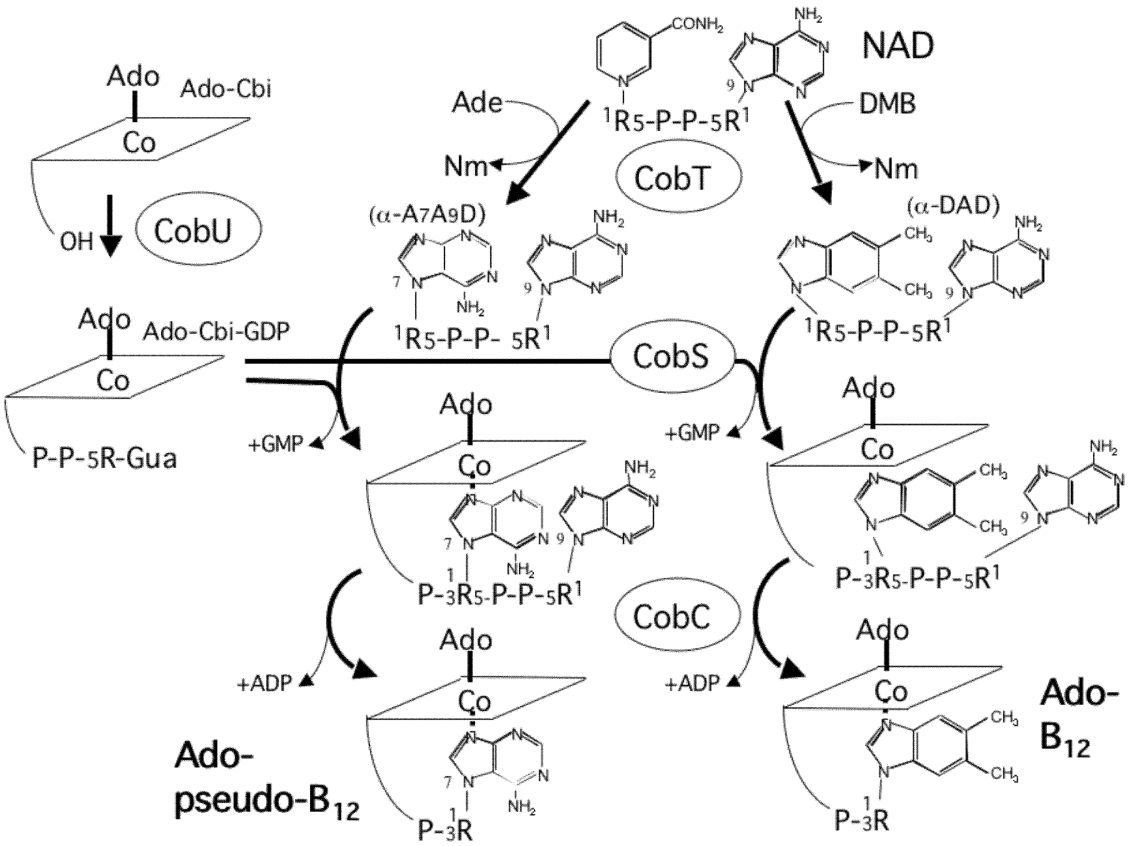

FIG. 7. Proposed pathway for completion of Ado-pseudo-B_{12} and Ado-Cbl. In this pathway, the reaction at the left forming AdoCbi-P-P-5R1-Gua is well established. The lower ligand base (adenine or DMB) is exchanged for the nicotinamide (Nm) moiety of NAD to form the donor dinucleotide (either α-7AAD or α-DAD). It is proposed that CobS catalyzes an attack on the pyrophosphate of the activated corrin precursor by the 3′OH of the ribose nearest the critical base (adenine or DMB) of the dinucleotide. This forms the proposed penultimate intermediate whose ribose has substitutions at positions 1, 3, and 5. Removal of AMP (remainder of the donor dinucleotide) from the 5′ position leaves the complete corrinoid. Ade, adenine; Co, corrinoid; Gua, guanine.

entire CobT product (α-A^7A^9D or α-DAD) is attached directly to the corrinoid precursor by the CobS enzyme. The pyrophosphate of Ado-Cbi-GDP activates the corrin precursor and is attacked by the free 3′OH of ribose from α-DAD or α-A^7A^9D, releasing GMP from the corrin precursor. This generates a corrinoid intermediate whose ribose has three substitutions. Its position 3 is linked by a phosphodiester to the aminopropanol side chain of the corrin. Its C-1 carbon is attached to the base (Ade7 or DMB) that will be the α-axial ligand of the complete corrinoid. Its C-5 position remains attached to ADP (the rest of the incoming α-DAD or α-A^7A^9D dinucleotide). The final step is removal of this ADP by CobC to produce the finished cofactor. Whenever CobT uses NaMN as substrate, the produced mononucleotide (DMB-R-P or A^7-R-P) is transferred by CobS to the activated corrin, and CobC removes the phosphate.

Sources of DMB in *S. enterica*. While genes for synthesis of DMB have been discovered in *Sinorhizobium meliloti* and *Rhodospirillum rubrum* (4, 15, 54), none has been identified among the many B_{12}-deficient mutants isolated in *S. enterica* (10, 18, 19, 45). Two redundant factors may have conspired to allow such mutants to escape detection.

DMB appears to be a minor contaminant in standard agar, and extremely little (about 100 molecules per cell) is sufficient for production of methionine by MetH, the genetic assay used to identify cobalamin mutants. Purified (Noble) agar was used here to show that ethanolamine synthesis required the addition of DMB, since on standard agar, cells showed some growth on ethanolamine without added DMB. The compound contaminating agar is not a complete corrinoid, since Cbi is needed under all aerobic conditions tested. In the purest mineral salts medium (without agar), wild-type *S. enterica* (grown aerobically with Cbi) makes B_{12} (20) but makes only about 100 molecules per cell (2), an amount sufficient for methionine synthesis. It seems likely that this minimal level of DMB synthesized by *S. enterica* could be made either enzymatically or chemically by the "facile oxidative cascade" suggested by Maggio-Hall et al. (29). The amount of DMB contaminating agar is likely to be sufficient to allow methionine production in mutants totally unable to make DMB.

The *bluB* gene, shown to synthesize DMB in other bacteria, has homologues in *S. enterica* genes (*ydjA*, *mdaA*, and *nfnB*) with 25% sequence identity and 41% similarity and similar molecular weight. Deletions mutants for each individual gene and one double mutant (*mdaA nfnB*) were constructed and found unimpaired in their ability to grow using corrinoid-dependent MetH to supply their methionine requirement (aerobically with Cbi but no added DMB). However, this finding cannot be securely interpreted, because one might expect cells

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries

to form pseudo-B_{12} if their pathway to DMB synthesis is blocked.

Our results suggest that *S. enterica* makes pseudo-B_{12} cofactors whenever corrinoid demand exceeds the minimal supply of DMB. That is, adenine may be installed whenever the DMB level is too low. Thus, mutations that prevent DMB synthesis are not expected to block corrinoid production but rather to cause a shift to pseudo-B_{12} synthesis and cause no growth defect. Since *S. enterica* corrinoid-dependent enzymes can use both forms, it will be difficult to genetically identify or characterize mutations in the DMB pathway unless a way can be found to block production or insertion of adenine as an α-axial ligand.

Normal source of free adenine for synthesis of pseudo-B_{12} coenzymes. While adenine levels are increased in the mutants described here, we do not know the source of adenine when wild-type cells make pseudo-B_{12} cofactors. Deletion of the *amn* gene did not prevent pseudo-B_{12} production, making it clear that this is not an essential source; however, the position of the *amn* gene adjacent to the *cob* operon (4.5 kb from CobT) still makes it an attractive candidate for contributing to adenine production in view of the frequent clustering of genes contributing to a single function (24, 25, 35). An interesting possibility is that CobT has a secondary activity of removing adenine from some adenine nucleotide and this activity is inhibited by DMB. Genetic evidence supports the idea that CobT has a second activity that contributes to synthesis of the lower ligand base (7). Such a second activity might resolve the curious situation that CobT has a 100-fold-lower K_m and a 100-fold-higher K_{cat} for the NAD^+ precursor NaMN than those for NAD^+, strongly suggesting that NaMN is the relevant acceptor of the lower nucleotide base (12, 13). The vastly higher intracellular concentration of NAD^+ led to the conclusion that NAD^+ might be a major contributor (30). If CobT has a second activity and produces adenine, its kinetic parameters might be very different when measured in the presence of the adenine donor molecule.

ACKNOWLEDGMENTS

This work was supported by NIH grant GM34804 (J.R.R. and P.J.A.), NIEHS Superfund Basic Research Program 5 P42 ES04699, and NIEHS Center for Children's Environmental Health and Diseases Prevention NIEHS P01 ES11269 (J.L. and B.D.H.).

REFERENCES

1. **Ailion, M., T. A. Bobik, and J. R. Roth.** 1993. Two global regulatory systems (Crp and Arc) control the cobalamin/propanediol regulon of *Salmonella typhimurium*. J. Bacteriol. **175:**7200–7208.
2. **Andersson, D. I., and J. R. Roth.** 1989. Mutations affecting regulation of cobinamide biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **171:**6726–6733.
3. **Bobik, T. A., M. Ailion, and J. R. Roth.** 1992. A single regulatory gene integrates control of vitamin B_{12} synthesis and propanediol degradation. J. Bacteriol. **174:**2253–2266.
4. **Campbell, G. R. O., M. E. Taga, K. Mistry, J. Lloret, P. J. Anderson, J. R. Roth, and G. C. Walker.** 2006. *Sinorhizobium meliloti bluB* is necessary for production of 5,6-dimethylbenzimidazole, the lower ligand of B_{12}. Proc. Natl. Acad. Sci. USA **103:**4634–4639.
5. **Carkeet, C., S. R. Dueker, J. Lango, B. A. Buchholz, J. W. Miller, R. Green, B. D. Hammock, J. R. Roth, and P. J. Anderson.** 2006. Human vitamin B_{12} absorption measurement by accelerator mass spectrometry using specifically labeled ^{14}C-cobalamin. Proc. Natl. Acad. Sci. USA **103:**5694–5699.
6. **Chen, P., M. Ailion, T. Bobik, G. Stormo, and J. Roth.** 1995. Five promoters integrate control of the *cob/pdu* regulon in *Salmonella typhimurium*. J. Bacteriol. **177:**5401–5410.
7. **Chen, P., M. Ailion, N. Weyand, and J. Roth.** 1995. The end of the *cob* operon: evidence that the last gene (*cobT*) catalyzes synthesis of the lower ligand of vitamin B_{12}, dimethylbenzimidazole. J. Bacteriol. **177:**1461–1469.
8. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
9. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. A manual for genetic engineering. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
10. **Escalante-Semerena, J. C., M. G. Johnson, and J. R. Roth.** 1992. The CobII and CobIII regions of the cobalamin (vitamin B_{12}) biosynthetic operon of *Salmonella typhimurium*. J. Bacteriol. **174:**24–29.
11. **Escalante-Semerena, J. C., S.-J. Suh, and J. R. Roth.** 1990. *cobA* function is required for both de novo cobalamin biosynthesis and assimilation of exogenous corrinoids in *Salmonella typhimurium*. J. Bacteriol. **172:**273–280.
12. **Friedmann, H. C.** 1965. Partial purification and properties of a single displacement trans-N-glycosidase. J. Biol. Chem. **240:**413–418.
13. **Friedmann, H. C., and D. L. Harris.** 1965. The formation of alpha-glycosidic 5′-nucleotides by a single displacement trans-N-glycosidase. J. Biol. Chem. **240:**406–412.
14. **Giranda, V. L., H. M. Berman, and V. L. Schramm.** 1989. Crystallographic quaternary structural analysis of AMP nucleosidases from *Escherichia coli* and *Azotobacter vinelandii*. J. Biol. Chem. **264:**15674–15680.
15. **Gray, M. J., and J. C. Escalante-Semerena.** 2007. Single-enzyme conversion of $FMNH_2$ to 5,6-dimethylbenzimidazole, the lower ligand of B_{12}. Proc. Natl. Acad. Sci. USA **104:**2921–2926.
16. **Hoffmann, B., M. Oberhuber, E. Stupperich, H. Bothe, W. Buckel, R. Konrat, and B. Krautler.** 2000. Native corrinoids from *Clostridium cochlearium* are adeninylcobamides: spectroscopic analysis and identification of pseudovitamin B_{12} and factor A. J. Bacteriol. **182:**4773–4782.
17. **Hurwitz, J., L. A. Heppel, and B. L. Horecker.** 1957. The enzymatic cleavage of adenylic acid to adenine and ribose 5-phosphate. J. Biol. Chem. **226:**525–540.
18. **Jeter, R. M., B. M. Olivera, and J. R. Roth.** 1984. *Salmonella typhimurium* synthesizes cobalamin (vitamin B_{12}) de novo under anaerobic growth conditions. J. Bacteriol. **159:**206–213.
19. **Jeter, R. M., and J. R. Roth.** 1987. Cobalamin (vitamin B_{12}) biosynthetic genes of *Salmonella typhimurium*. J. Bacteriol. **169:**3189–3198.
20. **Johnson, M. G., and J. C. Escalante-Semerena.** 1992. Identification of 5,6-dimethylbenzimidazole as the Co alpha ligand of the cobamide synthesized by *Salmonella typhimurium*. Nutritional characterization of mutants defective in biosynthesis of the imidazole ring. J. Biol. Chem. **267:**13302–13305.
21. **Keck, B., M. Munder, and P. Renz.** 1998. Biosynthesis of cobalamin in *Salmonella typhimurium*: transformation of riboflavin into the 5,6-dimethylbenzimidazole moiety. Arch. Microbiol. **171:**66–68.
22. **Keck, B., and P. Renz.** 2000. *Salmonella typhimurium* forms adenylcobamide and 2-methyladenylcobamide, but no detectable cobalamin during strictly anaerobic growth. Arch. Microbiol. **173:**76–77.
23. **Kleckner, N., J. Roth, and D. Botstein.** 1977. Genetic engineering in vivo using translocatable drug-resistance elements. New methods in bacterial genetics. J. Mol. Biol. **116:**125–159.

23a. **Kräutler, B.** 1999. B_{12} electrochemistry and organometallic synthesis, p. 332. *In* R. Banerjee (ed.), Chemistry and biochemistry of B_{12}. John Wiley and Sons, New York, NY.

24. **Lawrence, J.** 1999. Selfish operons: the evolutionary impact of gene clustering in prokaryotes and eukaryotes. Curr. Opin. Genet. Dev. **9:**642–648.
25. **Lawrence, J. G., and J. R. Roth.** 1996. Selfish operons: horizontal transfer may drive the evolution of gene clusters. Genetics **143:**1843–1860.
26. **Leung, H. B., K. L. Kvalnes-Krick, S. L. Meyer, J. K. deRiel, and V. L. Schramm.** 1989. Structure and regulation of the AMP nucleosidase gene (*amn*) from *Escherichia coli*. Biochemistry **28:**8726–8733.
27. **Leung, H. B., and V. L. Schramm.** 1980. Adenylate degradation in *Escherichia coli*. The role of AMP nucleosidase and properties of the purified enzyme. J. Biol. Chem. **255:**10867–10874.
28. **Leung, H. B., and V. L. Schramm.** 1984. The structural gene for AMP nucleosidase. Mapping, cloning, and overproduction of the enzyme. J. Biol. Chem. **259:**6972–6978.
29. **Maggio-Hall, L. A., P. C. Dorrestein, J. C. Escalante-Semerena, and T. P. Begley.** 2003. Formation of the dimethylbenzimidazole ligand of coenzyme B_{12} under physiological conditions by a facile oxidative cascade. Org. Lett. **5:**2211–2213.
30. **Maggio-Hall, L. A., and J. C. Escalante-Semerena.** 2003. Alpha-5,6-dimethylbenzimidazole adenine dinucleotide (alpha-DAD), a putative new intermediate of coenzyme B_{12} biosynthesis in *Salmonella typhimurium*. Microbiology **149:**983–990.
31. **Maggio-Hall, L. A., and J. C. Escalante-Semerena.** 1999. In vitro synthesis of the nucleotide loop of cobalamin by *Salmonella typhimurium* enzymes. Proc. Natl. Acad. Sci. USA **96:**11798–11803.
32. **Matthews, R. G.** 2001. Cobalamin-dependent methyltransferases. Acc. Chem. Res. **34:**681–689.
33. **Nahvi, A., J. E. Barrick, and R. R. Breaker.** 2004. Coenzyme B_{12} riboswitches are widespread genetic control elements in prokaryotes. Nucleic Acids Res. **32:**143–150.

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries

34. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:**736–747.
35. **Osterman, A., and R. Overbeek.** 2003. Missing genes in metabolic pathways: a comparative genomics approach. Curr. Opin. Chem. Biol. **7:**238–251.
36. **O'Toole, G. A., and J. C. Escalante-Semerena.** 1993. *cobU*-dependent assimilation of nonadenosylated cobinamide in *cobA* mutants of *Salmonella typhimurium*. J. Bacteriol. **175:**6328–6336.
37. **O'Toole, G. A., J. R. Trzebiatowski, and J. C. Escalante-Semerena.** 1994. The *cobC* gene of *Salmonella typhimurium* codes for a novel phosphatase involved in the assembly of the nucleotide loop of cobalamin. J. Biol. Chem. **269:**26503–26511.
38. **Price-Carter, M., J. Tingey, T. A. Bobik, and J. R. Roth.** 2001. The alternative electron acceptor tetrathionate supports B_{12}-dependent anaerobic growth of *Salmonella enterica* serovar Typhimurium on ethanolamine or 1,2-propanediol. J. Bacteriol. **183:**2463–2475.
39. **Renz, P.** 1999. Biosynthesis of the 5,6-dimethylbenzimidazole moiety of cobalamin and of the other bases found in natural corrinoids, p. 557–576. *In* R. Banerjee (ed.), Chemistry and biochemistry of B_{12}. John Wiley & Sons, Inc., New York, NY.
40. **Richter-Dahlfors, A. A., and D. I. Andersson.** 1992. Cobalamin (vitamin B_{12}) repression of the Cob operon in *Salmonella typhimurium* requires sequences within the leader and the first translated open reading frame. Mol. Microbiol. **6:**743–749.
41. **Richter-Dahlfors, A. A., S. Ravnum, and D. I. Andersson.** 1994. Vitamin B_{12} repression of the *cob* operon in *Salmonella typhimurium*: translational control of the *cbiA* gene. Mol. Microbiol. **13:**541–553.
42. **Roessner, C. A., and A. I. Scott.** 2006. Fine-tuning our knowledge of the anaerobic route to cobalamin (vitamin B_{12}). J. Bacteriol. **188:**7331–7334.
43. **Rondon, M. R., and J. C. Escalante-Semerena.** 1992. The *poc* locus is required for 1,2-propanediol-dependent transcription of the cobalamin biosynthetic (*cob*) and propanediol utilization (*pdu*) genes of *Salmonella typhimurium*. J. Bacteriol. **174:**2267–2272.
44. **Roth, J. R., J. G. Lawrence, and T. A. Bobik.** 1996. Cobalamin (coenzyme B_{12}): synthesis and biological significance. Annu. Rev. Microbiol. **50:**137–181.
45. **Roth, J. R., J. G. Lawrence, M. Rubenfield, S. Kieffer-Higgins, and G. M. Church.** 1993. Characterization of the cobalamin (vitamin B_{12}) biosynthetic genes of *Salmonella typhimurium*. J. Bacteriol. **175:**3303–3316.
46. **Schramm, V. L.** 1976. Comparison of initial velocity and binding data for allosteric adenosine monophosphate nucleosidase. J. Biol. Chem. **251:**3417–3424.
47. **Schramm, V. L.** 1974. Kinetic properties of allosteric adenosine monophosphate nucleosidase from *Azotobacter vinelandii*. J. Biol. Chem. **249:**1729–1736.
48. **Schramm, V. L., and F. A. Fullin.** 1978. Kinetics of adenosine monophosphate nucleosidase inactivation by phosphate and protection by substrate and allosteric activator. J. Biol. Chem. **253:**2161–2167.
49. **Schramm, V. L., and G. H. Reed.** 1980. Interaction of Mn^{2+} and $MnATP^{2-}$ with the allosteric sites of AMP nucleosidase. J. Biol. Chem. **255:**5796–5801.
50. **Sheppard, D. E., J. T. Penrod, T. Bobik, E. Kofoid, and J. R. Roth.** 2004. Evidence that a B_{12}-adenosyl transferase is encoded within the ethanolamine operon of *Salmonella enterica*. J. Bacteriol. **186:**7635–7644.
51. **Shi, W., K. S. Tanaka, T. R. Crother, M. W. Taylor, S. C. Almo, and V. L. Schramm.** 2001. Structural analysis of adenine phosphoribosyltransferase from *Saccharomyces cerevisiae*. Biochemistry **40:**10800–10809.
52. **Stupperich, E., H. J. Eisinger, and B. Krautler.** 1988. Diversity of corrinoids in acetogenic bacteria: *P*-cresolylcobamide from *Sporomusa ovata*, 5-methoxy-6-methylbenzimidazolylcobamide from *Clostridium formicoaceticum* and vitamin B_{12} from *Acetobacterium woodii*. Eur. J. Biochem. **172:**459–464.
53. **Stupperich, E., and E. Nexo.** 1991. Effect of the cobalt-N coordination on the cobamide recognition by the human vitamin B_{12} binding proteins intrinsic factor, transcobalamin and haptocorrin. Eur. J. Biochem. **199:**299–303.
54. **Taga, M. E., N. A. Larsen, A. R. Howard-Jones, C. T. Walsh, and G. C. Walker.** 2007. BluB cannibalizes flavin to form the lower ligand of vitamin B_{12}. Nature **446:**449–453.
55. **Trzebiatowski, J. R., and J. C. Escalante-Semerena.** 1997. Purification and characterization of CobT, the nicotinate-mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase enzyme from *Salmonella typhimurium* LT2. J. Biol. Chem. **272:**17662–17667.
56. **Trzebiatowski, J. R., G. A. O'Toole, and J. C. Escalante-Semerena.** 1994. 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. J. Bacteriol. **176:**3568–3575.
57. **Tucker, B. J., and R. R. Breaker.** 2005. Riboswitches as versatile gene control elements. Curr. Opin. Struct. Biol. **15:**342–348.
58. **Zhang, Y., S. E. Cottet, and S. E. Ealick.** 2004. Structure of *Escherichia coli* AMP nucleosidase reveals similarity to nucleoside phosphorylases. Structure **12:**1383–1394.

Downloaded from http://jb.asm.org/ on December 30, 2016 by University of North Texas Libraries