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Characterization of the Cobalamin (Vitamin B_{12}) Biosynthetic Genes of *Salmonella typhimurium*

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Salmonella typhimurium **synthesizes cobalamin (vitamin B_{12}) de novo under anaerobic conditions. Of the 30 cobalamin synthetic genes, 25 are clustered in one operon, *cob*, and are arranged in three groups, each group encoding enzymes for a biochemically distinct portion of the biosynthetic pathway. We have determined the DNA sequence for the promoter region and the proximal 17.1 kb of the *cob* operon. This sequence includes 20 translationally coupled genes that encode the enzymes involved in parts I and III of the cobalamin biosynthetic pathway. A comparison of these genes with the cobalamin synthetic genes from *Pseudomonas denitrificans* allows assignment of likely functions to 12 of the 20 sequenced *Salmonella* genes. Three additional *Salmonella* genes encode proteins likely to be involved in the transport of cobalt, a component of vitamin B_{12}. However, not all *Salmonella* and *Pseudomonas* cobalamin synthetic genes have apparent homologs in the other species. These differences suggest that the cobalamin biosynthetic pathways differ between the two organisms. The evolution of these genes and their chromosomal positions is discussed.**

Cobalamin (vitamin B_{12}) is an evolutionarily ancient cofactor (9, 44, 46) and one of the largest, most structurally complex, nonpolymeric biomolecules described. Vitamin B_{12} is an essential nutrient for many animals and must be acquired by ingestion (29). It is generally believed that plant taxa neither synthesize nor utilize cobalamin (40), although studies have disputed this conclusion (79). Bacteria are the primary producers of cobalamin. The structure, role in catalysis, and biosynthesis of cobalamin have been intensely investigated (8, 83, 92). As shown in Fig. 1, cobinamide is derived from the extensive modification of uroporphyrinogen III (Uro III), a precursor of heme, siroheme, chlorophylls, and corrins. The conversion of Uro III to cobinamide constitutes part I of the cobalamin synthetic pathway. This process entails extensive methylation of the porphyrin ring, amidation of carboxyl groups, removal of a ring carbon, insertion and reduction of the cobalt atom, and addition of the adenosyl moiety and the aminopropanol side chain. Part II of the pathway entails the synthesis of dimethylbenzimidazole (DMB), probably from flavin precursors. The DMB moiety provides the Coα (lower) axial ligand of the centrally bound cobalt atom (59) (Fig. 1). Part III of the pathway involves the covalent linkage of cobinamide, DMB, and a phosphoribosyl moiety derived from an NAD precursor to complete the formation of cobalamin. Although adenosylcobalamin is the product of the biosynthetic pathway, forms of cobalamin with methyl or adenosyl groups as the Coβ (upper) axial ligands of cobalt (91, 96) (Fig. 1) serve as coenzymes. When transported into the cell, cobalamin precursors are adenosylated before they are integrated into the cobalamin synthetic pathway (43).

Salmonella typhimurium synthesizes vitamin B_{12} de novo only under anaerobic conditions (57). Although cobalamin is a known cofactor for numerous enzymes mediating methylation, reduction, and intramolecular rearrangements (91, 96), only four vitamin B_{12}-dependent enzymes are known in *Salmonella* spp. None of these enzymes is vital or appears to have a unique value under the anaerobic conditions required for cobalamin synthesis. These enzymes are as follows. (i) Homocysteine methyltransferases catalyze the final step in methionine synthesis. Both a cobalamin-dependent (*metH*) enzyme and a cobalamin-independent (*metE*) enzyme are encoded in the genomes of *Salmonella* spp. and other enteric bacteria (23, 93, 98). (ii) Ethanolamine ammonia lyase degrades ethanolamine to acetaldehyde and ammonia. *Salmonella* spp. can use ethanolamine as a carbon and/or nitrogen source under aerobic conditions when exogenous cobalamin or cobinamide is provided; anaerobically, *Salmonella* spp. can use endogenously produced cobalamin to degrade ethanolamine to provide a very poor source of nitrogen (22, 86, 87). (iii) Propanediol dehydratase converts propanediol to propionaldehyde. Propanediol cannot be fermented or oxidized anaerobically by *Salmonella* spp. Since propionaldehyde can be oxidized only under aerobic growth conditions (56, 75, 104), *Salmonella* spp. require an exogenous corrinoid to use propanediol as a carbon and energy source. (iv) Queuosine synthetase catalyzes the last step in the synthesis of queuosine, a hypermodified nucleoside found in four tRNAs: $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{His}$, and $tRNA^{Tyr}$ (74). Queuosine is not essential for growth under laboratory conditions, and its functions are unknown (45, 74).

Superficially, none of the cobalamin-dependent functions in *Salmonella* spp. appears to justify the synthesis of the cofactor. Methionine can be made both aerobically and anaerobically by use of the cobalamin-independent methyltransferase (*metE*). Neither ethanolamine nor propanediol can serve as a carbon source under the anaerobic conditions required for cobalamin synthesis. Queuosine appears to be a nonessential tRNA modification. Therefore, the only apparent value of vitamin B_{12} in otherwise wild-type cells is in the support of very poor anaerobic growth with ethanolamine as

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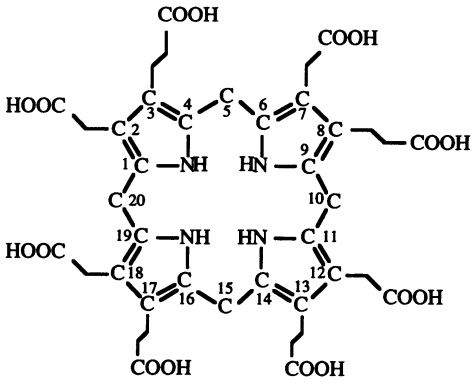
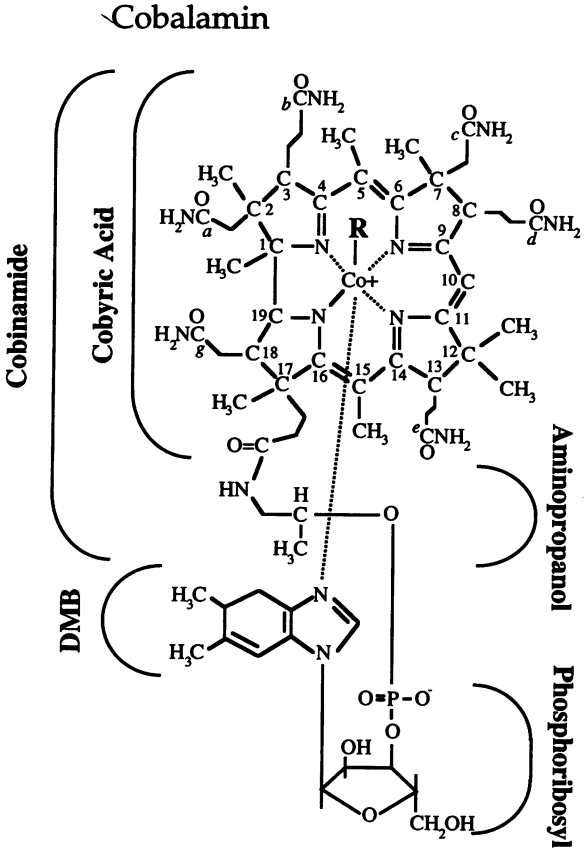


Uroporphyrinogen III



Cobalamin

FIG. 1. Structures of Uro III and cobalamin. Central carbon atoms are numbered; peripheral amidated carboxyl groups are lettered. R groups include CN (cyanocobalamin), CH_3 (methylcobalamin), and adenosyl (adenosylcobalamin).

a sole nitrogen source. The significance of vitamin B_{12} is probably not due to an undiscovered cobalamin-dependent enzyme, since deletion mutants lacking the entire cobalamin synthetic operon grow normally on defined laboratory media under both aerobic and anaerobic conditions; the only detectable mutant phenotype is the failure to utilize ethanolamine as a nitrogen source under anaerobic growth conditions. Since the *Salmonella cob* operon is induced by propanediol, utilization of this compound is likely to provide the major function of cobalamin in these species.

TABLE 1. Strain list

Strain	Genotype	Plasmid
TT10939	*metE205 ara-9 Δphs-204 cbi-141*	
TT10940	*metE205 ara-9 Δphs-204 cbi-142*	
TT10941	*metE205 ara-9 Δphs-204 cbi-143*	
TT10943	*metE205 ara-9 Δphs-204 cbi-145*	
TT10947	*metE205 ara-9 Δphs-204 cbi-149*	
TT10950	*metE205 ara-9 Δphs-204 cbi-152*	
TT11465	*metE205 ara-9 cob-64*::MudJ	
TT15023	*metE205 ara-9* CRR299 (*his cob*)	p41-1
TT17364	*metE205 ara-9* CRR299 (*his cob*)	p51-3
TT17365	*metE205 ara-9* CRR299 (*his cob*)	pJE1
TT17366	*metE205 ara-9* CRR299 (*his cob*)	pJE2

Salmonella mutants unable to synthesize cobalamin fall into three phenotypic classes, corresponding to the three parts of the cobalamin synthetic pathway described above (17, 57, 58). Mutants defective for part I of the pathway synthesize cobalamin only when provided with the corrin intermediate cobinamide (Fig. 1); part II mutants synthesize cobalamin only when provided with DMB; and part III mutants fail to make vitamin B_{12} even when provided with both precursors. Most mutations map in a single operon, *cob*, located at minute 41 on the *Salmonella* chromosome (17, 58). Within this operon, mutations of each phenotypic class (I, II, and III) are physically clustered; that is, part I genes are segregated from part II genes, which are segregated from part III genes. Regulation of the *cob* operon is mediated by the *pocR* gene product (17, 42, 85) and is further influenced by the level of cyclic AMP (42) and the redox state of the cell interior (4, 5). The latter control is mediated by the *arc* global regulatory system (6). In addition, the operon is transcriptionally regulated by adenosylcobalamin (17, 39, 57, 58).

Cobalamin synthetic genes have also been characterized for *Bacillus megaterium* (18, 110) and *Pseudomonas denitrificans* (19–21, 31–33). Extensive enzymology studies have determined the functions of, substrates for, and reaction orders of many of the cobalamin synthetic genes in *P. denitrificans* (10–16, 19, 21, 31–33, 36–37, 100–103). We describe here the nucleotide sequences of parts I and III of the *Salmonella cob* operon. A comparison of these sequences with those of the cobalamin synthetic genes from *P. denitrificans* allows assignment of likely functions to the majority of the inferred proteins. The *cob* operon provides a system for examining the evolution of operon structure, the evolution of a complex metabolic pathway, and the regulation of a large operon of translationally coupled genes.

MATERIALS AND METHODS

Bacterial strains and nomenclature. All strains were derived from *S. typhimurium* LT2 (Table 1). Isolation and genetic mapping of *cob* mutations have been described (17, 58). Although mutations involved in cobalamin synthesis have been assigned the *cob* designation, insufficient letters remain to designate all biosynthetic genes with this nomenclature. Therefore, we have assigned the *cbi* designation to genes involved in the synthesis of cobinamide (part I of the pathway). To avoid confusion, a single series of allele numbers has been assigned to mutations located in both *cob* and *cbi* genes.

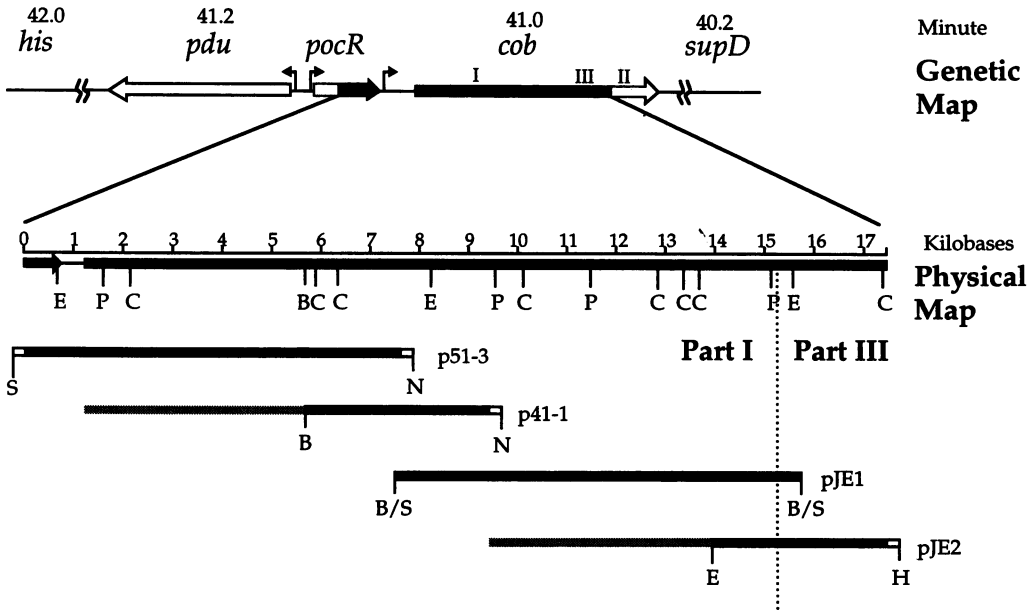


FIG. 2. Clones used for Multiplex sequencing. The genetic map depicts the *pocR* regulon (17, 85) and its position on the *S. typhimurium* genetic map. The physical map denotes the sequenced region, extending from a point within the *pocR* gene through the entire part III region. Letters denote restriction enzymes: B, *Bam*HI; B/S, *Bam*HI cloning site regenerated by insertion of the DNA fragment initially cleaved by *Sau*3A; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Nhe*I; S, *Sph*I. Clones p51-3, p41-1, pJE1, and pJE2 were isolated as described in the text. Plasmid sequences included in the Multiplex analysis are shown as solid bars, portions not included in the Multiplex analysis are shown as shaded bars, and vector sequences included in the Multiplex analysis are shown as open bars.

Cloning of *cob* genes. A plasmid library of *S. typhimurium* LT2 DNA with sized inserts cloned in pBR328 was kindly provided by R. Mauer and C. Miller. Plasmids were mobilized by bacteriophage P22 transduction as described previously (35). All transduction recipients used in initial clone identification carried three mutations: a *recA* mutation was included to prevent recombination, a *metE* mutation rendered methionine synthesis cobalamin dependent, and a mutation located within the *cob* operon at minute 41 blocked cobalamin synthesis. Clones carrying portions of the *cob* operon were selected by their ability to complement the *cob* mutation, allowing cobalamin synthesis and methionine-independent growth under anaerobic conditions. Two clones carrying portions of the CobI region, p51-3 and p41-1, were selected by D. Andersson; two clones carrying portions of the CobIII region, pJE1 and pJE2 (41), were selected by J. Escalante. Restriction mapping was performed by conventional methods.

Fragment selection and preparation. The DNA fragments included in the Multiplex sequencing procedure (25) are indicated in Fig. 2. Fragments were chosen to provide sufficient overlap between adjacent clones. Plasmid inserts were separated from vector sequences by restriction endonuclease digestion and gel electrophoresis. The inserts of plasmids p41-1 and pJE2 were further digested, and the indicated subfragments were recovered. Each DNA fragment was independently polymerized by blunt-end ligation following end repair with Klenow DNA polymerase. The polymerized fragments were sonicated to 400 bp in length (420 ± 100 bp), and the pools of sheared fragments were cloned into various Multiplex vectors as described previously (25). Certain pools were also cloned into the Bluescript vector (Stratagene, Inc.).

DNA sequencing. Multiplex DNA sequencing, based on chemical degradation sequencing methods (67), was performed as described previously (25). Sufficient numbers of random fragment clones were established to provide an average coverage of 6.3-fold; actual coverage of various points in the sequenced region varied from 3- to 30-fold. The film-scanning software REPLICA (26) was used to read sequence film data with a Photometrics/Anorad camera configuration and a VAX 3200 workstation. Contiguous fragments were assembled by use of a modification of the GCG Fragment Assembly Program (38, 95), which accommodates both larger projects and interactions with REPLICA (24). The high-resolution film image and base assignment data were accessible for proofreading and periodic consultation.

Dideoxy sequencing reactions (90), done with both T7 DNA polymerase and Sequenase version 2.0 (U.S. Biochemical Corp.) and with Mn^{2+} and/or 7-deaza-GTP (97), were performed to resolve compressions and other ambiguities in the Multiplex data. Primer-template combinations were chosen with the aid of software described above. Oligonucleotide primers included standard Multiplex tags and *cob*-specific sequences. Templates included Multiplex plasmid clones, primary plasmid clones, and templates amplified via the polymerase chain reaction (88, 89) and purified and sequenced as described previously (63).

Computer protocols. The TBLASTN program was used to compare all *cbi*- and *cob*-encoded inferred proteins with the entire GenBank and EMBL data bases, with each data base sequence being translated in all six frames, by use of a 150 MIPS Silicon Graphics 4D/280 computer at the National Center for Biotechnology Information, Bethesda, Md. (3, 60). Additional searches were done by use of the TFASTA program (77), the PLSEARCH program (94), and the GCG program package (38). Hydropathy plots were obtained from the DNA Strider program (C. Marck) by the method of Kyte and Doolittle (62).

TABLE 2. Genes of the *Salmonella cob* operon

Gene	Start[a]	Codon[b]	Stop[a]	Codon[b]	Length of:		N terminus confirmed[c]	*Pseudomonas* comparison			
					Gene	Peptide		Mean[d]	Identity[e]	Similarity[e]	Gene
cbiA	1288		2667		1,379	458		21.5	33.1	58.5	*cobB*
cbiB	2664		3623		960	319		19.6	36.1	64.2	*cobD*
cbiC	3634		4266		633	210	Yes	19.4	35.5	50.5	*cobH*
cbiD	4266		5405	TAA	1,140	379		19.3			
cbiE	5399		6004		606	201	Yes	21.0	31.8	52.2	*cobL* (N-terminal domain)
cbiT	5994		6572		579	192	Yes	19.6	31.8	57.0	*cobL* (C-terminal domain)
cbiF	6556		7329		774	257	Yes	20.9	40.6	60.6	*cobM*
cbiG	7310		8365		1,056	351		20.1			
cbiH	8365		9090		726	241	Yes	19.6	41.7	59.6	*cobJ*
cbiJ	9087	GTG	9878	TAA	792	263		20.0			
cbiK	9881		10675		795	264	Yes	18.6			
cbiL	10672		11385		714	237	Yes	21.0	32.3	59.4	*cobI*
cbiM	11382		12119	TAA	738	245		18.9			
cbiN	12121		12402		282	93		19.2			
cbiQ	12389		13066	TAA	681	226		19.7			
cbiO	13075		13890		816	271		18.6			
cbiP	13887		15407		1,521	506		18.1	43.1	63.5	*cobQ*
cobU	15407		15949		543	180		19.3	43.0	63.0	*cobP*
cobS	15946		16689		744	247		20.1	33.9	60.8	*cobV*
cobT	16686		17442+		756+	252+		19.8	30.7	56.6	*cobU*

[a] Coordinates of GenBank sequence STYVB12AA (L12006).
[b] Start and stop codons are ATG and TGA, respectively, unless otherwise noted.
[c] The N-terminal amino acid sequence was verified following gene expression from a multicopy plasmid (84).
[d] Mean percent identity of the amino acid sequence to that encoded by 30 *P. denitrificans* genes.
[e] Percent nucleotide identity and percent amino acid similarity were determined by use of the GCG program GAP (38). Each *P. denitrificans* homolog bore amino acid identities at least 3 standard deviations larger than the mean percent identity.

Nucleotide sequence accession number. The nucleotide sequence described in this paper has been submitted to GenBank and has been assigned the locus STYVB12AA and the accession number L12006.

RESULTS

Isolation of *cob* gene clones. The sized plasmid library of R. Mauer and C. Miller was screened as described in Materials and Methods. Four plasmids harboring portions of the *cob* operon were identified by complementation. Plasmid p51-3 was isolated by its ability to complement mutation *cbi-141* (TT10939); this plasmid complemented most mutations defective in part I of the pathway but failed to complement part I mutations *cbi-142*, *cbi-145*, *cbi-149*, and *cbi-152*. Plasmid p51-3 neither complemented nor recombined with *cob-64*, a mutation defective in part III. We inferred that this plasmid included the promoter-proximal end of the operon but lacked some genes for part I functions. Plasmid p41-1 was isolated by its ability to complement mutation *cbi-143* (TT10941); this plasmid complemented the part I mutations that were not complemented by plasmid p51-3. Plasmid p41-1 neither complemented nor recombined with *cob-64*, a mutation defective in part III. Plasmid pJE1 complemented one-half of the part III mutations tested, including *cob-64*; plasmid pJE2 complemented all part III mutations (41). Although plasmid pJE2 did not complement part II mutations located downstream of the part III region (Fig. 2), plasmid recombination could repair several promoter-proximal part II mutations. Therefore, plasmid pJE2 contained a portion of a gene from the part II region. The overlaps among the plasmids were confirmed by restriction mapping (Fig. 2).

Nucleotide sequence. Fragments of these four plasmids were isolated by restriction endonuclease digestion and electrophoresis, and the nucleotide sequence of parts I and III of the *cob* operon, encompassing 17,442 bp, was determined by Multiplex sequence analysis and conventional sequencing methods. The *cob* operon transcription initiation point has been localized to nucleotide 824 of the determined sequence (81). On the basis of this transcription initiation point and the genetic evidence that the entire *cob* locus is a single operon (17), the portion of the *cob* operon reported here includes 20 open reading frames (ORF) dedicated to cobalamin synthesis (Table 2). (An additional ORF extends from the first nucleotide of the sequence to nucleotide 690; this ORF encodes the C-terminal portion of the *pocR* regulatory gene [17, 85] and will be discussed below.) Genes assigned to part I of the pathway are assigned *cbi* designations (synthesis of cobinamide); genes assigned to part III of the pathway are assigned *cob* designations. The N-terminal amino acid sequences of many of the predicted proteins have been determined following the expression of an individual ORF on multicopy plasmids (84); these sequences agree well with the deduced amino acid sequences. Moreover, the molecular weights of the proteins estimated by gel mobility show good agreement with the sizes of the predicted proteins.

The nucleotide sequences at the borders of these genes are presented in Table 3. The ATG start codon and the TGA stop codon are used by 19 of 20 and 15 of 20 genes, respectively; these frequencies are congruent with the sequences of known *Salmonella* genes. The 19 intergenic spaces are uniformly small. Ten of 19 gene junctions have overlapping start and stop codons; that is, they share at least 1 nucleotide. Five genes appear to have start codons entirely contained within the coding sequence of the preceding gene, an arrangement also leaving no intergenic space. At the remaining four junctions, 1 to 10 bases separate adjacent genes. For several of the genes whose start codons were inferred to overlap substantially with the preceding gene, the assigned start was verified by N-terminal polypeptide se-

TABLE 3. Translational coupling of *Salmonella cob* genes

5′ Gene	Upstream region[a,b]	Start of 3′ gene[b]	3′ Gene
	AACAGGATCAGGGTA	ATG	*cbiA*
cbiA	GGCGAGGCGCGTATT	ATGA	*cbiB*
cbiB	CCTGAGGACGACAGT	ATG	*cbiC*[c]
cbiC	CCTCCGGGAGGCCTG	ATG	*cbiD*
cbiD	TGCTAAGGAGCTGCA	ATGCTAA	*cbiE*[c]
cbiE	GCAGTGGTGACCTTG	ATGAAAGATGA	*cbiT*[c]
cbiT	AGAAGGAAGAAAACC	ATGTCAGAGACATTTGA	*cbiF*[c]
cbiF	CAGCGGACTTTAGCC	ATGAATAACGTAAAGCCTGA	*cbiG*
cbiG	AGGAGTTGCACAGTG	ATG	*cbiH*[c]
cbiH	GCGAGGTTACACGCT	GTGA	*cbiJ*
cbiJ	AGCTGAGGAGTAAAA	ATG	*cbiK*[c]
cbiK	GGTAGAGGAGGCGGC	ATGA	*cbiL*[c]
cbiL	GTGGGAGTATGCAGA	ATGA	*cbiM*
cbiM	TACAAGGACATTAAA	ATG	*cbiN*
cbiN	GGTAAACAACGCCGTG	ATGACCGGGCTTGA	*cbiQ*
cbiQ	CCTGTAAGGACTATT	ATG	*cbiO*
cbiO	ATTCAGGGAGGCGTC	ATGA	*cbiP*
cbiP	TCAGGAGCCGGTATG	ATG	*cobU*
cobU	TGGAGTCAAAATTAA	ATGA	*cobS*
cobS	TCTGCTGGCTCTGTT	ATGA	*cobT*

[a] Region immediately upstream of the start of the 3′ gene. Potential Shine-Dalgarno sites are indicated by boldface type.
[b] Sequences of the 5′ gene are underlined.
[c] The N-terminal amino acid sequence was verified (84).

quencing (84) (Tables 2 and 3). The juxtaposition of genes within the *cob* operon is highly suggestive of translational coupling of the gene products.

Sequence homology. Protein sequences were deduced for each ORF in the *cob* operon and used to search DNA sequence data bases as described above. The 20 *Salmonella* genes fell into three classes. (i) Twelve deduced *Salmonella cob* proteins exhibited a high degree of similarity to deduced *cob* proteins from *P. denitrificans*; the genes are listed in Table 2. In each case, the *Salmonella* protein was significantly more similar to a particular *Pseudomonas* protein than to any other protein in the data base. (ii) Two deduced *Salmonella cob* proteins (CbiO and PocR) were similar to proteins of known function but not to any *Pseudomonas cob* protein. (iii) The remaining seven *Salmonella cob* proteins exhibited no significant similarity to any reported protein. However, two of these proteins were likely to be membrane bound, as determined by hydropathy profiles. The high degree of similarity between numerous *Salmonella* and *Pseudomonas* cobalamin synthetic genes is consistent with a common evolutionary origin of these two groups of genes. The failure to identify homologs for all *Salmonella* and all *Pseudomonas cob* genes suggests that differences may exist between the cobalamin biosynthetic pathways of these two organisms.

Figure 3 diagrams the map locations of all known cobalamin synthetic genes in *S. typhimurium* as well as the arrangement of their homologs in the four *Pseudomonas* DNA fragments. Figure 3 also depicts previously described *Salmonella* genes that are located outside the *cob* operon and that are homologs of the *Pseudomonas cob* genes. The *cysG* gene encodes the Uro III methylase shared between cobalamin and siroheme syntheses (108); the *cobA* gene encodes a cobalamin adenosyltransferase (43). The *Salmonella cobA* gene is homologous to the *btuR* gene of *Escherichia coli* (65). Cobalamin synthetic genes without known interspecies homologs are circled in Fig. 3.

Transport proteins. Three of the *Salmonella* genes without *Pseudomonas* homologs, *cbiN*, *cbiQ*, and *cbiO*, are located together at the distal end of the part I region of the *cob* operon (Fig. 3). The deduced amino acid sequence of each gene was used to search the GenBank and EMBL data bases translated in six frames as described above. All proteins most similar to the CbiO protein were members of energy-dependent membrane transport systems (Table 4). Each homologous protein, including CbiO, bore the sites conserved among proton ATPases ($G^{N}_{S}GXGK^{S}_{T}T$ and DEPTX XLD). These data suggest that the CbiO protein belongs to a family of ATP-dependent membrane transport proteins. It is likely that the CbiO protein is involved in the transport of cobalt, since several of the most similar proteins also transport metal ions. Moreover, several part I biosynthetic mutations in this region of the *cob* operon are corrected by the addition of excess cobalt. (It should be noted, however, that one transport protein of this family with an unidentified substrate, locus Bfi1 of *Bacillus firmus* [Table 4], was located immediately upstream of a homolog of methylmalonyl-coenzyme A mutase [54]. Since this enzyme requires vitamin B_{12} as a cofactor, it is plausible that the adjacent transport protein may mobilize cobamides or cobalt.)

Adjacent to the *cbiO* gene are two genes that may encode additional components of a membrane transport system. Hydropathy plots of the deduced *cbiQ* and *cbiO*-encoded proteins are shown in Fig. 4. Typical of some transport proteins, the CbiO protein does not possess strongly hydrophobic membrane-spanning domains (Fig. 4). In other such cases, additional proteins provide membrane anchor peptides and potential membrane pores and also serve to stabilize the ATP-hydrolyzing protein at the membrane (e.g., the HisP, OccP, NocP, SfuC, and DrrA systems; Table 4). The hydropathy plots of the deduced amino acid sequences of the *cbiN* and *cbiQ* genes are typical of proteins with multiple membrane-spanning domains. The CbiN protein has two very hydrophobic domains, and the CbiQ protein has seven potential membrane-spanning domains (Fig. 4). No other protein encoded by the *cob* operon exhibited a hydropathy profile indicative of a membrane-spanning polypeptide. We propose that the *cbiN*, *cbiQ*, and *cbiO*

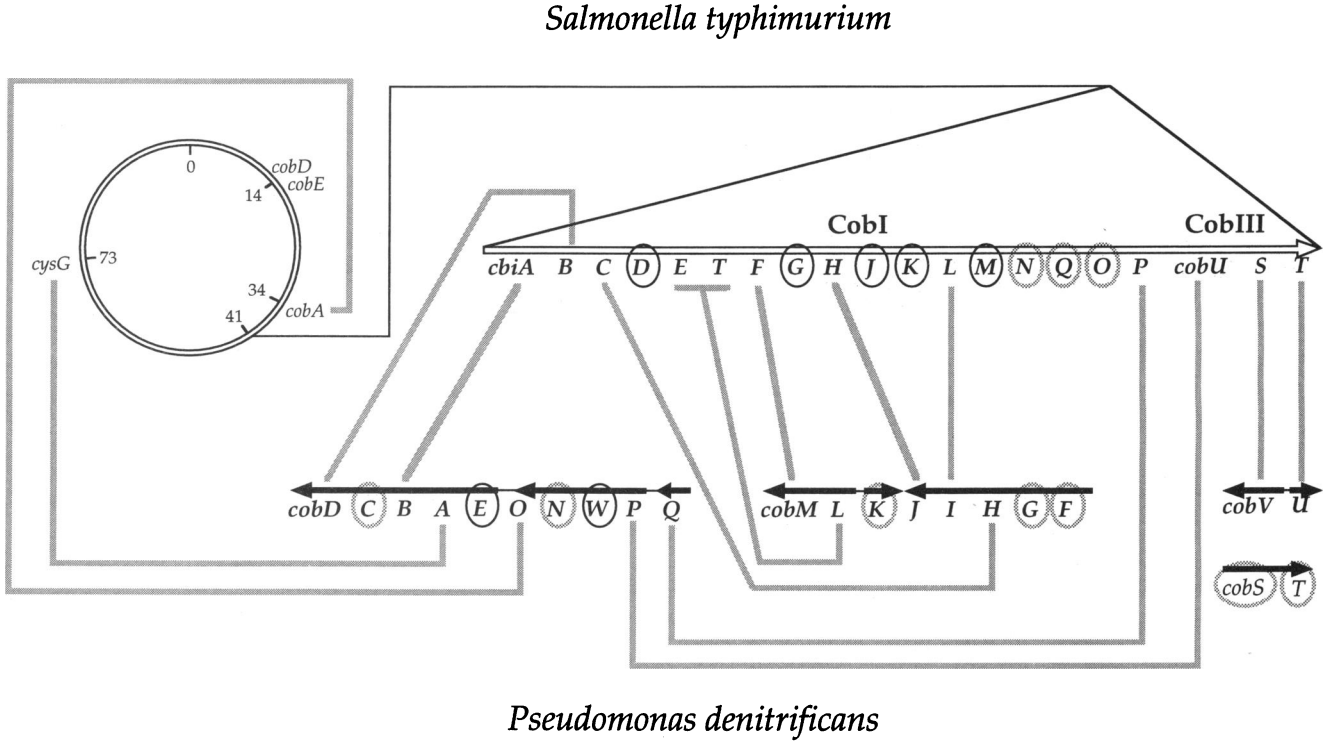


FIG. 3. Cobalamin synthetic genes of *S. typhimurium* and *P. denitrificans* (19, 21, 31–33). Homologs are connected by thick grey lines (Table 2). Genes without homologs are circled. Genes in grey circles have identified functions; genes in black circles do not have identified functions.

genes encode components of an active transport mechanism for cobalt ions, possibly chelated by additional molecules.

It is not surprising that the *cbiN*- and *cbiQ*-encoded proteins did not show strong similarity to other proteins in the data base. If these proteins serve as anchor peptides, they would not bear highly conserved domains, like the ATP binding domain of the CbiO protein, which would allow the identification of distantly related homologous proteins. Similarly, a direct comparison of the sequences of the CbiQ and CbiN proteins with those of the membrane-spanning members of related transport systems (e.g., HisQ, OccQ, and NocQ) did not reveal sufficient similarities to support homology. This result is also not surprising, as these peptides evolved more rapidly than the ATPases (e.g., CbiO, HisP,

TABLE 4. Membrane transport proteins similar to CbiO

Gene[a]	Organism	Substrate	Alignment[b]			
			Length	Identity	Similarity	GenBank
ThiA	*Thiobacillus ferrooxidans*	?	224	29	55	M58480
occP	*Agrobacterium tumefaciens*[c]	Octopine	231	28	55	M80607
potA	*Escherichia coli*	Polyamine	236	28	54	M64519
sfuC	*Serratia marcescens*	Iron	218	28	54	M33815
drrA	*Streptomyces peucetius*	Duanorubicin	222	28	53	M73758
glnQ	*Bacillus stearothermophilus*	Glutamine	215	26	52	M61017
fecE	*Escherichia coli*	Iron dicitrate	252	28	51	M26397
nocP	*Agrobacterium tumefaciens*[c]	Nopaline	235	28	51	M77785
rbsA	*Escherichia coli*	Ribose	212	27	51	M13169
mglA	*Escherichia coli*	Methylgalactoside	216	24	51	M59444
fhuC	*Escherichia coli*	Iron	226	29	50	M12486
nosF	*Pseudomonas stutzeri*	Copper	214	30	49	X53676
malK	*Escherichia coli*	Maltose	206	30	49	J01648
msmK	*Streptococcus mutans*	α-Galactosides	212	26	49	M77351
hisP	*Salmonella typhimurium*	Histidine	236	28	48	J01805
Bfi1	*Bacillus firmus*	?	207	26	48	X59424

[a] A gene encoding a protein similar to the *S. typhimurium* CbiO protein. The ThiA and Bfi1 designations indicate ORF with unassigned functions and identified in GenBank entries M58480 (upstream of the *ntrA* gene) and X59424 (upstream of a homolog of methylmalonyl-coenzyme A mutase), respectively (see the text).

[b] Alignment of the encoded sequence to the deduced amino acid sequence of the *Salmonella cbiO* gene. The length of the aligned sequences, percent amino acid identity, and percent amino acid similarity are provided, as is the GenBank accession number for the DNA sequence indicated.

[c] Plasmid-borne sequences.

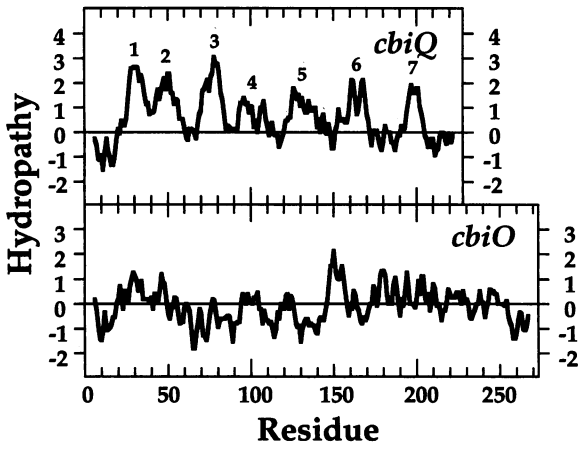


FIG. 4. Hydropathy plots of potential *S. typhimurium* cobalt transport proteins. Hydropathy values were calculated by the method of Kyte and Doolittle (62). Potential transmembrane domains are numbered.

OccP, and NocP). Most of the transport systems of this family include a periplasmic binding protein. It is possible that either the CbiN protein or an unidentified protein, such as CbiM, provides this function.

DISCUSSION

Assigning gene functions. Functions may be assigned to many of the genes in the *Salmonella cob* operon by examination of the *Pseudomonas* homologs listed in Table 2. Most steps in the biosynthesis of cobalamin in *P. denitrificans* have been elucidated (10–16, 19, 21, 31–33, 36, 37, 100–103); these steps and the relevant intermediates are listed in Fig. 5. The reactions of part I of the biosynthetic pathway entail the synthesis of cobinamide (reactions 1 to 13). This process begins with Uro III, an intermediate in the heme biosynthetic pathway (Fig. 1). Subsequent reactions include methylation of eight ring carbon atoms (reactions 1, 2, 3, and 6), amidation of six peripheral carboxyl groups (reactions 9 and 12), ring contraction by the elimination of carbon C-20 (reaction 4), reduction of the precorrin ring (reaction 5), decarboxylation of a peripheral acetyl group (attached to carbon C-12) and associated methyl migration (from carbon C-11 to carbon C-12; reactions 7 and 8), insertion and reduction of a cobalt atom (reaction 10), and addition of an aminopropanol side chain to carbon C-17 (reaction 13). The exact intermediates and order of reactions between precorrin-3 and precorrin-6x, steps 3 and 4, have not been determined; the hypothetical molecule precorrin-6w is shown for clarity. Steps 6 and 7 are performed by a bifunctional enzyme in *P. denitrificans*; *Salmonella* spp. have separate enzymes for these two reactions, each homologous to a different portion of the *Pseudomonas* peptide. Therefore, the hypothetical molecule precorrin-8w is added on the assumption of a two-step process in *Salmonella* spp. The adenosylation of cobamide in *P. denitrificans* occurs as reaction 11. Thereafter, all biosynthetic intermediates are indicated as the adenosylated molecules. The timing of cobalt insertion and adenosylation has not been determined for *Salmonella* spp.; if it occurs earlier in the *Salmonella* biosynthetic pathway, additional precursor molecules may be adenosylated.

The reactions of part II of the pathway entail the synthesis of DMB, utilized in reaction 16. Genetic studies have indicated that part II genes are located immediately promoter-distal to the sequence analyzed here (41, 57). The reactions of part III of the pathway entail the covalent linkage of adenosylcobinamide, DMB, and a phosphoribosyl moiety derived from an NAD precursor (reactions 14 to 16 in Fig. 5). Reactions 14 and 15 are catalyzed by a bifunctional enzyme encoded by the *Pseudomonas cobP* gene; the *Salmonella* homolog of this protein is encoded by the *cobU* gene. The incorporation of DMB to form cobalamin is a multistep process in both organisms.

Salmonella homologs have been identified for the *Pseudomonas* proteins catalyzing the majority of the reactions required for the synthesis of cobinamide (part I reactions in Fig. 5). The eight methylation reactions are catalyzed by six *Pseudomonas* enzymes, encoded by the *cobA*, *cobI*, *cobJ*, *cobM*, *cobL* (5′ portion), and *cobF* genes; five of these genes have *Salmonella* homologs: *cysG*, *cbiL*, *cbiH*, *cbiF*, and *cbiE*, respectively. The two *Pseudomonas* amidase genes, *cobB* and *cobQ*, have *Salmonella* homologs: *cbiA* and *cbiP*, respectively. Proteins catalyzing the decarboxylation and methyl migration reactions, encoded by *cobL* (C terminus) and *cobH*, also have *Salmonella* homologs, encoded by *cbiT* and *cbiC*, respectively. Furthermore, all three part III genes in *Pseudomonas* spp., *cobP*, *cobV*, and *cobU*, have *Salmonella* homologs: *cobU*, *cobS*, and *cobT*, respectively. The *Pseudomonas cobP* and *Salmonella cobU* genes both encode bifunctional enzymes with cobinamide kinase and cobinamide phosphate guanylyltransferase activities.

The *cobT* sequence reported here is incomplete (Table 2); the first 252 codons of the *cobT* gene were carried on plasmid pJE2 and are characterized here. The CobT protein is homologous to the *Pseudomonas* CobU protein, which encodes the DMB:nicatinamide phosphoribosyltransferase and is essential for cobalamin synthesis in *P. denitrificans*. Since plasmid pJE2 complements all CobIII mutations in *Salmonella* spp., several explanations are possible. (i) The CobT protein is not essential for cobalamin synthesis in *Salmonella* spp. While CobT may be used when exogenous DMB is supplied, endogenous ribosyl-DMB is formed by an alternative pathway. (ii) No mutants defective in *cobT* gene expression can be isolated. This possibility is unlikely, especially since CobIII insertions in the *cobU* and *cobS* genes would be polar on the *cobT* gene, requiring its expression from complementing plasmids. (iii) The truncated CobT protein produced by plasmid pJE2, bearing the N-terminal 252 amino acids, is fully functional. Currently, we cannot discriminate among these alternatives.

Evolution of methylases. As detailed above, the conversion of Uro III to cobalamin involves the addition of eight methyl groups; in all cases, *S*-adenosylmethionine is the methyl group donor. Two of these methyl groups are added by the *Pseudomonas* CobA protein (12), which is homologous to the enteric CysG enzyme (41.4% identical and 62.1% similar to the *Escherichia coli* enzyme). Of the remaining five *Pseudomonas* methylase genes, only four have *Salmonella* homologs; there is no *Salmonella* homolog for the *Pseudomonas cobF* gene. However, the sequences of the putative methylases of both organisms are more closely related to one another than to other sequences in the data base (data not shown), implying a common ancestor for all of the methylase genes involved in cobalamin biosynthesis.

To test this hypothesis, we applied UPGMA (unweighted pair group with arithmetic means) algorithms (71) to the methylase amino acid sequences from both organisms. When analyzed separately, the relationships among the

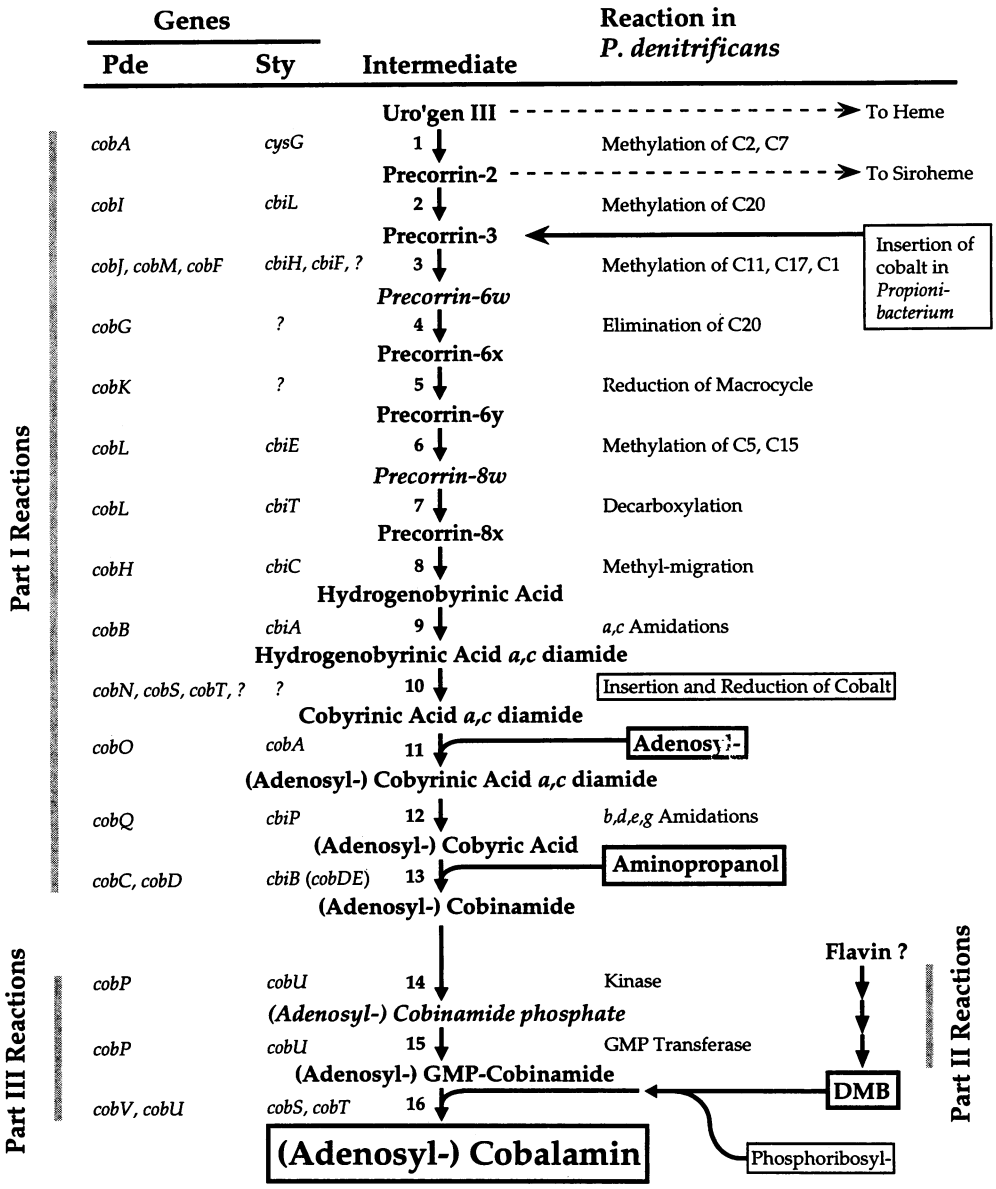


FIG. 5. Cobalamin biosynthetic pathways. Pathways were constructed by use of identified intermediates and known reaction sequences in *P. denitrificans* (10–16, 19, 21, 31–33, 36, 37, 100–103). Differences in the timing of cobalt insertion (69, 70) are indicated. Intermediates precorrin-6w and precorrin-8w have been added for clarity (see the text). Pde, *P. denitrificans*; Sty, *S. typhimurium*. Uro'gen III = Uro III.

Pseudomonas proteins were congruent with the relationships among their *Salmonella* homologs (Fig. 6); when analyzed in tandem, each *Salmonella* gene was grouped with its *Pseudomonas* homolog (Fig. 6). These data support the hypothesis of a common ancestor for all of the methylase genes involved in cobalamin biosynthesis. The various methylase genes were clearly established prior to the divergence of the *Pseudomonas* and *Salmonella* methylase genes. The divergence of the *Salmonella* and *Pseudomonas* homologs is demonstrated by the coincident divergence of the methylase genes as shown in Fig. 6. The 11 methylase genes share two domains of unusually high similarity (Fig. 7). The first domain may correspond to an *S*-adenosylmethionine binding site because of some similarities with the adenosyl binding site of *S*-adenosylmethionine synthetases (53); the second domain may be specific for binding of the corrin ring. Since it is clear that the methylase genes represent an ancient gene family, the lack of a *Salmonella* homolog of the *cobF* methylase is perplexing. Explanations include the following. (i) A homolog of the *Pseudomonas cobF* gene is located in the *Salmonella* chromosome but not in the sequenced region. This possibility is not likely since, aside from *cobA*, all part I mutations prior to reaction 13 have been localized to this region. (ii) The *Pseudomonas cobF* gene was recruited following the divergence of the two operons. This possibility seems unlikely, since the high similarity of the CobF protein to the other methylases would require an unacceptable amount of convergent evolution (Fig. 7). (iii) An ancestral bifunctional methylase, retained in the *Salmonella* operon, was duplicated and diverged to separate substrate specifici-

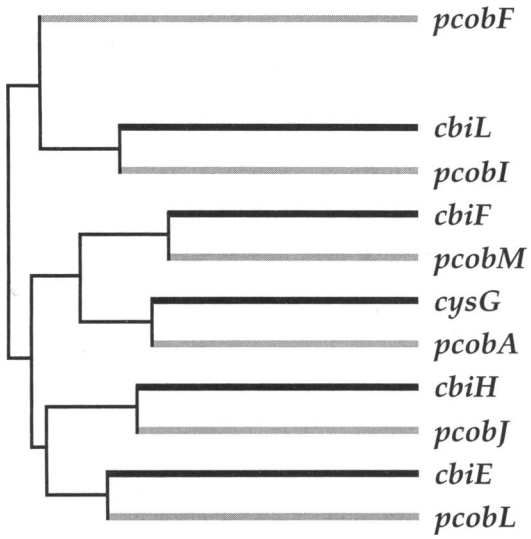


FIG. 6. Relationships among methylases involved in cobalamin synthesis. The dendrogram was constructed by use of UPGMA algorithms (71). Gene designations prefixed with "p" are *P. denitrificans* genes; other genes are from *S. typhimurium*, except for *cysG*, which is from *E. coli*. Thin lines indicate the evolution of the ancestral methylase; thick black lines indicate the divergence of the *S. typhimurium* proteins; thick grey lines indicate the divergence of the *P. denitrificans* proteins.

TABLE 5. Cobalamin synthetic genes without homologs

Species	Gene	Proposed function
P. denitrificans	*cobF*	Methylation
	cobG	Elimination of C-20
	cobK	Reduction of the corrin ring
	cobN	Insertion of cobalt
	cobS	Insertion of cobalt
	cobT	Insertion of cobalt
	cobC	Addition of aminopropanol
	cobE	Unknown—essential for cobalamin synthesis
	cobW	Unknown—essential for cobalamin synthesis
S. typhimurium	*cbiD*	Unknown
	cbiG	Unknown
	cbiJ	Unknown
	cbiK	Unknown
	cbiM	Unknown
	cbiN	Cobalt transport
	cbiQ	Cobalt transport
	cbiO	Cobalt transport

ties in the *Pseudomonas* operon. This possibility is unlikely since, in the absence of accelerated evolution, the divergence of a duplicated bifunctional protein in *P. denitrificans* would yield, at best, an unresolved trifurcation on the dendrogram presented in Fig. 6. (iv) The *cobF* homolog was lost from the *Salmonella* operon, and its function was replaced either by a recruited methylase, not identified because of a lack of sequence similarity, or by the expanded substrate specificity of an existing methylase. This possibility is plausible in that the methylation of the C-1 carbon atom may have required a substantially different protein if the substrate molecule had bound cobalt (as discussed below). If the methylase was recruited, it would have been encoded by one of the genes lacking a known function: *cbiD*, *cbiG*, *cbiJ*, *cbiK*, or *cbiM*. Alternatively, since carbon atoms C-1 and C-11 are situated symmetrically in Uro III (Fig. 1), they may have been methylated by a bifunctional enzyme in *Salmonella* spp., either the CbiH or the CbiF protein. For the reasons detailed above, we propose that the recruitment of an unrelated methylase or the evolution of a bifunctional methylase in *Salmonella* spp. represents the most likely explanation for the lack of a *Salmonella* homolog of the *Pseudomonas cobF* gene.

Genes without homologs. Cobalamin synthetic genes from either organism that lack homologs in the other organism are of special interest; these genes are listed in Table 5. Al-

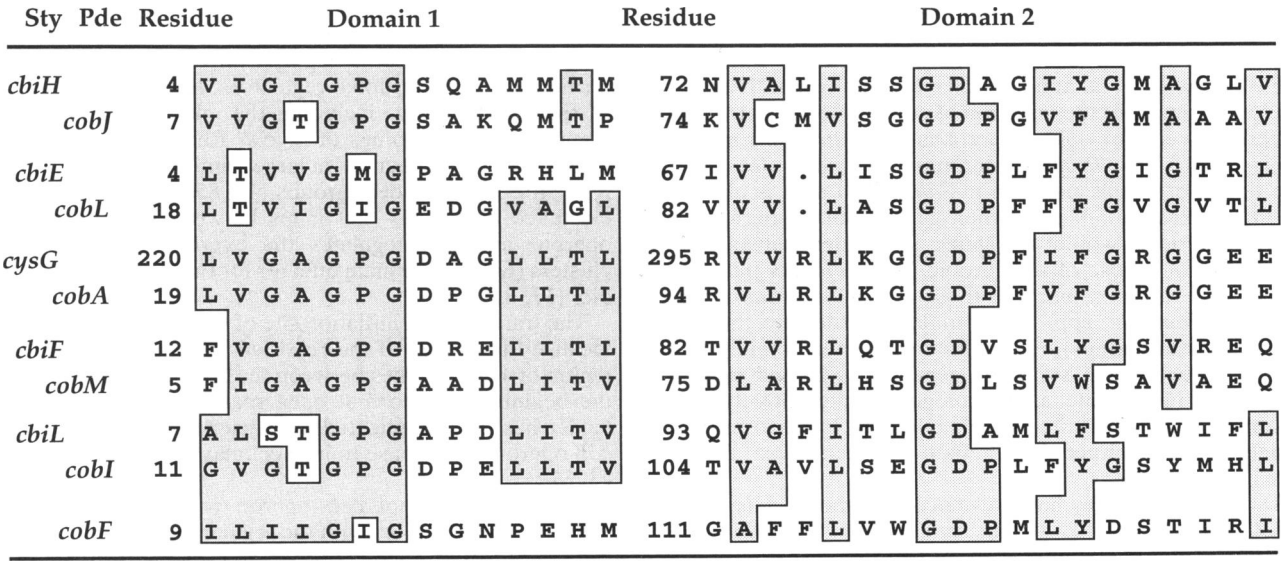


FIG. 7. Conserved domains among corrin methylases. Gene designations are as in Fig. 6. Positions with a majority of identical or highly similar amino acids are shaded. Similar amino acids are shadedi dots represent deleted residues. Sty, *S. typhimurium*; Pde, *P. denitrificans*.

though the *Pseudomonas* genes *cobE* and *cobW* are essential for cobalamin synthesis, neither deduced protein has an identified function. Therefore, it is difficult to interpret the lack of homologs for these genes in the *Salmonella* gene cluster. As discussed above, three of the *Salmonella* genes, *cbiN*, *cbiQ*, and *cbiO*, are likely to encode an active cobalt transport system. Growth conditions used during the isolation of *Pseudomonas cob* mutants may have precluded the cloning and identification of potential homologs of these genes. The remaining gene products without apparent homologs are likely to catalyze similar subsets of reactions in each of the two organisms. Seven *Pseudomonas cob* genes with characterized functions lack homologs among the five uncharacterized *Salmonella cob* genes. One *Pseudomonas* gene, *cobF*, encodes a methylase (discussed above); another *Pseudomonas* gene product, CobC, is involved in the synthesis and addition of aminopropanol, required for the conversion of cobyric acid to cobinamide (discussed below).

The remaining five *Pseudomonas* genes without identified *Salmonella* homologs correspond to three specific reactions: (i) the reduction of the corrin macrocycle (*cobK*; reaction 5 in Fig. 5), (ii) the insertion of cobalt (*cobNST*; reaction 10), and (iii) the elimination of the C-20 carbon atom (inferred to be catalyzed by the *cobG* gene product; reaction 4). If the synthesis of cobalamin is an evolutionarily ancient process (9, 44, 46), it is likely that the ancestral pathway evolved under anaerobic conditions. Accordingly, the suite of proteins encoded by the *Salmonella cob* operon may resemble the ancestral state and therefore are able to function only under anaerobic conditions. The aerobically functional pathway in *P. denitrificans* could have recruited new proteins able to perform oxygen-sensitive cobalamin synthetic reactions under high oxygen tensions. Alternatively, the selection for aerotolerance in the *Pseudomonas* lineage may have led to a more rapid rate of evolution among oxygen-sensitive enzymes; the wider divergence of these proteins may have prevented the detection of sequence similarity.

The reactions listed above entail reduction (reaction 5 [CobK] in Fig. 5), insertion of the cobalt ion in the macrocycle, and removal of a carbon atom from the corrin ring (reaction 4 [CobG]). The reduction of cobalt in *P. denitrificans* involves the product of an as-yet-uncharacterized gene (36a). It is plausible that these reactions might be accomplished by different means under aerobic and anaerobic conditions; the proteins with likely aerotolerant functions, e.g., methylases and amidases, have homologs in these two taxa. The timing of the insertion of cobalt into the corrin ring differs between the obligate aerobes *P. denitrificans* and the obligate anaerobe *Propionibacterium shermanii* (69, 70). If the timing of cobalt insertion in *Salmonella* spp. is like that in *Propionibacterium* spp., then gene products performing these functions would recognize substrates substantially different from those of the aerobic *Pseudomonas* pathway. Therefore, it is possible that these enzymes in *Salmonella* and *Pseudomonas* spp. are homologous (despite their sequence dissimilarities) but have evolved more rapidly than the other cobalamin synthetic gene products and may no longer be identifiable as homologous. Alternatively, one taxon may have recruited unrelated genes to perform these functions. We cannot discriminate between these alternatives.

Data base searches have revealed that the *Pseudomonas* CobG protein exhibits strong similarity to the *Salmonella* CysI protein, encoding sulfite reductase, and to spinach nitrite reductase. Both of these proteins bind siroheme, a derivative of precorrin-2 that is structurally similar to the substrate of the CobG protein (Fig. 5). It is unclear whether the similarity between the CobG protein and the two reductases reflects similar precorrin binding domains or shared reductase activities.

Synthesis and addition of aminopropanol. Reaction 13 in Fig. 5 involves the addition of the aminopropanol side chain to cobyric acid to form cobinamide (see also Fig. 1). The aminopropanol moiety links DMB to the corrinoid ring; the ultimate origin of aminopropanol during cobalamin synthesis remains unclear for both *Salmonella* and *P. denitrificans* Although radioactive tracer experiments have suggested that aminopropanol is derived from threonine (72), the enzymatic decarboxylation of threonine to aminopropanol has never been demonstrated. Genetic studies with both *S. typhimurium* (47) and *P. denitrificans* (30) have suggested that the process of aminopropanol addition may be more complicated, since at least three genes are involved in each organism (30, 47, 48).

In *S. typhimurium*, *cobD* mutations located at minute 14 (Fig. 3) synthesize cobalamin only when aminopropanol is provided (47); a similar mutant class has been found for *P. denitrificans* (30). A second enzyme involved in aminopropanol addition is encoded by the *Pseudomonas cobD* gene and its *Salmonella* homolog, *cbiB*. A third *Pseudomonas* enzyme, encoded by *cobC*, shares strong similarity with transaminases (including the *Salmonella* HisC enzyme) and exhibits a plausible pyridoxal phosphate binding site (data not shown). This *Pseudomonas* enzyme may be involved in the modification of threonine. An additional mutant class involved in aminopropanol addition in *S. typhimurium*, *cobE* mutations, is also located at minute 14. The nucleotide sequence of this region may reveal a *Salmonella* homolog of the pyridoxal phosphate binding *Pseudomonas* CobC enzyme.

Operon regulatory region. Regulatory elements for the *cob* operon lie between the 5′ end of the reported sequence and the beginning of the *cbiA* gene at bp 1288. This region includes the C-terminal portion of the *pocR* gene (bp 1 to 690), the main promoter of the *cob* operon (transcription starts at bp 824), and a 464-base untranslated mRNA leader sequence that is involved in transcriptional repression of the *cob* operon by adenosylcobalamin (AdoCbl).

The *pocR* gene was first inferred from examination of this sequence. The deduced polypeptide encoded in the first 690 bp of this sequence is highly similar to the C-terminal portions of transcriptional regulators, specifically, members of the AraC family, including the enteric bacterial AraC homologs and homologs of *E. coli* RhaS. We inferred that PocR was a member of a large family of transcriptional regulators. Independent groups (17, 85) have confirmed the existence of a locus that maps between the *cob* and *pdu* operons and that regulates the expression of both gene clusters (Fig. 2); the main effector for this control is propanediol (17, 85).

The transcription initiation site of the *cob* operon has been localized to bp 824 of the reported sequence (81). A leader region of 464 bases lies between the 5′ end of the mRNA and the beginning of the first open reading frame (*cbiA*). This leader has been implicated in transcriptional control of the *cob* operon in response to intracellular levels of AdoCbl (42, 43, 82).

The mechanism of *cob* operon repression by AboCbl shares several features with the regulation of the *E. coli btuB* gene. The leader regions of the *cob* (131 bp upstream of the translation start site) and *btuB* (99 bp upstream) operons share a conserved 17-nucleotide sequence (AAGCC$^{A}_{C}$GAA

GACCTGCC). A search of the entire nucleotide sequence data base revealed a single match to this sequence (15 of 17 nucleotides) 169 bp upstream of the *P. denitrificans cobP* gene. These sequences are not likely to be standard operator binding sites. Their size, nonpalidromic character, and location well within the mRNA leader regions are properties atypical of operators (28). As noted, all mutations that abolish repression of the *btuB* or *cob* operon affect the mRNA leader regions. This fact is consistent with the lack of a single repressor protein responsible for AdoCbl repression. The conserved nature of this 17-nucleotide sequence suggests that it may be involved in repression of these operons by AdoCbl. Observations of repression by AdoCbl raise the intriguing possibility of a direct interaction between AdoCbl and the leader mRNA. The three-plane structure of AdoCbl may allow intercalation; both the adenosyl group and peripheral amides could form hydrogen bonds. The lack of repression by cyanocobalamin (2, 39) is consistent with this model.

Translational coupling. On the basis of genetic studies of the *cob* operon, we have inferred a single, large operon whose expression is regulated by a single promoter under the control of the *pocR* regulatory gene (4, 5, 17, 41, 56–58, 85); this promoter has been localized to bp 824 (78) of the sequence reported here. DNA sequence analysis has revealed downstream of this promoter 20 open reading frames that are inferred to encode proteins involved in cobalamin biosynthesis (Table 2). The junctions between the genes of this operon are small, a fact suggestive of translational coupling, that is, restarting of the preloaded ribosome on an adjacent start site following translation termination (Table 3). This phenomenon has been observed frequently among bacterial operons (51, 52, 64, 76, 99). Translational coupling is most efficient among genes with overlapping stop and start signals (34, 106), and 10 of 19 *cob* gene junctions exhibit this property (Table 3). The remaining gene junctions vary between 1 and 17 nucleotides. Ribosome reinitiation varies in efficiency with increasing distance between adjacent stop and start signals (106) as well as the strength of the binding signal, the Shine-Dalgarno sites (49), and the use of nontraditional start sites (78). Lower frequencies of ribosome reinitiation may result in mRNA termination or degradation at those sites because of a lack of ribosome coverage (49, 61, 68, 107). We propose that various degrees of translational coupling, in concert with weak, internal, constitutive promoters (39), establish the relative level of expression of proximal and distal *cob* genes.

Evolution of operon structure. Cobalamin synthetic genes have been characterized in three organisms: *B. megaterium*, *P. denitrificans*, and *S. typhimurium*. In all three organisms, gene clustering is evident, but gene orders are quite different. The *Bacillus* cobalamin synthetic genes have been localized to two large linkage groups (18, 110). The *Pseudomonas* cobalamin synthetic genes have been found on four DNA fragments (19, 21, 31–33); the locations of these fragments in the *Pseudomonas* chromosome are unknown. The clustering of cobalamin synthetic genes is most striking in *S. typhimurium*, in which more than 20 genes have been found in a single operon, and genes within this operon have been grouped according to function. Comparison of the *Salmonella* operon with the *Pseudomonas* chromosomal fragments (Fig. 3) reveals little evidence for the conservation of gene arrangements between these organisms. Either these gene arrangements have been independently generated from unlinked genes since the divergence of the gene sets, or gene order in established clusters is subject to continual rearrangement.

In many bacterial operons encoding multiple biosynthetic enzymes, the first gene in the operon encodes the initial enzyme in the biosynthetic pathway and is subject to feedback inhibition by the end product; this is true of the *his*, *trp*, *leu*, and *thr* operons of *E. coli* and *S. typhimurium* (27, 80, 105, 109). The *cob* operon of *S. typhimurium*, and the two gene clusters of *P. denitrificans* do not follow this pattern. The first reaction in *P. denitrificans* is catalyzed by the product of the *cobA* gene (Fig. 5), the fifth gene in its cluster (Fig. 3). In *S. typhimurium* the promoter-proximal *cbiA* gene encodes an amidase that acts rather late in the biosynthetic pathway (Fig. 5). The first enzyme in the pathway dedicated exclusively to coenzyme B_{12} synthesis is precorrin-2 methylase; this enzyme is encoded by the 12th gene in the *Salmonella* operon, *cbiL*. Only if the *Salmonella* pathway has a substantially different sequence of reactions could the CbiA-catalyzed amidation be an early reaction. Gene clusters may evolve by gene rearrangement, insertion, deletion, and operon fusion (1, 55, 73). These processes appear to have occurred during the evolution of the *Pseudomonas* and *Salmonella* gene clusters; these operons provide an example of the remarkable plasticity of operon organization evident among eubacteria.

Summary. Twenty cobalamin synthetic genes of *S. typhimurium* have been isolated and characterized. These genes constitute the majority of a large operon of translationally coupled genes located at minute 41 on the *Salmonella* chromosome. The functions of 15 of 20 gene products have been deduced and involve most of the enzymes involved in cobinamide synthesis, all the enzymes used in the synthesis of cobalamin from cobinamide and DMB, and three proteins that may constitute an ATP-dependent cobalt transport system. Although most of the *S. typhimurium* and *P. denitrificans cob* genes have homologs in the other organism, several genes are unique to each taxon. We suggest that these differences reflect variations in the coenzyme B_{12} biosynthetic pathways between the two organisms, possibly because of differences in the natural growth conditions under which the two organisms synthesize cobalamin.

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