COBALAMIN (COENZYME B\textsubscript{12}): Synthesis and Biological Significance

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KEY WORDS: B\textsubscript{12}, propanediol, metabolism, \textit{Salmonella} spp., corrinoids

ABSTRACT
This review examines deoxyadenosylcobalamin (Ado-B\textsubscript{12}) biosynthesis, transport, use, and uneven distribution among living forms. We describe how genetic analysis of enteric bacteria has contributed to these issues. Two pathways for corrin ring formation have been found—an aerobic pathway (in \textit{P. denitrificans}) and an anaerobic pathway (in \textit{P. shermanii} and \textit{S. typhimurium})—that differ in the point of cobalt insertion. Analysis of B\textsubscript{12} transport in \textit{E. coli} reveals two systems: one (with two proteins) for the outer membrane, and one (with three proteins) for the inner membrane. To account for the uneven distribution of B\textsubscript{12} in living forms, we suggest that the B\textsubscript{12} synthetic pathway may have evolved to allow anaerobic fermentation of small molecules in the absence of an external electron acceptor. Later, evolution of the pathway produced siroheme, (allowing use of inorganic electron acceptors), chlorophyll (O\textsubscript{2} production), and heme (aerobic respiration). As oxygen became a larger part of the atmosphere, many organisms lost fermentative functions and retained dependence on newer, B\textsubscript{12} functions that did not involve fermentation. Paradoxically, \textit{Salmonella} spp. synthesize B\textsubscript{12} only anaerobically but can use B\textsubscript{12} (for degradation of ethanolamine and propanediol) only with oxygen. Genetic analysis of the operons for these degradative functions indicate that anaerobic degradation is important. Recent results suggest that B\textsubscript{12} can be synthesized and used during anaerobic respiration using tetrathionate (but not nitrate or fumarate) as an electron acceptor. The branch of enteric taxa from which \textit{Salmonella} spp. and \textit{E. coli} evolved appears to have lost the ability to synthesize B\textsubscript{12} and the ability to use it in propanediol and glycerol degradation. \textit{Salmonella} spp., but not \textit{E. coli}, have acquired by horizontal transfer the ability to synthesize B\textsubscript{12} and degrade propanediol. The acquired ability to degrade propanediol provides the selective force that maintains B\textsubscript{12} synthesis in this group.
INTRODUCTION

Several questions surround the cofactor cobalamin (coenzyme B\textsubscript{12}). Its large size and chemical complexity (84, 131) have made the study of its biosynthesis a challenge (9, 10, 146, 147). Cobalamin’s uneven distribution among modern life-forms and its proposed prebiotic origins raise questions regarding its general biological significance. Although cobalamin is made by some bacteria and is essential to humans (it was discovered by its ability to cure pernicious anemia), it seems to play no role in the metabolism of plants, fungi, or some bacteria (46, 97, 107, 123). Why do some organisms continue to use this cofactor, whereas others flourish without it?
The discovery of B12 synthesis and use in bacteria with well-developed genetic systems has made it possible to apply genetic methods to these broad questions. The synthesis, transport, physiological importance, and evolution of this complex cofactor can be studied in the single organism *Salmonella typhimurium*. We review here the general area of B12 metabolism research, with an emphasis on biological uses revealed by genetic studies of enteric bacteria.

**Structure of Cobalamin**

The magnitude of the synthetic problem can be seen in Figure 1, which diagrams the structure of one form of B12: 5′ deoxyadenosylcobalamin (Ado-B12).

Ado-B12 has a molecular weight of 1580, and at least 25 enzymes are uniquely involved in its synthesis. The Ado-B12 molecule has three parts: a central ring, an adenosyl moiety, and a nucleotide loop. The central ring is structurally and biosynthetically related to those of heme and chlorophyll. The ring found in B12 differs from related rings by its lack of the carbon bridge between the A and D porphyrins (see Figure 1), by the ring oxidation state, by the distribution of ring decorations (methyl, acetamide, and propionamide), and by the central cobalt atom. Ado-B12 also has a 5′ deoxyadenosyl moiety serving as its upper (Coα) axial ligand; the 5′ carbon of this ribose group is joined by a covalent bond to the cobalt within the corrin ring. Homolytic cleavage of the cobalt-carbon bond is central to catalysis of intramolecular rearrangement reactions (154). Cobalt’s lower (Coβ) axial ligand is the N-7 of dimethylbenzimidazole (Dmb). The Dmb moiety is attached covalently to the corrin ring as part of a nucleotide loop. The nucleotide, 3′ phosphoribosyl-Dmb, is linked through its phosphate to an aminopropanol moiety that is attached to a propionyl group extending from the D porphyrin of the corrin ring.

Distinct forms of cobalamin exist with upper and/or lower ligands that are different from those described above. The cofactor for methyltransferases is methylcobalamin as cofactor, in which the deoxyadenosyl moiety is replaced by a methyl group (145). Cobalamin is prepared commercially with a cyano group as the Coβ ligand; this form (CN-B12) is not found in nature but frequently is used as a nutrient for humans and for bacterial mutants. Alternative forms of B12 with different bases in place of Dmb have been identified in various Bacteria and Archaea (35, 63, 72, 156, 157). These corrinoids appear to be isofunctional; no correlation has been made between the nucleotide used as the lower ligand and the function or distribution of a corrinoid (156).

**Ancient Origins of Cobalamin**

Many authors have suggested that B12 was synthesized prebiotically (13) and may have been important to catalysis in the “RNA world.” In some bacteria, B12 biosynthesis begins with an aminoacyl-tRNA molecule, and an RNA molecule
Figure 1  Structure of 5′ deoxyadenosylcobalamin, coenzyme B₉. Ring carbons are numbered, and sites of amidations are lettered in italic lower case. Porphyrin rings are designated with capital letters. Name, abbreviation, and source of each moiety are noted.
may play an intimate role in regulating expression of biosynthetic and transport genes (see below). Porphyrins such as those contributing to the B\textsubscript{12} structure have been synthesized nonenzymatically in so-called primitive earth experiments (85), and Eschenmoser has commented eloquently on how the complex B\textsubscript{12} structure can be formed by energetically favorable reactions (62).

The cobalamin biosynthetic pathway, and the pathways common to heme, chlorophyll, and siroheme synthesis, may reflect the evolution of energy metabolism (see Figure 2). It seems likely that this pathway first evolved to produce B\textsubscript{12}, a view supported by considering the structure of UroIII (62), the common precursor of heme, siroheme, chlorophyll, and cobalamin. UroIII is asymmetric in the sense that one of the porphyrin rings is reversed with respect to the others. The reversal may be important for the carbon elimination (ring contraction) that occurs later in cobalamin synthesis (see below). This arrangement indicates that the entire pathway developed initially to serve B\textsubscript{12} synthesis and later added the branches to siroheme, heme, and chlorophyll.

We suggest below that the initial significance of B\textsubscript{12} was to support anaerobic fermentation of small molecules by generating internal electron sinks. Later, siroheme allowed inorganic molecules to be used as electron acceptors, which is seen in siroheme’s modern role as a cofactor for sulfite and nitrite reductases. Still later arrivals may have been chlorophyll and heme, which allowed biological formation of molecular oxygen and use of oxygen as a respiratory electron acceptor.

**Distribution of B\textsubscript{12} Among Living Forms**

According to a widely accepted historical view, B\textsubscript{12} synthesis is restricted to some Bacteria and Archaea. Many animals (including humans) and protists require B\textsubscript{12} but apparently do not synthesize it. Plants and fungi are thought to neither synthesize nor use B\textsubscript{12} in their metabolism (58). Figure 3 superimposes the distribution of B\textsubscript{12} on a prominent view of the evolutionary relationships between these life-forms.

Figure 3 represents, at best, general conclusions. Some possible exceptions have been documented among algae and legume taxa (119, 170, 171). Some reports of B\textsubscript{12} in plants and fungi may not have considered adequately the difficulty of excluding bacterial contamination. As better assays are made in a variety of organisms and more rigorous nutritional tests are made, more exceptions may surface.

The distribution pattern shown in Figure 3, even with some exceptions, raises questions regarding the biochemical significance of B\textsubscript{12} and the metabolic differences among life-forms that allow some to escape the need for B\textsubscript{12}. Such questions should be kept in mind as we review the general nature of B\textsubscript{12}-dependent reactions. Later we use studies of bacterial mutant phenotypes and
Figure 2  Overview of B12 synthetic pathway. Parts I, II, and III are portions of the cobalamin biosynthetic pathway defined by nutritional behavior of mutants (see below). More detailed views of this pathway may be seen in Figure 6 (Part I) and Figure 7 (Parts II and III). The boxed reaction highlights activities provided by the single CysG protein of *S. typhimurium*/*E. coli* (65, 152); the shaded portion indicates activities of the homologous (CobA) protein of *P. denitrificans* (19, 48). The two pathways leading to Ado-Cbi are the aerobic pathway of *P. denitrificans* (top arrow) and the anaerobic pathway of *S. typhimurium* (bottom arrow). General functions of cofactors are indicated above and below the pathway.
regulatory behavior of B12 synthetic enzymes to illustrate additional puzzles regarding the biological significance of B12 within enteric bacteria.

**B12-DEPENDENT REACTIONS**

In trying to understand the evolutionary history of B12, we have looked for fundamental properties of B12-catalyzed reactions that might explain the evolution and later loss of B12 synthesis from various groups. Fermentation may be that common feature. Many of the known B12-dependent reactions in a variety of bacteria support anaerobic fermentation of small molecules. Fermentation, as used in this review, refers to anaerobic growth without an exogenous electron acceptor. In this situation, redox reactions must be internally balanced, and ATP must be produced by substrate-linked phosphorylations. Some reduced compound is excreted to get rid of excess reducing equivalents. In enteric bacteria most fermentable carbon sources are large sugars.

We propose that the original significance of B12, and its remaining primary role in many modern bacteria, may be to support fermentation of small molecules by catalyzing molecular rearrangements that generate both an oxidizable compound and an electron sink for use in balancing redox reactions. In enteric bacteria, this role is seen in the B12-dependent degradation of ethanolamine, propanediol, and glycerol (see Figure 4). In these reactions, the B12-mediated rearrangement generates an aldehyde that can be oxidized and provide ATP; the oxidation reactions can be balanced by reducing a portion of the

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**Figure 3** Distribution of cobalamin synthesis and use among living forms. Wedges designate in a general way the evolutionary and current importance of oxygen to organisms in each group.
aldehyde to an alcohol, which is excreted. The B_{12}-dependent reaction forms the reducible compound by a rearrangement, essentially an internal redox reaction.

In nonenteric bacteria, the B_{12}-dependent amino mutases (specific for glutamic acid, lysine, leucine, or ornithine) catalyze mechanistically similar reactions that support fermentation of these amino acids. In methanogens, B_{12} plays a role as a carrier of methane and in essence helps provide a means for getting rid of reducing equivalents.

Thus the earliest use of B_{12} may have been to support anaerobic, fermentative growth at the expense of small molecules. Additional reactions (such as methionine synthesis and nucleotide reduction) appeared as secondary uses. After oxygen and aerobic respiration appeared on earth, many organisms no longer needed to perform fermentations and lost some of their original enzymatic capabilities. The secondary uses such as methyl transfer remained important and enforced a continued requirement for B_{12}. Obligate aerobes and animals appear to require B_{12} to perform these nonfermentative functions. In humans, two B_{12}-dependent reactions are known. Methionine synthetase, a methyl transferase, is presumed to be important primarily in recycling folate and secondarily in producing methionine. Methyl malonyl CoA mutase may serve mainly to remove toxic products of lipid breakdown.

B_{12}-Dependent Reactions in Enteric Bacteria

The enzymes listed below are found in one or more species of enteric bacteria. The catalyzed reactions are diagrammed in Figure 4.

PROPANEDIOL DEHYDRATASE This enzyme, which converts 1,2-propanediol to propionaldehyde, is found in virtually all enteric bacteria tested except Escherichia coli. Some bacteria ferment propanediol by oxidizing a portion of the propionaldehyde to provide carbon and energy while reducing the rest to provide an electron sink for balancing redox reactions. In Salmonella species this process provides energy but no carbon source. Propanediol is encountered frequently by bacteria, because it is produced during anaerobic catabolism of the common methylpentoses, rhamnose and fucose.

ETHANOLAMINE AMMONIA LYASE This enzyme converts ethanolamine to acetaldehyde and ammonia. Under some conditions, the produced acetaldehyde can serve as a carbon and energy source via acetyl-CoA. Ethanolamine is frequently encountered in nature as part of common lipids, phosphatidyl ethanolamine and phosphatidyl choline.

GLYCEROL DEHYDRATASE This enzyme converts glycerol to β-hydroxypropiionaldehyde, which can be reduced to 1, 3 propanediol. This reaction balances the reducing equivalents generated by glycerol dehydrogenase.
Figure 4  Cobalamin-dependent pathways known for enteric bacteria. Only the first reaction for each pathway employs cobalamin as a cofactor. For each B₁₂-dependent enzyme, the S. typhimurium genetic designation is given. THF indicates tetrahydrofolate.
Glycerol dehydratase is common in enterics but is absent from both *Salmonella* spp. and *E. coli* (100).

**METHIONINE SYNTHETASE** This enzyme transfers a methyl group from methyltetrahydrofolate to homocysteine as the final step in synthesis of methionine and is probably the best-known B<sub>12</sub>-dependent reaction (57, 162). The lack of this reaction underlies many aspects of human B<sub>12</sub>-deficiency disorders (3).

In *Salmonella* spp. and in *E. coli*, the B<sub>12</sub>-dependent methyl transfer reaction is catalyzed by the MetH enzyme, and both organisms produce a second enzyme (MetE) that can catalyze the same reaction without B<sub>12</sub> (40, 44, 57, 151). The MetH enzyme is used preferentially when B<sub>12</sub> is available; the alternative MetE enzyme is induced in response to accumulated homocysteine when the MetH enzyme is inactive (175). In bacteria with an alternative, B<sub>12</sub>-independent enzyme, methionine synthesis is unlikely to be the major selective force maintaining B<sub>12</sub> synthetic capacity.

**EPOXYQUEOSINE REDUCTASE** This enzyme performs the last step in formation of the hypermodified tRNA base, queuosine, found in tRNA<sup>tyr</sup>, tRNA<sup>his</sup>, tRNA<sup>asn</sup>, and tRNA<sup>asp</sup> (73). The modified base is not essential for bacterial growth under laboratory conditions (113). Although the final reaction has been reported to require B<sub>12</sub> (73), it proceeds in anaerobically grown *E. coli* cells, which do not make B<sub>12</sub> (100). We suggest that the reaction may be stimulated by B<sub>12</sub>, perhaps indirectly, in vivo, but can be catalyzed without this cofactor.

**Other Prominent B<sub>12</sub>-Dependent Reactions**

**ACETYL-COA SYNTHESIS** In many anaerobic bacteria, methyl-corrinoids are involved in acetyl-CoA synthesis via the Wood/Ljungdahl pathway (125, 155, 174). In this pathway, a methyl group is transferred from methyltetrahydrofolate via a methyl-corrinoid/iron sulfur protein to CO-dehydrogenase, which synthesizes acetyl-CoA from this methyl group, CO, and coenzyme A (69). A corrinoid plays an analogous role in the energy-yielding metabolism of acetogenic bacteria (103, 125), which synthesize aceticate from 2 CO<sub>2</sub> as a means of generating a terminal electron sink.

**METHYL TRANSFER IN THE METHANE-PRODUCING ARCHAEA** Methyl-corrinoids are essential for formation of methane by the strictly anaerobic methane-producing Archaea (68, 155). Corrinoid proteins play a role in the transfer of methyl groups from methanogenic substrates to the thiol group of the methanogen-specific cofactor, coenzyme M. Different enzymes mediate methyl transfer from alternative methanogenic substrates such as acetate (67), methylamines (36), methanol (95), pyruvate (25) and methyltetrahydromethanopterin, an intermediate of methanogenesis from formate and CO<sub>2</sub> (121, 163). The latter
reaction is analogous to methionine synthesis in that the methyl group is transferred from an intermediate pterin to a thiol group via methyl-B₁₂ (121).

Considerable energy is released ($\Delta G'$ of approximately $-30\text{kJ/mol}$) by transfer of a methyl group to coenzyme M from methyltetrahydromethanopterin (a natural structural and functional analog of methyltetrahydrofolate) (81, 111, 155, 163). The energy of the transfer to coenzyme M can be recovered by coupling the methyl transfer to extrusion of a sodium ion, which eventually leads to generation of a proton motive force (11, 23). The transferase that achieves this feat is an integral membrane protein (70) composed of eight different subunits (81).

**RIBONUCLEOTIDE REDUCTASES** Ribonucleotide reductases generate the deoxyribonucleotides needed for DNA synthesis. Four classes of this reductase are known, each with a different cofactor requirement and quaternary structure; this variability is unusual for enzymes that play such key metabolic roles. The Ado-B₁₂-dependent reductases belong to Class II and are found primarily in microorganisms (16, 17). The reaction mechanism and active site structure of the corrinoid-dependent reductase appears remarkably similar to that of the Ado-B₁₂-independent Class I enzyme of *E. coli* (26). In this reductase, Ado-B₁₂ serves as a free-radical generator. Other reductases form radicals by alternative means (126).

**DEGRADATION OF $\beta$-HYDROXY ETHERS AND $\beta$-HYDROXY AMINES** Corrinoids are implicated in the anaerobic degradation of several compounds thought to be generally recalcitrant to degradation in the absence of oxygen. The compounds include polyethylene glycol (77), triethanolamine (78), and possibly phenoxyethanol (76). The significant chemical feature of these compounds is a hydroxyl group $\beta$ to an ether, a tertiary amine, or a secondary amine. These reactions are related to those catalyzed by diol dehydratases in that they are intramolecular redox reactions that involve the migration of a hydroxyl group.

**METHYLMALONYL-COA MUTASE** Methylmalonyl-CoA mutase interconverts (R)-methylmalonyl-CoA and succinyl-CoA. Higher animals require the mutase for degradation of odd-chain-length fatty acids and certain branched-chain amino acids. In humans, mutase deficiency results in an often fatal methylmalonic acidemia (101), and in certain neuropsychiatric symptoms. These symptoms may result from synthesis of abnormal myelin lipids in the presence of accumulated propionyl- and methylmalonyl-CoA (3, 124).

In certain bacterial fermentations, succinate is converted to propionate via the mutase rather than being excreted (128). This pathway allows conservation of a biotin-activated CO₂ that is derived from succinate. In *Streptomyces*
In constructing Ado-B₁₂, multiple components are synthesized individually and then assembled (see Figure 2). This pattern is curiously more common for vitamins than for other metabolites. The largest component of B₁₂ is the corrinoid ring, synthesized from uroporphyrinogen III (UroIII), a precursor common to heme, siroheme, F₄₃₀, and cobalamin. Two distinct pathways have been identified (in different bacteria) for conversion of UroIII to the intermediate adenosylcobinamide (Ado-Cbi), which has the fully modified corrin ring with an attached aminopropanol side-chain (see Figure 1). The major difference between the two pathways is in the time of insertion of cobalt (see below).

The nucleotide loop is assembled by first activating the aminopropanol side-chain of Cbi to form GDP-Cbi. The Coα axial ligand, dimethylbenzimidazole (Dmb), is synthesized separately and converted to a nucleotide (DmbMN) by addition of ribose derived from nicotinic acid mononucleotide (NaMN), an intermediate in the synthesis of NAD. Ultimately, Dmb nucleoside is added to the end of the activated isopropanol side-chain to form the nucleotide loop and complete the synthesis of Ado-B₁₂.

Great progress has been made recently in defining the nature of the cobalamin synthetic pathway. This progress is largely the result of genetic identification of biosynthetic genes. Genes were cloned by complementation of these mutants, and cloned sequences were then used to produce enzymes for biochemical analysis. This approach was pursued in *Pseudomonas denitrificans* and in *Salmonella typhimurium* (10, 147). Synthetic genes also have been cloned from *Bacillus megaterium* (34, 173).

### Genetic Analysis of B₁₂ Synthesis in *S. typhimurium*

*S. typhimurium* has genes for biosynthesis and for transport of cobalamin and uses a B₁₂ cofactor in at least three reactions. Figure 5 shows the positions of relevant genes in this organism’s genetic map. *S. typhimurium* mutants defective in B₁₂ synthesis (*cob, cbi*) were isolated in a parental *metE* mutant, since simple cobalamin-deficient mutants have no easily detectable growth phenotype (see below). Strains of *S. typhimurium* with a *metE* mutation require methionine unless they can synthesize (or are given) cobalamin, which is required by the alternative methionine synthetase, MetH. Since *S. typhimurium* synthesizes B₁₂ only during anaerobic growth (86), a simple *metE* mutant can grow anaerobically without methionine by producing its own B₁₂ and using the MetH enzyme. Starting with such a *metE* strain, mutants unable to synthesize...
B$_{12}$ can be identified anaerobically as methionine auxotrophs correctable by B$_{12}$ or one of its precursors (see Table 1).

Mutants can be assigned to one of the three parts of the pathway by anaerobic nutritional tests. These parts are designated I, II, and III in Figure 2, and gene positions are shown in Figure 5. A mutant for Part I of the pathway can make B$_{12}$ if supplied with cobinamide (Cbi). A mutant for Part II can make B$_{12}$ if Dmb is provided. Mutants for Part III lack ability to join Cbi and Dmb and fail to make B$_{12}$ even when both intermediates are provided (see Table 1). Under aerobic conditions, a $cob^+$ metE mutant behaves like a Part I mutant because early steps in Part I of the pathway are sensitive to oxygen (148).

Most of the B$_{12}$ synthetic genes are located in a single, 20-gene operon that maps near minute 44 of the S. typhimurium chromosome (86, 88, 140). Mutations with defects in Part I of the pathway affect one of the first 17 genes, designated cbi. Mutations with a Part III defect affect the next two genes, cobU and cobS (59, 117). Mutations in the last gene of the operon, cobT, cause a Part II defect (42, 166). The CobT protein appears to have multiple activities and acts in both Parts II and III of the pathway (see below).

More detailed functional assignments were allowed by identification of S. typhimurium homologues of P. denitrificans cob genes (140), whose biochemical roles had been defined (described below). Within the Part I region is a three-gene cluster (cbiNQO) that is likely to encode a cobalt transport system (140). This assignment was made by sequence comparisons to known transport genes and by finding that the phenotype of mutations mapping in this region can be corrected by a high concentration of cobalt (D Walter, M Ailion, & J Roth, unpublished data).

As more mutations were classified, several (cobA, cobB, cobC, cobD, and cysG) were found to map outside of the main operon. These unlinked biosynthetic genes appear to contribute to assimilation of exogenous B$_{12}$ precursors or to play secondary roles in some process other than cobalamin de novo synthesis.

### Table 1  Growth phenotypes of S. typhimurium mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Aerobic additions</th>
<th>Anoxic additions</th>
<th>Growth on methionine-deficient medium$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Met</td>
<td>Cbi</td>
</tr>
<tr>
<td>wild type</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>metE</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>metE metH</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>metE cob (Part I)</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>metE cob (Part II)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>metE cob (Part III)</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

$^a$ A plus sign indicates growth on the indicated medium; a minus sign indicates the lack of growth.
Figure 5  Genes involved in cobalamin synthesis and use in *S. typhimurium*. Map positions are according to Sanderson et al (142). Cobalamin-dependent pathways are positioned on the inside of the map; cobalamin biosynthetic and transport genes are positioned on the outside. Genes involved in cobalamin transport are shaded. Arrows indicate direction of transcription. For the centrally positioned *eut* and *pdu* operons, raised letters indicate genes with an aerobic mutant phenotype; the dashed arrow indicates the unsequenced portion of the *pdu* operon. The functions of genes for Parts I, II, and III of the synthetic pathway (*cbi* and *cob*) are indicated in Figure 2.

Thus the main *cob* operon contains only genes needed for de novo B12 synthesis; the unlinked genes may have additional functions that are important even when B12 is not being synthesized de novo. The exact functions of some of these unlinked genes is not yet clear. We outline our present understanding of their roles later.

**Genetic Analysis of B12 Synthesis in *P. denitrificans***

A large set of cobalamin-defective mutants was isolated both in *Pseudomonas putida* and in *Agrobacter tumefaciens*; the mutants were identified by their inability to degrade ethanolamine unless provided with cobalamin (38). Genomic libraries of *P. denitrificans* were screened for clones that complemented the various mutants. Analysis of the clones revealed four gene clusters (37, 39, 47–49). The locations of these clusters on the *P. denitrificans* chromosome are unknown. The mutants were isolated in the presence of Dmb so that no mutants with simple defects in its synthesis could have been expected. Nevertheless, if the synthesis of Dmb proves to be accomplished by a single multifunctional
enzyme as described below, it is likely that mutants for the Dmb synthetic gene were isolated by virtue their secondary defect in Part III of the pathway.

Comparison of Genes from Salmonella and Pseudomonas spp.
When DNA sequences of cob genes from P. denitrificans were compared to those from S. typhimurium, many pairs of homologous genes were identified; however, each data set included multiple genes without a homologue in the other species (140). This situation could be explained if each of the two gene identification efforts had missed a different set of genes. It seems more likely that the unmatched genes reflect substantial differences in the biochemistry of the synthetic pathways in the two organisms.

BIOSYNTHESIS OF COBALAMIN
In a monumental effort combining biochemical and genetic work, a detailed picture of the complete biosynthesis of the corrinoid ring in P. denitrificans has been developed by studying the activities of proteins produced from individually cloned genes (18–22, 37, 49–51, 164). A similar approach has been taken in Propionibacterium shermanii and S. typhimurium (135, 143, 149, 152). The results reveal two distinct pathways for corrin ring synthesis; this chemical work has been reviewed recently (10, 147).

Multiple Biosynthetic Pathways to the Corrin Ring
The most extensive analysis of corrinoid ring synthesis was done using P. denitrificans, which is able to make B12 in the presence of oxygen. In contrast, P. shermanii (an anaerobe) and S. typhimurium (a facultative anaerobe) make B12 only under anaerobic conditions. This difference in lifestyle is reflected in the nature of the B12 biosynthetic pathways.

The aerobic pathway of P. denitrificans requires at least 20 steps to convert UroIII to Ado-Cbi. These reactions, Part I of the pathway, can be seen in Figure 6. Cobalt insertion, reduction, and adenosylation (Reactions 15–17) occur late in this reaction sequence. This pathway not only proceeds in the presence of oxygen, but includes one step (Reaction 4) that requires oxygen (in vitro) for reoxidation of the enzyme (10). In contrast, both S. typhimurium and P. shermanii can make Ado-Cbi only in the absence of oxygen. In the anaerobic pathway of these organisms, the initial reactions are oxygen-sensitive and cobalt insertion occurs early in the pathway (109, 110). Thus the cobalamin biosynthetic pathway of one organism may depend on oxygen, whereas the pathway of the others is toxified by oxygen.

In view of these differences, it is not surprising that the two pathways might use nonhomologous enzymes to catalyze some analogous reactions. Most
Figure 6  Part I of the aerobic cobalamin biosynthetic pathway. The *P. denitrificans* genes that encode relevant enzymes are listed below each reaction; homologous genes of *S. typhimurium* are in parentheses. Reaction 4 entails the incorporation of an oxygen atom, probably from water, during the reductive lactone formation (10). In this reaction, the enzyme is reduced and can only turn over in the presence of oxygen (52). Reaction 5, the methylation of C17, initiates spontaneous ring contraction (Reaction 6) between C1 and C19 (52). Reaction 7, methylation at C11, leads to the extrusion of acetate (Reaction 8), which can occur spontaneously (10). Reaction 16, reduction of cobalt, is catalyzed by the cobyrinic acid a,c-diamide reductase, whose gene has not been cloned (21). SAM: S-adenosyl methionine; SAH: S-adenosyl homocysteine. No homologues have been identified for any of the unassigned genes in the lower right corner of the figure.)
intermediates in the aerobic pathway lack cobalt and, therefore, the upper axial ligand, whereas those in the anaerobic pathway contain cobalt and possibly adenosine. Figure 6 presents the aerobic pathway used by \textit{P. denitrificans}. Note that the \textit{P. denitrificans} genes without a \textit{S. typhimurium} homologue include those involved in the oxygen-dependent step (Reaction 4) and the late cobalt insertion (Reaction 15). Other genes involved in Part I of the pathway but lacking an assigned function are listed in the lower right corner of the figure.

Additional B\textsubscript{12} pathways may be found since many enteric bacteria can make B\textsubscript{12} both aerobically (impossible for a pathway of the anaerobic type) and anaerobically (without the oxygen required for the aerobic type) (100). Alternatively these enterics may use the aerobic pathway but are able to regenerate their enzyme for Reaction 4 without molecular oxygen.

The First Enzyme in B\textsubscript{12} Synthesis

The first two methylations of UroIII are performed by the CysG enzyme of \textit{S. typhimurium}/\textit{E. coli} and by a homologous protein (CobA) of \textit{P. denitrificans}. These reactions generate Precorrin-2, the precursor of both B\textsubscript{12} and siroheme (see Figures 2 and 6).

The \textit{S. typhimurium} and \textit{E. coli} CysG enzymes are longer than the homologue from \textit{P. denitrificans}, possessing an additional N-terminal region of 201 amino acids. This region appears to be required for catalysis of the additional reactions (ring oxidation and iron insertion) needed for synthesis of siroheme (152). Thus in \textit{S. typhimurium} and \textit{E. coli}, a single protein catalyzes four distinct reactions (see boxed reactions in Figure 2). Certain \textit{S. typhimurium} cysG mutations eliminate B\textsubscript{12} synthesis but allow continued siroheme production (65). Since these mutants are corrected by exogenous cobalt, CysG protein may also catalyze insertion of cobalt, probably into Factor-2, and thus seems to be responsible for the first reaction unique to B\textsubscript{12} synthesis by the anaerobic pathway. The multifunctional CysG enzyme of \textit{S. typhimurium} is positioned so as to control the flux of UroIII into three pathways. No evidence has been presented that the activities of this protein vary in response to intracellular conditions. The shorter \textit{P. denitrificans} homologue (CobA; aerobic pathway) appears to catalyze only the two methylation reactions needed to form Precorrin-2; cobalt insertion occurs later in this pathway and is supported by distinct proteins.

Addition of the Deoxyadenosyl Moiety

Adenosyl transfer is required at several points in cobalamin metabolism. During biosynthesis, adenosine is added to a biosynthetic intermediate, leading to synthesis of the intermediate Ado-Cbi. Any assimilated corrinoids are likely to require adenosylation, since the carbon-cobalt bond of cobalamin is unstable and likely to be lost in the extracellular environment. Adenosylation may
also be required by enzyme-bound cofactors lacking the upper ligand. Such enzymes may also recycle cofactors that have lost the adenosyl group as a result of damage by the inherently reactive radicals involved in catalysis.

In *S. typhimurium*, adenosyl transferase mutants (*cobA*) were identified as Part I mutants that map outside of the main operon. Under aerobic conditions *cobA* mutants cannot use the biosynthetic intermediate Cbi as a precursor but can grow when Ado-Cbi is supplied, which suggests a defect in adenosylation. This nutritional pattern was seen when synthesis of B12 was scored by the activity of the MetH enzyme, a methyl transferase that requires methyl-B12, not Ado-B12. The results suggest that, under aerobic conditions, adenosylation is a prerequisite for the reactions of Part III of the pathway (see Figure 7); that is, the CobUS enzymes use only Ado-Cbi as a substrate.

Under anaerobic conditions, *cobA* mutations can use Cbi (nonadenosylated) as a precursor for synthesis of a B12 form that will support the MetH enzyme. Anaerobiosis may allow the CobU protein to adenosylate Cbi or to process Cbi to form nonadenosylated B12 (116); the effect of anaerobic conditions may be simply to permit higher induction of the operon. In *P. denitrificans*, a gene homologous to *cobA* was identified after purification and partial sequencing of a cobalamine adenosyl transferase. This sequence was used to identify the relevant gene from among the sequenced biosynthetic genes (49a).

The CobA enzyme of *S. typhimurium*, in addition to its role in de novo synthesis, appears to adenosylate assimilated CN-B12. This conclusion was reached because a *cobA* mutation prevents repression of the *cob* operon by exogenous CN-B12 but does not block repression by Ado-B12 (61, 158, 159). Similarly in *E. coli*, the BtuR protein (equivalent to CobA of *S. typhimurium*) was identified by a mutation that causes constitutive expression of the *btuB* (B12 transport) gene in cells growing in the presence of a normally repressive level of CN-B12; the *btuR* mutation does not prevent repression by Ado-B12. The regulatory mechanisms of the *cob* operon and the *btuB* gene appear to respond only to Ado-B12, which cannot be made from CN-B12 without the CobA adenosyl transferase. These regulatory mechanisms are discussed later. Although adenosylation of enzyme-bound corrinoids has not been demonstrated, genetic evidence suggests that such adenosyl transferases are encoded within the *eut* and *pdu* operons. These operons are also described later.

The Problem of Cobalt Reduction
Cobalt must be reduced to the CoI state prior to addition of an adenosyl group during biosynthesis or in assimilation of a nonadenosylated corrinoid. Cobalt can be reduced chemically in vitro (e.g., by methyl viologen) or biochemically by a system that depends on reduced NADP and a flavodoxin (21, 79, 115). Although proteins able to perform this reduction have been observed, no mutants
Figure 7  Parts II and III of the cobalamin biosynthetic pathway. Listed genes are those of *S. typhimurium*; homologous genes from *P. denitrificans* are denoted in parentheses. Uncertainties regarding alternative routes from Dmb to Ado-B_{12} are described in the text.
defective in cobalt reduction have been identified to demonstrate biological relevance. The failure to recover such mutants may suggest the existence of multiple reductases or may indicate that a single reductase is also essential to other functions.

**Source of the Aminopropanol Side-Chain**

Isotope tracer studies in *P. shermanii* and *Streptomyces olivaceus* suggest that the 1-amino-2-propanol side-chain of B₁₂ is formed from threonine (98, 104). A simple decarboxylation reaction would generate free 1-amino-2-propanol, which could be attached to cobyric acid (Cby) to form Cbi; however, this decarboxylation has not been demonstrated despite multiple attempts. An alternative route requires oxidation of threonine to amino-ketobutyric acid, followed by decarboxylation to aminoacetone and reduction to yield aminopropanol (112). A third possibility is that threonine may be attached directly to the ring and modified in place (74). The latter possibility would involve no free aminopropanol.

Two classes of mutants appear to be defective in addition of the aminopropanol group: the *cbiB* and *cobD* genes of *S. typhimurium* and the homologous *cobD* and *cobC* genes of *P. denitrificans* (48, 140; C Grabau, unpublished data). These genes have been assigned to Reactions 19 and 20 in the pathway (see Figure 6) based on their ability to use Cbi but not Cby as a B₁₂ precursor. The *A. tumifaciens cobD* and *cobC* mutants accumulate cobyric acid (48). *S. typhimurium cobD* mutants can make B₁₂ if aminopropanol is provided, which suggests that they are unable to synthesize free aminopropanol (80). However, if threonine is normally attached to the ring before decarboxylation, the growth response of *cobD* mutants could be the result of a scavenging pathway for use of exogenous aminopropanol.

**Synthesis of Dimethylbenzimidazole**

The synthetic pathway for Dmb has remained elusive. In *P. shermanii*, Dmb is derived from riboflavin, but the synthetic pathway is unknown. A proposed pathway involves five reactions but requires the presence of oxygen (127). This may make sense for *P. shermanii* (an aerotolerant anaerobe), which is reputed to make Dmb only when exposed to oxygen. However, *Salmonella* spp. make B₁₂ with a Dmb lower ligand during extended growth under anaerobic conditions, which suggests that Dmb synthesis can occur without oxygen (89). *S. typhimurium* mutants were isolated that could make B₁₂ only if supplied with Dmb. These mutants affect a single gene (*cobT*) mapping at the distal end of the *cob* operon (42, 166). The simplest interpretation of these results is that the single CobT protein catalyzes the complete synthesis of Dmb. There are several reasons to believe that the situation is more complex.
First, it seems unlikely that any single enzyme could synthesize Dmb from its probable (but unproved) precursor, riboflavin (see Figure 7). More disturbing, there are mutants of *P. putida* that can be complemented by the *cobU* gene of *P. denitrificans* (a homologue of *Salmonella cobT* gene). These mutants show a phenotypic defect in Part III of the pathway, the joining of Dmb and Cbi (37). Thus two proteins of similar size, showing 33% identity (57% similarity) over their entire length, appear to have different mutant phenotypes in two organisms (42). The *P. denitrificans* enzyme, consistent with its mutant phenotype, was shown to catalyze transfer of ribose phosphate from NaMN to Dmb (37). The *S. typhimurium* CobT enzyme also catalyzes this Part III reaction (166), even though its mutant phenotype shows proficiency in Part III of the pathway. Thus homologous enzymes in two species appear to catalyze the same reaction but have distinct mutant phenotypes; the Part II phenotype of the *S. typhimurium* mutant does not fit with the known enzymological defect.

Escalante-Semerena and coworkers offered an explanation for this discrepancy by proposing that *S. typhimurium* (but not *P. putida*, which was used for the mutant hunts) possesses a second enzyme capable of catalyzing phosphoribosyl transfer to Dmb (166). The alternate enzyme, they suggested, might have a poor affinity for Dmb and thus could act only when a high level of Dmb was supplied exogenously. Their argument was supported indirectly by identification of two classes of mutations (*cobC* and *cobB*); either of these mutations, when combined with a *cobT* mutation, causes a defect in Part III of the pathway. (Single *cobT*, *cobB*, or *cobC* mutations do not eliminate Part III.) It was suggested that the CobC and CobB enzymes together duplicate the CobT role in Part III of the pathway and thus mask the Part III phenotype expected for *cobT* mutations. Apparently this alternative method for making Dmb nucleosides is not present in *P. putida*. Although this hypothesis explains the phenotype of *cobT* mutants, it does not explain why an enzyme (CobB) would be made that couldn’t use ambient levels of Dmb or why the hypothetical Dmb synthetic genes were not affected by any of the many *S. typhimurium cob* mutations.

Later some *cobT* mutants were found to be satisfied by a very low level of exogenous Dmb, whereas others required a 1000-fold higher concentration (42). Deletions of the *cobT* gene require a very high level of Dmb and gain a Part III defect when combined with a *cobB* mutation, showing a CobT role in Part III. Certain point mutants (*cobT*) satisfied by little Dmb retain their Part II phenotype even in the presence of a *cobB* mutation, suggesting that they are proficient in Part III and have a simple (Part II) defect in Dmb production (42).

These results suggested that the normal CobT enzyme might catalyze the complete synthesis of Dmb in addition to performing the ribosyl phosphate
transfer. Although this theory would explain the mutant phenotypes, it still leaves a single enzyme to perform a complex set of mechanistically difficult reactions needed for Dmb synthesis. Possible activities of the *S. typhimurium* *cobB* and *cobC* genes are discussed below.

**Formation of the Dmb Nucleoside: The Phosphate Problem**

The CobT enzyme of *S. typhimurium* (and the homologous *P. denitrificans* CobU enzyme) have been shown to catalyze the transfer of Ribose-PO₄ from nicotinic acid mononucleotide (NaMN; an intermediate in NAD synthesis) to Dmb (37, 166). This reaction generates DmbMN, which has a phosphate on the 5′ carbon of ribose. This phosphate poses a problem. Since it is not a part of normal Ado-B₁₂, it must be removed at one of three points in the pathway:

1. The 5′ phosphate could be removed from NaMN before transfer of the ribose to Dmb; it is not clear whether nicotinic acid ribonucleoside (NaR) has ever been tested as a substrate for the ribose transfer reaction.

2. The phosphate could be removed from DmbMN prior to B₁₂ completion.

3. DmbMN could be transferred intact to activated Ado-Cbi yielding Ado-B₁₂-PO₄, whose phosphate could be removed as the last synthetic step.

These possibilities are included in Figure 7 with possible *S. typhimurium* gene names. We suspect that in *S. typhimurium*, several of these routes may coexist.

Evidence supports the third possibility in *P. denitrificans*. The CobU enzyme can use NaMN as a phosphoribose donor to produce DmbMN; the CobV enzyme DmbMN can join to Cbi to form Ado-B₁₂-PO₄ (37). This observation suggests that the phosphate could be carried through the joining step, although the joining enzyme is also capable of using Dmb-ribose, which is present in great excess over the nucleotide in cells of *P. denitrificans* (37).

Escalante and coworkers have provided evidence that *S. typhimurium*’s CobC enzyme is a phosphatase that converts DmbMN to Dmb-ribose (DmbR) (118); however, the activity measured was very low, and *P. denitrificans* has no known homologue of the CobC protein. If the CobC enzyme serves the suggested function and is the only route to B₁₂, *S. typhimurium cobC* mutants should show a Part III phenotypic defect, but they do not. Rather, *cobC* mutations show a curious partial defect in B₁₂ synthesis that can be corrected by any of the following single nutrients: Cby, Cbi, aminopropanol, or Dmb (M Ailion, D Walter, C Grabau, & J Roth, unpublished data). We suspect that the *cobC* mutation may eliminate only one of several alternative ways of removing the phosphate (see Figure 7).
Completion of Ado-\(B_{12}\)

To join Ado-Cbi and the Dmb nucleoside, the aminopropanol group of Ado-Cbi is first activated by two reactions (see Figure 7). The end of the aminopropanol side-chain is phosphorylated (to Ado-Cbi-PO\(_4\)), followed by transfer of guanosine phosphate from GTP, with displacement of pyrophosphate, to form Ado-Cbi-GDP (Ado-Cbi-guanosine pyrophosphate) (49). These two reactions are catalyzed by a single enzyme, CobP of \(P.\) denitrificans or CobU of \(S.\) typhimurium (49, 117). These reactions activate the end of the aminopropanol side-chain for attachment of the Dmb ribonucleoside at its \(3'\) position, catalyzed by CobV of \(P.\) denitrificans or CobS of \(S.\) typhimurium (37, 117). Attachment generates the completed Ado-\(B_{12}\). As noted above, the joining reaction might use NaMN and yield phosphorylated Ado-\(B_{12}\).

TRANSPORT OF \(B_{12}\)

The transport of cobalamin into bacterial cells poses two problems. First, the size of cobalamin far exceeds the limit for passage through outer membrane porins of enteric bacteria. Thus entry of \(B_{12}\) requires some specific outer-membrane transport system. Second, cobalamin may be present in extremely low quantities in the environment. To transport significant quantities of the cofactor, this outer-membrane transport system must be able to scavenge \(B_{12}\) with high affinity and move it into the periplasmic space; other systems can then move it across the inner membrane.

The mechanism of \(B_{12}\) transport has been studied extensively in \(E.\) coli (28) and includes one system for transport across the outer membrane and one for transport across the inner membrane (see Figure 8). Transport mutants were isolated by using MetE mutants, which depend on transported exogenous \(B_{12}\) for growth on methionine-deficient medium. Mutants were isolated that require an abnormally high level of exogenous cobalamin to support growth. Transport-deficient mutants fell into four classes (see Table 2) (7, 55, 56).

Transport Across the Outer Membrane

Strains with a \(btuB\) mutation transport \(B_{12}\) only when it is supplied at very high concentration; these mutations map at minute 90 of the genetic map. Mutations with a similar phenotype affect the TonB protein, which participates in several outer-membrane transport systems (see below). The transport defect of \(btuC\) and \(btuD\) mutants is corrected by a more modest excess of \(B_{12}\) (56); these mutations represent two complementation groups that map at minute 37.

Transport through the outer membrane requires the BtuB protein acting with TonB. This system has a high affinity for vitamin \(B_{12}\) and its many derivatives, including adenosylcobalamin and cobinamide (31, 96, 160, 161, 172). Without
Table 2  Phenotypes of cobalamin transport mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>$E. coli$ B$_{12}$ required for Met$^+$ (nM)</th>
<th>Map*</th>
<th>$S. typhimurium$ B$_{12}$ required for Met$^+$ (nM)</th>
<th>Map*</th>
</tr>
</thead>
<tbody>
<tr>
<td>metE</td>
<td>0.075</td>
<td>NA</td>
<td>0.075</td>
<td>NA</td>
</tr>
<tr>
<td>metE tonB</td>
<td>1000</td>
<td>28</td>
<td>1000</td>
<td>38</td>
</tr>
<tr>
<td>metE btuB</td>
<td>1000</td>
<td>90</td>
<td>1000</td>
<td>90</td>
</tr>
<tr>
<td>metE btuC</td>
<td>0.75</td>
<td>37</td>
<td>0.75</td>
<td>30</td>
</tr>
<tr>
<td>metE btuD</td>
<td>0.75</td>
<td>37</td>
<td>0.75</td>
<td>30</td>
</tr>
<tr>
<td>metE btuF</td>
<td>NA$^b$</td>
<td>NA</td>
<td>0.75</td>
<td>5</td>
</tr>
<tr>
<td>metE btuCED btuF</td>
<td>NA$^b$</td>
<td>NA</td>
<td>0.75</td>
<td>NA</td>
</tr>
<tr>
<td>metE btuB btuCED</td>
<td>ND$^c$</td>
<td>NA</td>
<td>100,000</td>
<td>NA</td>
</tr>
<tr>
<td>metE btuB btuF</td>
<td>NA$^b$</td>
<td>NA</td>
<td>100,000</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Map position in minutes of the tonB or btu gene.
$^b$Not applicable; btuF mutants are not known for E. coli.
$^c$Not done.

Figure 8  Transport of cobamides. Numbers indicate the inferred sequence of events based on work in E. coli (28). While periplasmic binding proteins are thought to act in this process, the BtuF protein is assigned this function only by general phenotypes of S. typhimurium mutants (see Table 2 and text).
the BtuB/TonB system, B\textsubscript{12} penetrates the outer membrane with extremely low efficiency. Once bound to the BtuB protein, B\textsubscript{12} is moved into the periplasm in an energy-dependent process that requires the TonB protein, which uses proton motive force to drive a structural alteration needed for transport (29, 33). Although the BtuB protein is thought to be associated physically with the TonB protein (12), it can bind B\textsubscript{12} even in \textit{tonB} mutant strains. Cotransport of calcium is required for successful passage of B\textsubscript{12} through the outer membrane (30, 32). The \textit{btuB} gene has been cloned and sequenced from \textit{E. coli} (82, 83) and \textit{S. typhimurium} (168).

The BtuB protein provides the binding sites for phage BF23 (8) and for Colicin E (91). The TonB protein also energizes outer-membrane transport systems for iron, including the FepA and FhuA systems (169). Thus the BtuB protein competes with these systems for TonB activity (92, 94).

**Transport Across the Inner Membrane**

Transport across the inner membrane is provided by the \textit{E. coli} BtuC and BtuD proteins, encoded by the \textit{btuCED} operon at minute 37 (7, 53, 54, 93). The \textit{btuC} and \textit{btuD} genes encode membrane proteins that resemble a family of transport proteins requiring a periplasmic binding protein (75). The BtuD protein has an ATP-binding site. The \textit{btuC} and \textit{btuD} mutant phenotypes are corrected by a modest increase in external B\textsubscript{12}. The BtuB/TonB system may concentrate B\textsubscript{12} in the periplasm, facilitating fortuitous transport of B\textsubscript{12} into the cytoplasm of mutants lacking the inner-membrane transport system.

The central gene of the \textit{btuCED} operon, \textit{btuE}, was found as an open reading frame in the operon DNA sequence but is not required for B\textsubscript{12} transport (134). The BtuE sequence is not clearly homologous to any known periplasmic binding protein and does not bear a signal sequence (75).

Although a periplasmic B\textsubscript{12} binding protein (expected for a system of the \textit{btuCD} type) has not been identified in \textit{E. coli}, the \textit{btuF} locus of \textit{S. typhimurium} may encode such a protein (N Clark, J Lawrence, & J Roth, unpublished data). \textit{S. typhimurium} has genetic loci corresponding to the \textit{btuB}, \textit{tonB}, and \textit{btuCED} loci of \textit{E. coli} (108; N Clark, J Lawrence, & J Roth, unpublished data).

In addition, the new \textit{btuF} transport gene of \textit{S. typhimurium} has a phenotype resembling that of \textit{btuC} and \textit{btuD} mutants (see Table 2). The double mutant combination of \textit{btuF} with \textit{btuC} or \textit{btuD} has a transport phenotype indistinguishable from those of the individual mutants, suggesting that all three genes contribute to transport of B\textsubscript{12} across the inner membrane. In contrast, the combination of a \textit{btuF} (or \textit{btuC} or \textit{btuD}) mutation with a \textit{btuB} mutation causes a transport defect that is more severe than those of the single mutants. This observation suggests two additive functions, one provided by BtuB/TonB (outer-membrane transport) and the other by the BtuC, BtuD, and BtuF proteins (inner-membrane
transport). The BtuCD transport system appears to involve a periplasmic binding protein (172), and BtuF function of \textit{S. typhimurium} is a good candidate for filling this role.

Although the \textit{btuCED} operon appears to be expressed constitutively; the \textit{btuB} gene is regulated as discussed below (6, 90, 106, 168). The cryptic plasmid of \textit{S. typhimurium} carries a cluster of genes that encode the proteins of a pilus; mutant forms of this region of the plasmid affect the rate at which B\textsubscript{12} crosses the outer membrane in the absence of BtuB function (132, 133).

**REGULATION OF B\textsubscript{12} SYNTHESIS IN \textit{S. TYPHIMURIUM}**

The regulatory behavior of any set of genes can provide clues to the role of those genes. Significant questions exist regarding the physiological importance of cobalamin to \textit{S. typhimurium} and \textit{E. coli} (see below). Understanding regulation of the \textit{S. typhimurium} \textit{cob} operon may shed light on use of B\textsubscript{12} in this organism.

**Control of the \textit{cob/pdu} Regulon**

The \textit{cob} operon (encoding B\textsubscript{12} synthetic enzymes) maps adjacent to the \textit{pdu} operon, which encodes enzymes for propanediol degradation. The two operons are both induced by propanediol using a single regulatory protein. These results indicate that the main role of B\textsubscript{12} in \textit{S. typhimurium} is in supporting catabolism of propanediol.

Two lines of evidence initially suggested that propanediol was involved in control of the \textit{cob} operon (24, 136). First, propanediol was found to induce transcription of \textit{cob-lac} fusions in cells growing on poor carbon sources. Second, single mutations mapping between the \textit{cob} and \textit{pdu} operons eliminate inducibility of both operons by propanediol. These mutations define the \textit{pocR} gene that encodes a regulatory protein of the AraC-family (43) (see Figure 9). The \textit{pduF} gene, encoding a diffusion facilitator for propanediol (43; P Chen & J Roth, unpublished data), was also found to lie in the region between the \textit{cob} and \textit{pdu} operons (43). There seem to be alternative routes of propanediol entry since \textit{pduF} mutations reduce inducibility of the \textit{cob} and \textit{pdu} operons only by virtue of their polar effect on expression of the \textit{pocR} gene (41).

Two global regulatory systems (Crp/Cya and ArcA/ArcB) affect inducibility of the \textit{cob} and \textit{pdu} operons (2, 4, 60). Both operons are activated aerobically and anaerobically by Crp protein and anaerobically by ArcA protein. Maximum inducibility is seen during anaerobic respiration of a poor carbon source; under these conditions the Crp and ArcA proteins act additively. The control of the regulon depends on five promoters, all located in the central region between the \textit{cob} and \textit{pdu} operons; four of these promoters are activated by the PocR protein (41).
The current model for control of this system includes the following features (see Figure 9): The cob and pdu operons are transcribed divergently from promoters indicated at the far right and left in the figure. These promoters are activated whenever both the PocR protein and propanediol are present. Global control of the two operons is exerted by varying the level of PocR protein. The pocR gene is transcribed by three promoters controlled both by global regulatory proteins and by autoinduction (41). The shortest transcript (from Ppoc) appears to be regulated only by Crp/cAMP. The P2 transcript clearly is autoregulated by PocR and, in addition, requires the ArcA protein, which signals a reduced cell interior (41).

Control of the P1 promoter is less certain but may involve either the Fnr protein (responding to a reduced cell interior) or the Crp protein (responding to a shortage of carbon and energy) in addition to the PocR activator. The involvement of these proteins in control of P1 is based heavily on the presence of appropriate binding sites upstream of that promoter.

We propose that the regulon has three states. In the off condition (during aerobic growth on glucose), all promoters are at their lowest level; PocR protein is produced by the basal expression levels of promoters P1, P2, and Ppoc. Cells enter a standby state when they grow under any set of global conditions appropriate for induction, but without propanediol. These conditions include aerobic growth on a poor carbon source and/or growth without oxygen. These conditions stimulate expression of both the PduF transporter and the PocR regulatory protein, but the cob and pdu operons remain uninduced. The standby expression of PocR can occur from the Ppoc and/or the P1 promoter without
The two proteins induced in the standby state (PduF and PocR) are those needed to sense propanediol. If propanediol appears, the P1 and P2 promoters are induced, increasing the level of PocR protein and placing the system in the on state. The resulting high level of PocR/propanediol complex induces expression of the cob and pdu promoters.

This regulatory pattern suggests several things about the role of B12 in Salmonella spp. physiology. Induction of the cob operon by propanediol (and its coinduction with the pdu operon) suggests that degradation of propanediol is a major function of cobalamin in Salmonella spp. Global control by Crp/cAMP suggests that B12 helps provide a carbon or energy source, consistent with the importance of B12 for propanediol degradation. Control by Arc (and perhaps Fnr) suggests that anaerobic expression is important.

Control of the cob Operon and the btuB Gene by Ado-B12

Independent of the control mechanisms described above, transcription of the cob operon, but not the pdu operon, is reduced in the presence of the end-product, Ado-B12. This control mechanism shares many features with that of the btuB gene, also repressed in response to Ado-B12. These two systems share sequence features with the P. denitrificans cobP gene, the first gene of a four-gene cob operon. Although the details of these three control mechanisms are uncertain, their common features suggest that all may use an mRNA leader sequence to sense Ado-B12 and to effect both translational and transcriptional controls.

The cob and btuB control mechanisms share the following features (see Figure 10):

1. Both are repressed by Ado-B12 and not by CN-B12 (105; M Ailion & J Roth, unpublished data).
2. Control seems to alter continuation of message synthesis rather than initiation; for example, repression by Ado-B12 is seen even when transcription is initiated at foreign promoters (5, 43, 129; M Ailion & J Roth, unpublished data).
3. Genetic analysis has revealed no protein that might mediate the repressive effects of Ado-B12; all mutations that eliminate control alter the mRNA leader region (106, 130).
4. The leader regions of the two mRNAs (and the region upstream of the P. denitrificans cobP gene) share some common sequence features (see Figure 10), including two areas of the leader (Box 1 and Box 2) and a hairpin
structure that occludes the translation initiation site of the adjacent structural gene.

5. In both the cob operon of *S. typhimurium* and the btuB gene of *E. coli*, translational control of the first gene seems to be an integral part of the control mechanism (106, 129, 130). In these two cases, fusions to distal points in the adjacent gene show both transcriptional and translational control. Fusions to an intermediate region of the gene show translational but not transcriptional control. More proximal fusions show no regulation (see Figure 10).

The above features suggest a mechanism in which a direct interaction occurs between the effector (Ado-B12) and the mRNA leader. This interaction may induce mRNA folding that stabilizes the hairpin, thereby blocking the translational start of the adjacent coding region; this hairpin exerts a translational control. The untranslated coding sequence may lead to message termination at a site within the structural gene, thus providing transcriptional control. Only fusions beyond the inferred transcription termination site can show transcriptional regulation. This model, suggested originally by Lundrigan and colleagues (106), was supported by Richter-Dahlfors and colleagues. This model accounts for the available data but has not been demonstrated fully. In particular, it remains

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**Figure 10** Features of genes repressed by Ado-B12. The three indicated transcripts are the cob operon (*S. typhimurium*), the btuB gene (*E. coli*), and the cobPWNO operon (*P. denitrificans*). Only the first two have been analyzed in detail; the cobPWNO features are inferred only by sequence inspection. The transcripts share two separated blocks of sequence similarity within the mRNA leader and a dyad symmetry that may sequester the translation initiation site. Regions required for control were inferred from effects of deletions on lac fusions to various points in the adjacent structural gene (106, 130). Bases within the first gene are numbered relative to the transcription start site for the cbiA and btuB genes. Numbers in parentheses indicate the number of base pairs between the indicated sequence elements.
THE SIGNIFICANCE OF B12 FOR ENTERIC BACTERIA

The B12 Paradox

The genetic analysis of B12 synthesis and use in S. typhimurium reveals a problem. Nearly 1% of the S. typhimurium genome is dedicated to synthesis or import of B12 (see Figure 5), yet a mutant defective only for cobalamin synthesis has no laboratory growth phenotype either aerobically or anaerobically. What selective pressures act in natural populations of Salmonella species to maintain this enormous genetic investment?

We have described 26 known genes involved in synthesis of cobalamin and 6 genes providing for its import. This list does not include several predicted functions that have not been identified genetically. Since roughly 4000 genes are likely to make up the S. typhimurium genome, nearly 1% of this genome is dedicated to B12 acquisition. In addition, this genome includes two large operons (described below) that encode the enzymes needed for B12-dependent utilization of ethanolamine and propanediol. Together these two operons appear to include about 30 genes (see Figure 5). Including the metH gene, a total of at least 63 genes are involved in synthesis and use of B12—representing a huge genetic investment.

In an otherwise wild-type strain, cob mutants have no growth phenotype under standard laboratory conditions. The genetics of B12 synthesis was made possible by use of a mutant (metE) in which methionine synthesis is dependent on B12. Even the two major B12-dependent catabolic pathways do not provide a strong selection for B12 synthesis. S. typhimurium synthesizes B12 only anaerobically, but it requires oxygen to use ethanolamine or propanediol as a sole carbon and energy source.

Even the presence of alternative electron acceptors (nitrate or fumarate) does not allow anaerobic use of these carbon sources. Aerobic use of propanediol or ethanolamine is seen only if B12 is provided exogenously, since there is no aerobic B12 synthesis. Thus S. typhimurium cannot use its endogenous B12 (made only anaerobically) to support degradation of propanediol or ethanolamine (requiring oxygen). Exceptions to this behavior are described below.

The large genetic investment in B12, together with the lack of laboratory phenotype for cob mutants, raises a paradox. It is clear that any gene not under natural selection will inevitably be lost as a result of mutation accumulation.
Figure 11  Metabolism of propanediol. Reactions indicated as proceeding without oxygen provide an electron sink and a source of ATP, but not a carbon source. Reactions indicated as proceeding with oxygen provide a carbon and an energy source by respiration using oxygen or tetrathionate (but not nitrate or fumarate) as an electron acceptor (T Bobik & J Roth, unpublished data). DHAP indicates dihydroxyacetone phosphate.

Maintenance of the large collection of genes involved in synthesis and use of B<sub>12</sub> requires that a strong selective force be imposed on these functions in natural *Salmonella* spp. populations. Standard laboratory phenotypes do not indicate what this selection might be. Understanding the conditions for this selection may provide significant insight into how *Salmonella* spp. live in the real world. Understanding this selection may also be fundamental to understanding the lifestyle of *Salmonella* spp.; the closely related bacterium *E. coli* can neither synthesize cobalamin de novo nor degrade propanediol.

The regulatory behavior of the *cob* operon suggested that B<sub>12</sub>-dependent utilization of propanediol (possibly under anaerobic conditions) must be a major aspect of the selection for B<sub>12</sub> production. The pathway for ethanolamine degradation shares many features with that for breakdown of propanediol. To approach the physiological importance of B<sub>12</sub>, we examine these two pathways.

**Metabolism of Propanediol**

The first step in this degradative pathway is the B<sub>12</sub>-dependent diol dehydratase, which produces propionaldehyde (see Figure 11). This aldehyde can
be oxidized to form propionyl-CoA or alternatively can be reduced to form propanol. By forming and excreting propanol, cells can balance internal redox reactions. Under anaerobic conditions, propionyl-CoA can be converted by a phosphotransacetylase to propionyl-phosphate, and then by a reversible acetyl-/propionyl-kinase to propionate, producing one molecule of ATP (167). According to this scheme, anaerobic metabolism of propanediol could provide an electron sink (propanol) and a source of ATP but no source of carbon since both propanol and propionate are excreted. Genes needed for conversion of propanediol to propionyl-CoA are encoded in the \textit{pdu} operon (87; T Bobik, Y Xhu, R Jeter, & J Roth, unpublished data; D Walter, M Ailion, & J Roth, unpublished data). This operon may also contain some of the additional genes encoding enzymes that contribute to anaerobic propanediol metabolism (e.g. propionyl-CoA phosphotransacetylase, propionate kinase, propanol dehydrogenase).

Under aerobic conditions, \textit{S. typhimurium} can use propionyl-CoA as both a carbon and an energy source, probably by a pathway that includes acrylyl-CoA and lactyl-CoA as intermediates in pyruvate production (66). An operon has been identified that appears to encode enzymes for the aerobic catabolism of propionyl-CoA (and exogenous propionate); this operon maps at minute 8 (J Tittensor, T Bobik, & J Roth, unpublished data).

\textbf{Metabolism of Ethanolamine}

The ethanolamine degradative pathway is directly analogous to that for propanediol. The B$_{12}$-dependent ethanolamine ammonia lyase produces acetaldehyde (in place of propionaldehyde), which can be oxidized to acetyl-CoA (in place of propionyl-CoA). The acetaldehyde can be also be reduced (to ethanol) to provide an electron sink. Acetyl-CoA can be converted in two reactions to acetate with production of ATP. As for propanediol, anaerobic use of ethanolamine provides ATP and an electron sink but no carbon source. Acetyl-CoA can be utilized aerobically via the TCA cycle. Mutants defective in aerobic use of ethanolamine map in the \textit{eut} operon. Ethanolamine degradation also provides a nitrogen source (see Figure 4), but the regulatory pattern of the operon suggests that this is not its main importance.

\textbf{Genetic Analysis of the \textit{pdu} and \textit{eut} Operons}

Genetic characterization of the propanediol (\textit{pdu}) and ethanolamine (\textit{eut}) operons has employed mutants defective for aerobic use of these compounds as carbon sources with exogenously provided B$_{12}$ (137, 138; D Walter, M Ailion, & J Roth, unpublished data). B$_{12}$ was provided because both propanediol and ethanolamine serve as carbon sources only in the presence of oxygen, which prevents B$_{12}$ synthesis. Complementation tests of the \textit{pdu} and \textit{eut} mutations...
revealed five genes in the pdu operon and six in the eut operon that apparently are needed for aerobic growth on the particular carbon source (diagrammed in Figure 5).

The affected pdu genes include those for propanediol dehydratase (pduCED) and for propionaldehyde dehydrogenase. The set of genes affected by eut mutations include those for the B_{12}-dependent enzyme ethanol ammonia lyase (eutBC) and for acetaldehyde dehydrogenase (eutE). Both operons appear to include genes (described below) that may encode B_{12} adenosyl transferases. The genetically identified genes encode functions expected to be necessary for aerobic degradation.

The pdu and eut operons include many genes that were not detected genetically. The pdu operon is about 15 kb in size based on PCR between transposons inserted in the operon, suggesting the existence of about 15 genes (J Lawrence & J Roth, unpublished data), only 5 of which were detected genetically. Only three of the five sequenced pdu genes have an aerobic phenotype, and these genes (pduCED) encode subunits of the diol dehydratase (T Bobik, Y Xhu, R Jeter, & J Roth, unpublished data). The sequence of the eut operon includes 15 genes (64, 153; E Kofoid & J Roth, unpublished data), only 6 of which were detected genetically. (One of these encodes the regulatory protein EutR.)

We suspect that for both operons, the discrepancy is the result of a large number of genes that are not needed under the aerobic conditions used (out of necessity) for mutant hunts. These extra genes are likely to be involved in anaerobic breakdown of these compounds under conditions that have not been fully defined. If conditions can be defined for anaerobic growth at the expense of propanediol and ethanolamine, these conditions may allow the extra genes to show a mutant phenotype. Such anaerobic conditions are likely to provide a selection for B_{12} synthesis that might apply to natural populations. Recent progress toward defining these conditions is described later.

**Regulation of B_{12}-Dependent Functions**

The eut operon is subject to global control by the Crp/cAMP system. The operon is induced by the simultaneous presence of both ethanolamine and Ado-B_{12}; this control is mediated by the EutR protein, a member of the AraC family of positive activator proteins (139, 150; E Kofoid & J Roth, unpublished data). The activator protein appears to recognize both effectors, but this has not been demonstrated directly.

The EutR activator is encoded within the operon and thus induces its own production. The autoinduction circuit avoids competition between the lyase and EutR for binding of a very small pool of Ado-B_{12}, estimated at about 100 molecules per anaerobically growing cell (5). Without autoinduction of EutR, increasing levels of lyase would bind all Ado-B_{12} and limit induction (150).
The global control by Crp/cAMP suggests that the operon is used mainly to provide a carbon and energy source. Inducibility by ethanolamine plus B$_{12}$ suggests that the genes of the operon are required for B$_{12}$-dependent ethanolamine degradation. The pdu operon is regulated coordinately with the cob operon, as described above.

**Homologues of Carboxysome Proteins**

Within both the eut and pdu operons are reading frames that encode homologues of proteins thought to be carboxysome skin proteins. The carboxysome is an organelle found in photosynthetic bacteria and in sulfur chemolithotrophs that use ribulose bisphosphate carboxylase (Rubisco) to fix CO$_2$. These organelles may support CO$_2$ fixation by concentrating CO$_2$ with Rubisco (inside the organelle) and excluding toxic O$_2$ (45, 120). Although such organelles have not been seen in enteric bacteria, the existence of these homologues warrants a closer look.

Two genes of the eut operon (eutM and eutK) and one of the pdu operon (pduA) encode homologues of the CcmK protein of *Synechococcus* sp. (43; E Kofoed & J Roth, unpublished data). The CcmK protein has been reported to be a shell protein of the carboxysome (122). Another eut gene (eutN) encodes a homologue of a different carboxysome structural protein (CcmL of *Synechococcus* sp.). In addition, the PduB and EutL proteins share significant homology to each other but not to other proteins in the database, suggesting a role common to the two pathways. The five proteins listed above are among those with no aerobic mutant phenotype. We suspect that *S. typhimurium* may assemble some carboxysome-like structure to allow anaerobic metabolism of propanediol and ethanolamine.

**Presence of Additional B$_{12}$ Adenosyl Transferases**

In addition to the general adenosyl transferase described above (CobA in *S. typhimurium*, CobO in *P. denitrificans*), additional versions of this enzyme may be encoded by the pdu and eut operons. These enzymes may adenosylate cofactor bound to a particular B$_{12}$-dependent enzyme. Their substrate could arise by binding of nonadenosylated cofactor or when the functional cofactor loses its adenosyl moiety.

A eutA mutant cannot degrade ethanolamine in the presence of a high level of CN-B$_{12}$; activity is restored if CN-B$_{12}$ is replaced by Ado-B$_{12}$ (138, 139). Lyase is strongly inhibited by CN-B$_{12}$ (15), and the eutA activity may be required to reduce the level of toxic CN-B$_{12}$. A cobA (B$_{12}$ adenosyl transferase) mutant cannot grow on ethanolamine with high CN-B$_{12}$, but it can grow if the level of CN-B$_{12}$ is reduced. This growth of a cobA mutant on low CN-B$_{12}$ is
eliminated by addition of a *eut*A mutation; for this reason *eut*A is presumed to encode an adenosyl transferase. Mutants in the *eut*D gene appear to be defective in an adenosyl transferase needed for optimal induction of the operon by CN-B₁₂ (150). We speculate that each B₁₂-dependent protein (lyase and regulatory protein) may have an associated adenosyl transferase for adenosylation of protein-bound corrinoids.

In the propanediol operon, *pdu*H mutations cause a defect in propanediol utilization on medium with CN-B₁₂, but the defect is corrected if Ado-B₁₂ is provided (D Walter, M Ailion, & J Roth, unpublished data). The gene may encode a transferase associated with the propanediol dehydratase.

**Possible Solutions to the B₁₂ Paradox**

Since B₁₂ is only made in the absence of oxygen, and its main use appears to be in propanediol metabolism, its main importance is likely to be found in some aspect of anaerobic catabolism of propanediol (and its sister compound ethanolamine). The known metabolism of propanediol and ethanolamine suggests that some benefit (for example, an electron sink and ATP production) might be derived from anaerobic catabolism even when it does not provide a carbon source (see Figure 11).

This theory was tested by measuring the effect of propanediol and ethanolamine on anaerobic growth when a carbon source is provided (as dilute casamino acids). With no added energy source, this amino acid mixture allows very poor anaerobic growth; both propanediol and ethanolamine stimulate the anaerobic growth rate and can do so using endogenously synthesized B₁₂ (T Bobik & J Roth, unpublished data). This is a selectable phenotype for B₁₂ synthesis that may be important under natural conditions.

In looking for a more striking anaerobic value for B₁₂, we considered various alternative electron acceptors. Although neither nitrate nor fumarate support anaerobic use of propanediol or ethanolamine, a less well-studied acceptor, tetrathionate, allows use of either propanediol or ethanolamine as an anaerobic carbon and energy source. This growth can be supported using endogenously synthesized B₁₂ (T Bobik & J Roth, unpublished data). Therefore, anaerobic tetrathionate medium provides conditions under which *S. typhimurium* can both synthesize B₁₂ and use it to support growth on these carbon sources.

If such growth conditions are common in the natural habitat of *Salmonella* species, respiration to tetrathionate could provide a selective value for synthesis of B₁₂ and solve the paradox of B₁₂ use. This observation, however, raises some serious questions of whether tetrathionate, or other polysulfides, are encountered in nature and how they are metabolized to support respiration of carbon sources that cannot be respired using nitrate or fumarate as electron acceptor.
Role of B12 in Supporting Growth of Salmonella Species
Within a Host Organism

Since S. typhimurium is discovered frequently by virtue of its pathogenicity, the role of B12 may be understandable only in terms of interactions between the bacterium and a metazoan host organism. This possibility has been addressed directly by testing the virulence of isogenic S. typhimurium strains with and without a functional cob operon (141). These experiments yielded the surprising result that strains unable to make B12 are more virulent than are a wild-type strain. This conclusion was seen both for oral and peritoneal routes of infection, suggesting a complex situation in which the host deals with an infecting bacteria differently depending on whether it produces B12. Perhaps by providing B12, S. typhimurium establishes a more benign relationship with a host—a relationship in which the invader is tolerated and to which S. typhimurium responds less aggressively.

Figure 12

Distribution of cobalamin synthesis and use among enteric bacteria. The dendrogram is based on Lawrence & Roth (100). B12 synthesis phenotypes are based on a bioassay of B12 production by cells grown with various nutritional supplements (see Table 1) needed to infer active parts of the pathway (Parts I, II, and III). Cobalamin-dependent growth phenotypes are: EA—ethanolamine utilization, PD—propanediol utilization, Gly—glycerol dehydratase activity. Arrow A indicates inferred evolutionary loss of functions (B12 Part I, PD, Gly); arrow B indicates acquisition of functions (B12 Parts I, II, III, and PD) by a single horizontal transfer.
EVOLUTION OF THE B₁₂ SYNTHETIC GENES IN ENTERIC BACTERIA

Analysis of the base sequence of the *S. typhimurium cob* and *pdu* operons suggested that these genes had been acquired by horizontal transfer since the divergence of *Salmonella* species and *E. coli* (99, 100, 140). Multiple isolates of *Salmonella* spp., *E. coli*, and other enteric bacteria were surveyed for their ability to synthesize B₁₂, for possession of sequences closely related to the *S. typhimurium cob* region, and for ability to perform various B₁₂-dependent metabolic functions (see Figure 12).

Virtually all *Salmonella* species tested behaved like *S. typhimurium*. They synthesized B₁₂ under anaerobic conditions and possessed a *cob* operon homologous to that of *S. typhimurium*. Virtually all natural isolates showed B₁₂-dependent degradation of propanediol. In contrast, no isolate of *E. coli* synthesized B₁₂ de novo, although most possessed genes (*cobUST*) that encoded Parts II and III of the B₁₂ synthetic pathway (99). No *E. coli* isolate showed B₁₂-dependent degradation of propanediol. Other enteric taxa synthesized B₁₂ both aerobically and anaerobically and were capable of B₁₂-dependent degradation of propanediol (100). Unlike *Salmonella* spp. and *E. coli*, these other enterics showed B₁₂-dependent degradation of glycerol. Although the other enterics synthesized B₁₂, their B₁₂ synthetic genes did not show strong homology to the *S. typhimurium cob* operon (<70% base sequence identity). These observations are summarized and interpreted in Figure 12.

We propose that the ancestor of most enteric bacteria synthesized B₁₂ and used it in degradation of propanediol, glycerol, and ethanolamine under both aerobic and anaerobic conditions. On the lineage leading to *E. coli* and *Salmonella* spp., the common ancestor lost both B₁₂ synthesis and the ability to degrade propanediol and glycerol. These losses are still apparent in modern *E. coli* isolates; however, the ancestor of modern *Salmonella* species reacquired by horizontal transfer a chromosomal fragment that includes a B₁₂ biosynthetic operon (functional only anaerobically) and the adjacent *pdu* operon. By inheriting these operons, *S. typhimurium* acquired B₁₂ synthetic ability and a selectable characteristic to ensure its maintenance.

These results suggest that the selection pressure to maintain B₁₂ synthesis varies with the lifestyle of the organism. *E. coli* seems to fill a niche that does not require full de novo B₁₂ synthesis, perhaps one in which B₁₂ (or Cbi) is prevalent, and ethanolamine (but not propanediol) is an important carbon source. For *Salmonella* spp., the ability to synthesize B₁₂ must be strongly selected; its main use may be to degrade propanediol under anaerobic conditions in the presence of a suitable alternative electron acceptor.
ACKNOWLEDGMENTS

This work was supported by research grant GM34804 from the National Institutes of Health.

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