

Anaerobic biosynthesis of the lower ligand of vitamin B₁₂

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Vitamin B₁₂ (cobalamin) is required by humans and other organisms for diverse metabolic processes, although only a subset of prokaryotes is capable of synthesizing B₁₂ and other cobamide cofactors. The complete aerobic and anaerobic pathways for the de novo biosynthesis of B₁₂ are known, with the exception of the steps leading to the anaerobic biosynthesis of the lower ligand, 5,6-dimethylbenzimidazole (DMB). Here, we report the identification and characterization of the complete pathway for anaerobic DMB biosynthesis. This pathway, identified in the obligate anaerobic bacterium *Eubacterium limosum*, is composed of five previously uncharacterized genes, *bzaABCDE*, that together direct DMB production when expressed in anaerobically cultured *Escherichia coli*. Expression of different combinations of the *bza* genes revealed that 5-hydroxybenzimidazole, 5-methoxybenzimidazole, and 5-methoxy-6-methylbenzimidazole, all of which are lower ligands of cobamides produced by other organisms, are intermediates in the pathway. The *bza* gene content of several bacterial and archaeal genomes is consistent with experimentally determined structures of the benzimidazoles produced by these organisms, indicating that these genes can be used to predict cobamide structure. The identification of the *bza* genes thus represents the last remaining unknown component of the biosynthetic pathway for not only B₁₂ itself, but also for three other cobamide lower ligands whose biosynthesis was previously unknown. Given the importance of cobamides in environmental, industrial, and human-associated microbial metabolism, the ability to predict cobamide structure may lead to an improved ability to understand and manipulate microbial metabolism.

vitamin B₁₂ | cobalamin | cobamide | 5,6-dimethylbenzimidazole | biosynthesis

Vitamin B₁₂ (cobalamin) is required by the majority of animals, protists, and prokaryotes, although B₁₂ and other cobamide cofactors are synthesized exclusively by a subset of prokaryotes (1). Cobalamin is a member of the cobamide family of cofactors that are composed of a central cobalt ion coordinated by a tetrapyrrolic corrin ring, an upper ligand, and a lower ligand covalently tethered to the corrin ring by a nucleotide loop (Fig. 1A) (1). The lower ligand of cobalamin is 5,6-dimethylbenzimidazole (DMB). Purines, phenolic compounds, and other substituted benzimidazoles have also been found as cobamide lower ligands (2).

Distinct oxygen-requiring (aerobic) and oxygen-sensitive (anaerobic) cobamide biosynthesis pathways have been characterized, and each contains ~30 genes (1). The biosynthetic pathway for DMB remained elusive until the discovery of the *bluB* gene, the product of which fragments reduced flavin mononucleotide (FMN_{H2}) to produce DMB in an oxygen-dependent reaction (3–6). Currently, the only unidentified genes in the cobamide biosynthesis pathway are those required for the anaerobic biosynthesis of DMB and other benzimidazoles (7). Based on previous isotope-labeling studies, performed mainly in the anaerobic bacterium *Eubacterium limosum*, the biosynthesis of DMB was proposed to branch from the purine biosynthetic pathway, similar to the pyrimidine ring of the cofactor thiamin pyrophosphate (Fig. 1B)

(2, 8–15). Here, we sought to identify the genes in *Eubacterium limosum* required for the biosynthesis of DMB.

Results

Identification of Putative DMB Biosynthesis Genes Adjacent to Cobalamin Riboswitches in *E. limosum*. We searched the published genome sequence of *E. limosum* strain KIST612 (16) for genes located downstream of the regulatory elements known as cobalamin riboswitches. These regulatory sequences are commonly found adjacent to genes involved in cobamide biosynthesis and transport, including *bluB* (3, 17). This analysis showed that the *E. limosum* genome contains 13 putative cobalamin riboswitches, of which 5 are associated with homologs of known cobalamin biosynthesis genes (Fig. 2A and *SI Appendix*, Fig. S1). One riboswitch sequence is located at the 5' end of a putative operon containing five genes of unknown function in addition to a homolog of *cobT*, which encodes the first enzyme necessary for the attachment of a lower ligand base to a cobamide precursor (Fig. 2A) (18). The first two ORFs in the operon are annotated as *thiC*, which encodes the enzyme 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) synthase, an oxygen-sensitive radical S-adenosylmethionine (SAM) enzyme that uses the purine intermediate 5-aminoimidazole ribotide (AIR) as a substrate for the biosynthesis of the pyrimidine ring of thiamin pyrophosphate (Figs. 1B and 2A) (8). The two *E. limosum thiC* homologs cluster in a phylogenetic clade together with other *thiC* homolog pairs found associated with *cobT* or other genes involved in B₁₂ biosynthesis or metabolism (Fig. 2B).

Significance

Vitamin B₁₂ is required by humans and a variety of other organisms for diverse metabolic processes, but is produced only by a subset of microorganisms. The anaerobic biosynthesis of the “lower ligand” of B₁₂, 5,6-dimethylbenzimidazole (DMB), is the only unknown component of the B₁₂ biosynthetic pathway. We report the identification of the *bzaABCDE* genes that are necessary and sufficient for the anaerobic biosynthesis of DMB. We have characterized the role of each of the *bza* genes and identified three intermediates in the pathway. This finding not only completes the B₁₂ biosynthesis pathway but also enables the sequence-based prediction of cobamides synthesized by anaerobic microorganisms.

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The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. [KT347435](#) (*E. limosum* ATCC 10825 *bza* operon) and [KT347436](#) (*M. thermoacetica* ATCC 39073 *bza* operon)].

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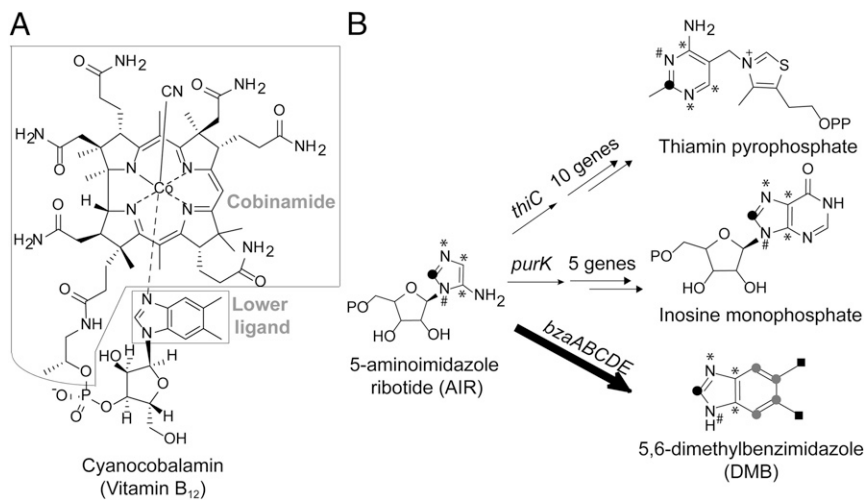


Fig. 1. Vitamin B₁₂ structure and the labeling pattern of DMB. (A) Structure of cyanocobalamin (vitamin B₁₂). Cbi and the lower ligand DMB are indicated. (B) AIR is a branch point in the biosynthesis of thiamin (8), purines (27), and benzimidazoles (as shown in this study). The symbols represent the origins of atoms in each product: ●, formyltetrahydrofolate; #, glutamine; *, glycine; shaded circle, erythrose 4-phosphate or threose; ■, methionine.

These gene products share 46 and 41% identity with each other and with ThiC, respectively (*SI Appendix*, Table S1). We focused our subsequent functional studies on this genomic locus. Based on the results described below, we have named these genes *bzaABCDE* (for benzimidazole biosynthesis, Fig. 2A).

Heterologous Expression of the *bzaABCDE* Genes in *Escherichia coli* Leads to DMB Biosynthesis. To examine the function of the *E. limosum bza* operon, we expressed the *bza* locus from *E. limosum* strain ATCC 10825 on a plasmid in *E. coli* and cultured the strain anaerobically. Although *E. coli* contains an incomplete cobamide biosynthesis pathway, it possesses the *cobUTSC* genes and therefore can form a cobamide from the advanced intermediate cobinamide (Cbi) (19, 20). In a mutant lacking the *metE* gene, *E. coli* relies on the cobamide-dependent methionine synthase encoded by *metH* (21). An *E. coli ΔmetE* strain containing the empty vector attached intracellularly purines to exogenously supplied Cbi to produce two purinyl cobamides, 2-methyladeninylcobamide (Factor A) and adeninylcobamide (pseudo-B₁₂ or pB₁₂), as revealed by HPLC analysis of corrinoide extracts (Fig. 3A). These purinyl cobamides were also detected when the *E. limosum cobT* gene was expressed in an *E. coli ΔmetE ΔcobT* mutant background, indicating that the *cobT* gene from the *bza* operon is functional (Fig. 3A). Notably, when the *E. limosum bza* operon was expressed in the *E. coli ΔmetE ΔcobT* strain, a cobamide was formed with a retention time and UV-visible (UV-Vis) spectrum identical to that of a standard of cyanocobalamin (vitamin B₁₂) (Fig. 3A and *SI Appendix*, Fig. S3). ¹H-NMR analysis of the purified cobamide confirmed its identity as cyanocobalamin (*SI Appendix*, Fig. S3). Furthermore, this cobamide could support the growth of *Salmonella enterica* provided with ethanolamine as the sole nitrogen source when grown anaerobically, a condition that has been shown to require exogenous B₁₂ (Fig. 3B) (1, 22–24).

To test whether the B₁₂ detected above was the result of de novo biosynthesis of DMB, *E. coli* expressing the *bza* operon was grown in a medium containing ¹⁵N-labeled ammonium chloride as the sole nitrogen source. A corrinoide extract of this culture was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM). A peak that coelutes with standard cyanocobalamin and corresponding to [¹⁵N₁, ¹⁵N₃-DMB]-B₁₂ was observed (top trace), whereas unlabeled B₁₂ was not detected (middle trace), confirming that DMB is synthesized de novo in this *E. coli* strain (Fig. 3C). Together, these results demonstrate that expression of the *E. limosum bza* operon is sufficient for anaerobic DMB biosynthesis in *E. coli*.

Functional Analysis of the Individual *bza* Genes and DMB Biosynthesis Intermediates. We next examined the functions of the individual *bza* genes by constructing a series of plasmids containing different subsets of the genes in the operon. These plasmids were introduced into the *E. coli ΔmetE ΔcobT* or the *E. coli ΔmetE* strain. LC-MS analysis of the corrinoide extracts from these strains showed that *bzaA* and *bzaB* were required together for the formation of the first

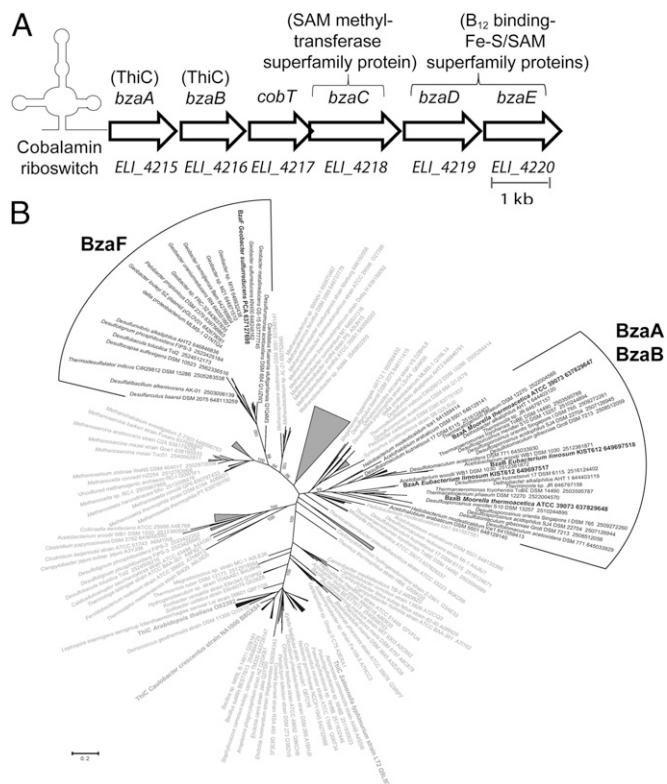


Fig. 2. Identification of the *E. limosum bza* genes. (A) *E. limosum bza* operon. Arrows represent ORFs. Gene locus tags as given by the National Center for Biotechnology Information (NCBI) database are shown below, and new gene names are shown above. Protein annotations in the NCBI database are shown in parentheses. (B) Phylogenetic tree of BzaA, BzaB, BzaF, and ThiC. BzaA, BzaB, and BzaF homologs are in black (BzaF is discussed below); ThiC homologs are in gray. Boldface indicates experimentally verified functions. An extended version of this figure is shown in *SI Appendix*, Fig. S2.

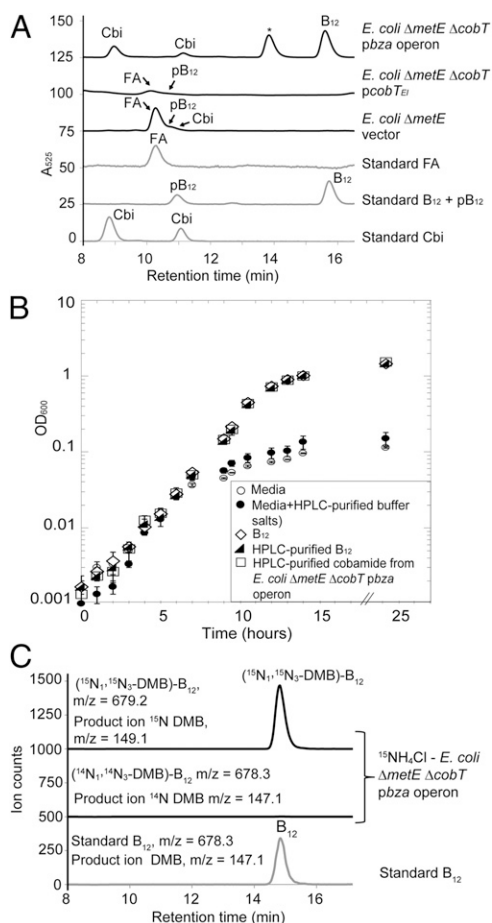


Fig. 3. Characterization of the *E. limosum* *bza* operon in *E. coli*. (A) HPLC chromatograms of corrinoid extracts of *E. coli* strains expressing *E. limosum* genes. Peaks confirmed by LC-MS/MS are labeled; an unidentified peak is marked with an asterisk (*). Standards are shown in gray. All cobamides shown are in their cyanated forms. (B) The *bza* operon product supports cobalamin-dependent growth in *S. enterica*. Growth curves of *S. enterica* cultured with ethanolamine as the sole nitrogen source are shown with added cobamide product of *E. coli* expressing the *E. limosum* *bza* operon (Upper chromatogram in A, 15.8 min) or the indicated controls. Error bars represent SD of triplicate samples. (C) The *bza* operon directs de novo DMB biosynthesis. LC-MS/MS with MRM analysis is shown for a corrinoid extract of the *E. coli* *metE cobT* strain expressing the *E. limosum* *bza* operon grown in media containing $^{15}\text{NH}_4\text{Cl}$.

intermediate, 5-hydroxybenzimidazole (5-OHBza), because strains expressing either gene alone were similar to a control strain containing the empty vector (Fig. 4 and *SI Appendix*, Fig. S4A). Expression of *bzaC*, which encodes a putative SAM-dependent methyltransferase, is required for methylation of this intermediate to form 5-methoxybenzimidazole (5-OMeBza) (Fig. 4 and *SI Appendix*, Fig. S4). The formation of the intermediate 5-methoxy-6-methylbenzimidazole (5-OMe-6-MeBza), and the final step, formation of DMB, additionally requires *bzaE* (Fig. 4). These results demonstrate that each of the *bza* genes is necessary for DMB biosynthesis. When these strains were cultured under aerobic conditions, benzimidazole formation was significantly reduced, suggesting that one or more oxygen-sensitive steps are involved in this pathway (*SI Appendix*, Fig. S4B). However, when *bzaC* was expressed alone in the presence of 5-OHBza, a small amount of 5-OMeBza was formed under both anaerobic and aerobic conditions, indicating that this step is likely not oxygen-sensitive (*SI Appendix*, Fig. S4B). Together, these results define the roles of each *bza* gene and the major intermediates in the pathway leading to the formation of DMB.

AIR Is the Precursor of 5-OHBza, the First Intermediate in DMB Biosynthesis. The results shown above demonstrate that 5-OHBza, the first benzimidazole intermediate, is formed from metabolites available in the *E. coli* cell. Given that (i) the imidazole ring of DMB shares a common metabolic origin with that of purines (2, 13), (ii) BzaA and BzaB are homologous to ThiC, and (iii) the ThiC enzyme uses the purine intermediate AIR as a substrate (8), we predicted that B₁₂ would not be produced in an *E. coli* strain lacking AIR (Fig. 1B). Indeed, expression of the *bza* genes in an *E. coli* $\Delta\text{metE } \Delta\text{cobT}$ strain lacking the AIR synthase encoded by the *purM* gene (25) did not lead to B₁₂ production, suggesting that AIR is a required precursor of DMB (Fig. 5A). In contrast, deletion of *purK*, which functions in the conversion of AIR to the subsequent intermediate in purine biosynthesis, *N*⁵-carboxyaminoimidazole ribonucleotide (NCAIR), had no effect on B₁₂ production (Fig. 5A) (26, 27). These results identify AIR as a precursor in the anaerobic B₁₂ biosynthesis pathway. Thus, AIR is a branch point in the biosynthesis of purines, thiamin, and DMB (Fig. 1B).

In addition to AIR, threose or erythrose was proposed to be a precursor for benzimidazole biosynthesis in *E. limosum* and *Clostridium barkeri* (Fig. 1B) (12, 14). To identify the complete set of substrates required for the biosynthesis of 5-OHBza, we purified a homolog of BzaA and BzaB from *Geobacter sulfurreducens* and assayed its activity in vitro. In *G. sulfurreducens* and several other bacteria, a single *thiC* homolog (hereafter called *bzaF*) is located adjacent to cobamide biosynthesis genes (Fig. 6) and is found in a phylogenetic clade distinct from ThiC, BzaA, and BzaB (Fig. 2B). We hypothesized that this gene would also function in 5-OHBza biosynthesis. We found that, like ThiC, purified BzaF protein contains an iron-sulfur (Fe-S) cluster and uses SAM as a cofactor. Unexpectedly, when only SAM and AIR were provided to BzaF, a product with a retention time, mass, and UV-Vis

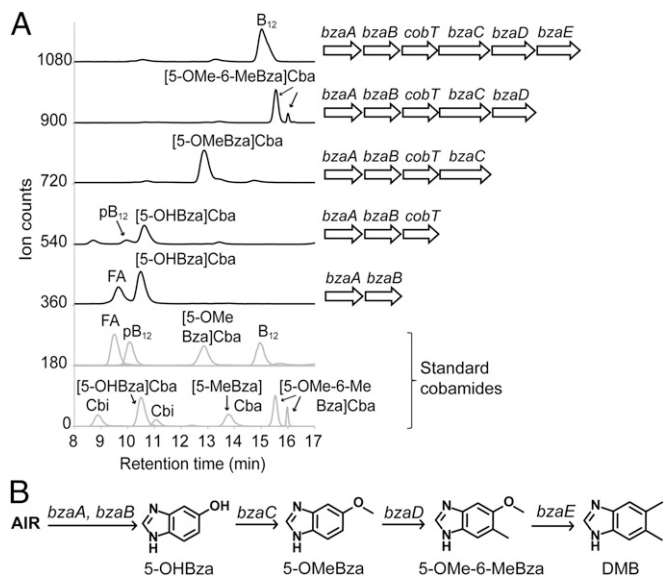


Fig. 4. Analysis of the functions of the individual *E. limosum* *bza* genes. (A) LC-MS/MS analysis of corrinoid extracts. *E. coli* strains expressing different subsets of the genes in the *bza* operon, as indicated on the Right, were grown as in Fig. 3A. The *bzaA-bzaB* plasmid was expressed in an *E. coli* ΔmetE background and the remaining constructs in a $\Delta\text{metE } \Delta\text{cobT}$ background. Standard cobamides are shown in the Lower two traces. (B) Model for the roles of the *bza* genes in DMB biosynthesis. The first intermediate, 5-OHBza, is synthesized from AIR (as shown below) by the *bzaA* and *bzaB* gene products. The *bzaC* gene product methylates 5-OHBza to form 5-OMeBza. Further methylation by the *bzaD* gene product converts 5-OMeBza to 5-OMe-6-MeBza, and *bzaE* acts next in the conversion of 5-OMe-6-MeBza to DMB.

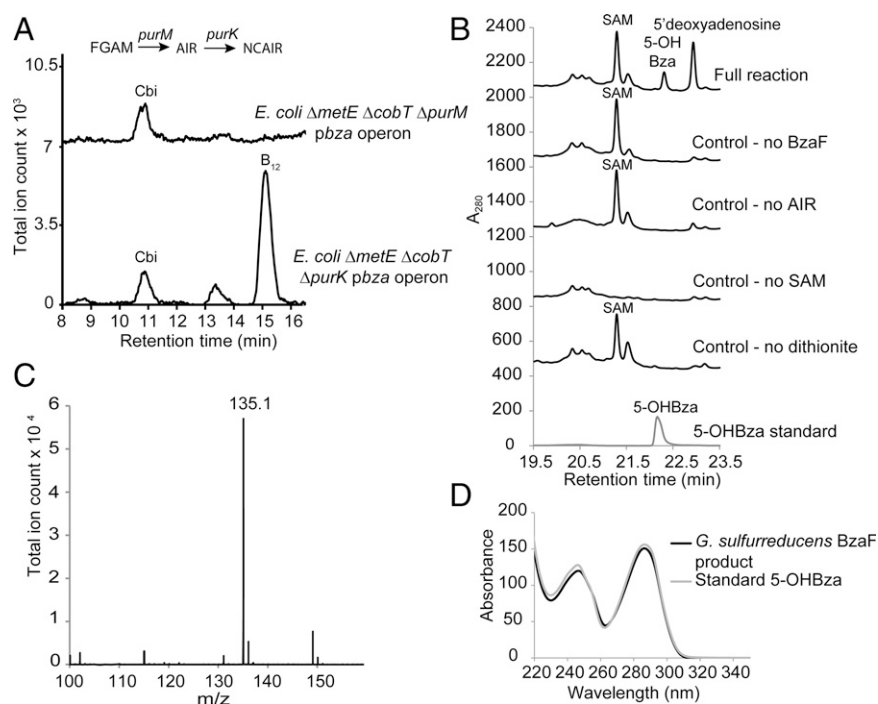


Fig. 5. AIR is the direct precursor of 5-OHBza. (A) LC-MS/MS analysis of corrinoid extracts of purine biosynthesis mutants. The indicated *E. coli* strains were grown with Cbi and ¹⁵NH₄Cl, and corrinoid extracts were analyzed for the presence of [¹⁵N₁, ¹⁵N₃-DMB]-B₁₂ (doubly charged *m/z* of 679.2, product ion DMB *m/z* 149.1). (B) In vitro analysis of 5-OHBza biosynthesis. HPLC analysis of the in vitro reconstitution reaction of *G. sulfurreducens* BzaF with AIR, SAM, and dithionite shows the formation of a product that coelutes with a standard of 5-OHBza. Control reactions with one component omitted are also shown. (C) The extracted ion chromatogram of 5-OHBza (*m/z* 135.1) from the LC-MS analysis of the in vitro reconstitution reaction of *G. sulfurreducens* BzaF with AIR, SAM, and dithionite. (D) UV-Vis spectroscopy of the BzaF reaction product. The HPLC-purified *G. sulfurreducens* BzaF reaction product is overlaid with that of a standard sample of 5-OHBza.

spectrum identical to that of an authentic standard of 5-OHBza was observed (Fig. 5 B–D). A detailed mechanistic analysis of another 5-OHBza synthase (HBI synthase), a homolog of BzaF from *Desulfuromonas acetoxidans*, reveals interesting mechanistic parallels with ThiC (28). Thus, similar to thiamin pyrimidine biosynthesis, AIR is the sole substrate required for synthesizing 5-OHBza.

bza Genes Are Present in Diverse Bacteria and Archaea and Can Be Used to Predict Cobamide Structure. Four other substituted benzimidazoles in addition to DMB have been reported as naturally occurring cobamide lower ligands in cultured anaerobic bacteria and archaea (SI Appendix, Fig. S5) (2). Our observation that three of these benzimidazoles are intermediates in the DMB biosynthetic pathway in *E. limosum* prompted us to examine whether homologs of the *bza* genes might be present in the genomes of other organisms that synthesize benzimidazolyl cobamides. Indeed, we found that the genomes of more than 25 bacteria and archaea contained putative *bza* operons (Fig. 6 and SI Appendix, Fig. S6). These organisms are phylogenetically diverse and are found in a variety of different environments. In the case of *Acetobacterium woodii*, which is reported to produce B₁₂, homologs of all of the *bza* genes are present and are arranged similarly to the *E. limosum* *bza* operon, suggesting that these genes also function in DMB production (Fig. 6) (29). Several other genomes were found in which a subset of the *bza* genes was present. In each of the organisms previously shown to produce a benzimidazolyl cobamide, the set of *bza* genes present in the genome was predictive of the structure of the cobamide produced (SI Appendix, Fig. S6). We further tested the ability of genome analysis to predict cobamide production by analyzing the cobamides synthesized by *G. sulfurreducens*. This bacterium has been shown to produce a cobamide, but the structure has not been determined (30). Because the genome of *G. sulfurreducens* contains *bzaF* but no other *bza* gene homologs, we expected it to produce [5-OHBza]Cba. Indeed, LC-MS analysis of a corrinoid extract from a *G. sulfurreducens* culture showed the production of [5-OHBza]Cba as the major corrinoid species (SI Appendix, Fig. S7). In addition, [5-OMeBza]Cba, the cobamide previously

reported to be produced by *Moorella thermoacetica*, was formed in an *E. coli* strain expressing the *M. thermoacetica* *bza* operon, which contains homologs of *bzaA*, *bzaB*, *cobT*, and *bzaC* (SI Appendix, Fig. S8) (31). Thus, we have identified not only the complete set of genes required for DMB biosynthesis in *E. limosum* and other B₁₂-producing anaerobes, but also those required for the biosynthesis of other cobamide-associated benzimidazoles.

Discussion

Although the B₁₂ biosynthetic pathway has been studied for several decades, resulting in the identification of over 40 genes involved in the anaerobic and/or aerobic biosynthesis pathways, the genes required for the anaerobic biosynthesis of the lower ligand remained elusive until now. With the present discovery, we have uncovered the last unknown component of the biosynthetic pathway for B₁₂.

Despite the lack of phylogenetic or enzymatic commonalities between the anaerobic and aerobic routes for DMB production, some parallels exist between the two pathways. First, both the *bza* genes and *bluB* are commonly found downstream of cobalamin riboswitches and clustered with B₁₂ biosynthesis genes in many bacterial genomes (3, 17) (SI Appendix, Fig. S6), suggesting a common mode of regulation of lower ligand biosynthesis and attachment. Second, both BzaA/B/F and BluB use the same substrate and cofactors as closely related enzymes—ThiC and nitroreductases, respectively—yet carry out a novel reaction (4, 8). It is interesting to note that, unlike the other components of the cobamide biosynthetic pathway, DMB biosynthesis has apparently evolved independently twice, underscoring the importance of DMB as a lower ligand. It remains unclear why some organisms synthesize benzimidazoles exclusively for use as cobamide lower ligands, given that lower ligands of the purine and phenolic classes are derived from other metabolic pathways.

The discovery of the anaerobic *bza* genes and analysis of their individual roles sheds light on several previously unresolved aspects of anaerobic benzimidazole biosynthesis. First, prior feeding studies with labeled substrates in *E. limosum* suggested that this pathway branches from the purine biosynthetic pathway; the present study pinpoints AIR as the direct precursor for 5-OHBza.

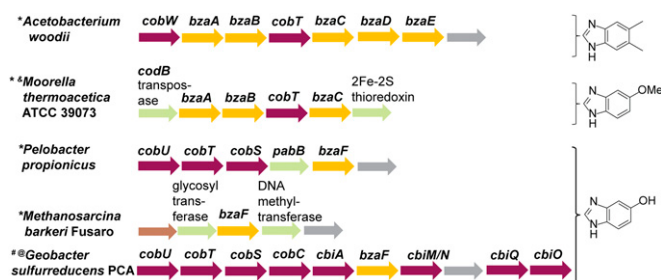


Fig. 6. The *bza* genes present in an organism are predictive of the cobamide that it produces. Genetic loci of organisms identified from the JGI database that contain *bza* genes adjacent to cobamide biosynthesis or metabolism genes are shown (an extended list is presented in *SI Appendix, Fig. S6*). The benzimidazole lower ligand that these organisms are predicted to synthesize is shown on the *Right*. The predictions verified in previous studies are labeled with an asterisk (*). Those verified by analysis of corrinoids (#), biochemical activity (@), or heterologous expression (&) are indicated.

Surprisingly, four carbon atoms from the ribose moiety of AIR are incorporated into the benzene ring component of DMB. Prior labeling studies implicated erythrose and threose as precursors of these carbon atoms, suggesting that the ribose moiety of AIR can be formed from these four-carbon sugars in *E. limosum* (12, 14). Second, our work agrees with feeding studies with ^{13}C -labeled benzimidazoles that implicated 5-OHBza as the precursor of all other benzimidazoles in *E. limosum* and *M. thermoacetica* (13, 32). However, we also show that 5-OMeBza and 5-OMe-6-MeBza, which were previously suggested to be side products of the pathway, are intermediates in DMB biosynthesis (13). A third insight from those studies was that DMB, although it is a symmetric molecule, is attached to the nucleotide loop exclusively via the nitrogen atom derived from glutamine (Fig. 1B) (10–12, 14, 15). This could mean either that the bond of this nitrogen to the ribose ring was formed on an asymmetric precursor or that a close association between enzymes in the pathway prevents the release of free DMB before phosphoribosylation by CobT. Because of the proximity of the *cobT* and *bza* genes in numerous genomes (*SI Appendix, Fig. S6*), we speculate that the phosphoribosylation step could be coupled to the biosynthesis of DMB or one of its precursors. Curiously, both BzaD and BzaE have putative B₁₂-binding domains, suggesting that the final two methylation steps could occur on a cobamide rather than a free base. A detailed mechanistic analysis of each of these enzymes will be required to investigate this further.

Because DMB and other substituted benzimidazoles share a common biosynthetic pathway, our findings enable the improved prediction of cobamide biosynthesis based on analysis of genome sequence. This is especially useful considering the increasing abundance of microbial genome and metagenome sequence data, coupled with persistent challenges associated with studying most microbes in pure culture, as well as the growing recognition of the role of microbes in human health and the environment. Given the importance of cobamides in environmental, industrial, and human-associated microbes, the identification of this pathway may lead to an enhanced ability to understand and manipulate microbial metabolism.

Materials and Methods

Materials. Cyanocobalamin (vitamin B₁₂), dicyanocobinamide (Cbi), DMB, and 5-OMeBza were purchased from Sigma-Aldrich. ^{15}N -ammonium chloride was purchased from Cambridge Isotopes Laboratories. 5-OHBza was synthesized as previously described (33). [5-OHBza]Cba, [5-OMeBza]Cba, [5-OMe-6-MeBza]Cba, pseudo-B₁₂, and Factor A were purified as described (34).

Bioinformatic Analysis.

Identification of cobalamin riboswitch-regulated operons: Nucleotide sequences of genomes of interest were searched to identify cobalamin riboswitches using Infernal v1.1 (35). A covariance model was built using the RF00174 seed alignment downloaded from Rfam (36) and Infernal's cmbuild function. After calibrating the covariance model with the cmcalibrate function, genomes were searched with the cmsearch function. Positive hits were those satisfying the default Infernal inclusion threshold of an *E*-value of 0.01 or less. The chromosomal locations of the putative cobalamin riboswitches were visualized and analyzed using the Joint Genome Institute's Integrated Microbial Genomes (IMG) web service (37).

Identification of *thiC* homologs: All archaeal and bacterial genomes of "Finished" status in IMG's system as of January 2, 2015, were searched. The function "pfam01964" was used to carry out a "Function Profile" against all finished genomes. Genomes with multiple *thiC* homologs were prioritized for examination of genomic context of *thiC* homologs. *thiC* homologs were screened to identify those that were contained within the same operon as cobalamin biosynthesis or transport genes.

Phylogenetic analysis of *thiC* homologs: The sequences of all *thiC* homologs from genomes that had multiple *thiC* homologs were downloaded from IMG and aligned to Pfam's PF01946 seed alignment using the "seed" option in Multiple Alignment using Fast Fourier Transform (MAFFT) v7.2 (38). The multiple sequence alignment was edited in Geneious v5.5 (39) to remove ambiguously aligned amino acids and large end gaps. After this editing step, sequences whose amino acid count was less than 60% of the length of the entire multiple sequence alignment were removed from the analysis. Seed sequences that were duplicates of sequences downloaded from IMG were also removed before phylogenetic analysis. The alignment was uploaded to the Cyberinfrastructure for Phylogenetic Research (CIPRES) web portal, and a maximum-likelihood (ML) tree was reconstructed using RAxML v8.1 (40). The amino acid substitution matrix used was the JTT matrix (41), with the Γ model of rate heterogeneity. RAxML's rapid bootstrap was performed with 1,000 replicates, and the best-scoring ML tree was saved.

Genetic and Molecular Techniques. *E. coli* strains $\Delta\text{cobT}::\text{Kan}^{\text{R}}$ (JW1969-1), $\Delta\text{metE}::\text{Kan}^{\text{R}}$ (JW3805-1), $\Delta\text{purM}::\text{Kan}^{\text{R}}$ (JW2484-1), and $\Delta\text{purK}::\text{Kan}^{\text{R}}$ (JW0511-1) were obtained from the Keio collection (42). The mutations were introduced into *E. coli* strain MG1655 by P1 transduction (43). For construction of double and triple mutants, the Kan^R cassette was removed by recombination of flanking FRT sites as described (44). Double and triple mutants were constructed by P1 transduction of the Kan^R cassette into deletion strains.

The *E. limosum* and *M. thermoacetica* *bza* operons were cloned into plasmid pTH1227 (45) by Gibson assembly (46) following PCR on genomic DNA with primers P604F/P619R (*E. limosum*) and P615F/P616R (*M. thermoacetica*). Additional constructs were created similarly with the primers in *SI Appendix, Table S2*. Strains and plasmids are in *SI Appendix, Table S3*.

The nucleotide sequence of *G. sulfurreducens* *bzaF* was synthesized and cloned into plasmid THT, a pET28 vector with a tobacco etch virus protease cleavage site for removing the His-tag.

Media and Culture Conditions. *E. coli* strains containing pTH1227 or its derivatives were grown at 37 °C under anaerobic conditions with 95–98% N₂ and 5–2% H₂ as headspace gas for 72–144 h in M9 glucose minimal medium supplemented with 100 μM isopropyl β -D-1-thiogalactopyranoside, 50 μM freshly prepared FeSO₄, 10 nM Cbi, and 25 mg/L tetracycline, unless otherwise indicated. Strains containing mutations in *purK* or *purM* were grown with 50 nM Cbi, 20 μM inosine, and 20 μM thiamin (unexpectedly, both strains could grow in the absence of added thiamin, although *purM* strains are expected to require thiamin). When indicated, $^{15}\text{NH}_4\text{Cl}$ was substituted for unlabeled NH₄Cl. For growth of *E. coli* under aerobic conditions, cultures were aerated at 250 rpm for 36–96 h. For corrinoid extractions, the strains were grown in 2-L cultures.

S. enterica serovar Typhimurium strain LT2, *G. sulfurreducens* PCA, *E. limosum* ATCC 10825, and *M. thermoacetica* ATCC 39073 were cultivated as described in *SI Appendix, SI Methods*.

Corrinoid Extraction and HPLC Analysis. Corrinoids were extracted from cell pellets, purified, and analyzed as described in *SI Appendix, SI Methods*.

Chemical Analysis. The UV-Vis spectra were collected during HPLC analysis using Agilent ChemStation software. Samples were further characterized on an Agilent 6410 liquid chromatograph-triple quadrupole mass spectrometer using an Agilent Zorbax SB-Aq column to identify cobamides based on their characteristic transitions and retention times (34). For corrinoid extracts from strains grown in $^{15}\text{NH}_4\text{Cl}$, the MS method was modified to detect the

following precursor and product ion transitions, respectively: 679.3, 149.1 (B₁₂); 673.3, 137.1 ([5-OHBza]Cba); 680.3, 151.1 ([5-OMeBza]Cba); 687.3, 165.1 ([5-OMe-6-MeBza]Cba); 675.3, 141.1 (pseudo-B₁₂); 682.3, 155.1 (Factor A). For NMR analysis, cobamides were purified by HPLC using an Agilent 1200 series fraction collector. A standard sample of cyanocobalamin and a blank buffer sample were also collected under the same HPLC purification conditions. These samples were analyzed using a Bruker Biospin Avance II 900 MHz NMR spectrometer equipped with a TXI cryoprobe accessory at the California Institute for Quantitative Biosciences Berkeley core facility.

Biochemical Characterization of *G. sulfurreducens* BzaF. His-tagged *G. sulfurreducens* PCA BzaF was expressed and purified as described in *SI Appendix, SI Methods*.

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