

## Supporting Information Appendix

### Methods:

**Cultivating *Geobacter sulfurreducens* PCA:** *G. sulfurreducens* was cultivated anaerobically with fumarate as the electron acceptor and acetate as the electron donor in the absence of added vitamins as previously described (1). Briefly, the medium contained basal salts, 40 mM fumarate and 20 mM acetate. The medium was flushed with 20% CO<sub>2</sub>:80% N<sub>2</sub>, inoculated inside an anaerobic chamber, and cultures were incubated at 30 °C for 72 h.

**Cultivating *Eubacterium limosum* ATCC 10825:** *E. limosum* ATCC 10825 was grown in modified Actinomyces broth composed of 17 g tryptone, 10 g yeast extract, 5 g dextrose, 5 g NaCl, 13 g K<sub>2</sub>HPO<sub>4</sub>, 2 g KH<sub>2</sub>PO<sub>4</sub>, 1 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g starch, 0.2 g MgSO<sub>4</sub> x 7 H<sub>2</sub>O, and 10 mg CaCl<sub>2</sub> x 2 H<sub>2</sub>O per liter. The medium was additionally supplemented with 1 ml of trace elements solution (SL-10) (2), 1 ml of Se/WO solution (4 mg Na<sub>2</sub>SeO<sub>3</sub> x 5 H<sub>2</sub>O, 8 mg Na<sub>2</sub>WO<sub>4</sub> x 2 H<sub>2</sub>O, and 0.5 g NaOH per liter), and 1 ml of Wolin's vitamin solution (with vitamin B<sub>12</sub> omitted) per liter (3). The medium was prepared under an atmosphere of N<sub>2</sub> gas and reduced with a 0.01% (w/v) cysteine-sulfide solution.

**Cultivating *Moorella thermoacetica* ATCC 39073:** *M. thermoacetica* ATCC 39073 was grown in reinforced Clostridial medium supplemented with Wolin's vitamin solution (with vitamin B<sub>12</sub> omitted) (3, 4).

### **Growth curve experiments with *Salmonella enterica* serovar Typhimurium strain LT2:**

Growth curves of *S. enterica* serovar Typhimurium strain LT2 were performed by inoculating triplicate 5 ml cultures of LB medium with single colonies and growing to saturation at 37 °C.

Cells were harvested by centrifugation at 12,800 x g and washed three times with a minimal medium containing glycerol as the carbon source and ethanolamine as the nitrogen source (5). Each cell suspension was diluted to an optical density at 600 nm (O.D.<sub>600</sub>) of 0.001 in 5 mL of the same medium containing the indicated supplements. The cultures were grown at 30 °C with aeration, and the O.D.<sub>600</sub> was measured at the indicated time points. Supplements included 10 nM cobamide product purified by HPLC from *E. coli*, HPLC-purified buffer salts, 10 nM HPLC-purified cyanocobalamin, or 10 nM cyanocobalamin.

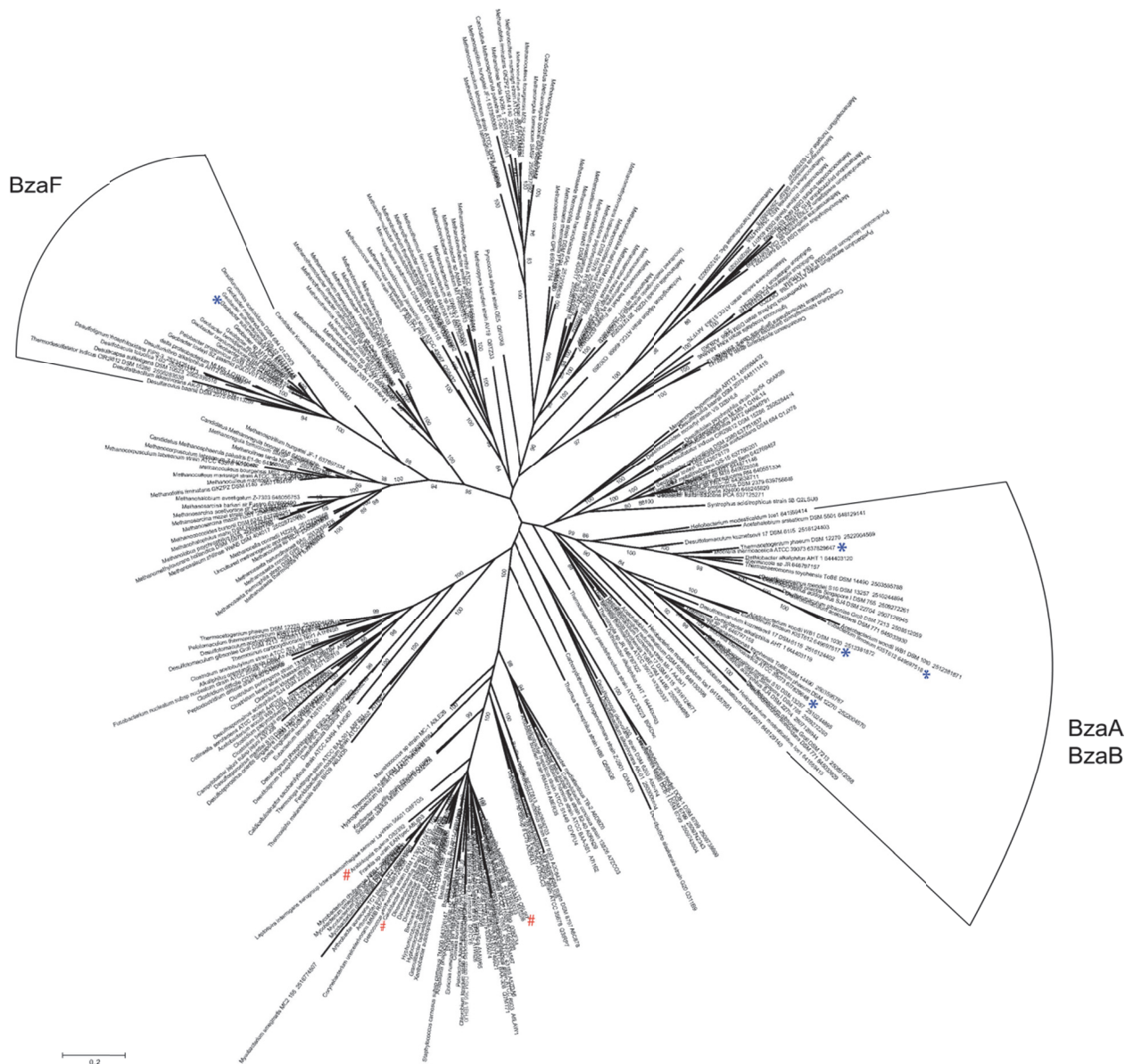
**Corrinoid extraction and HPLC analysis:** Corrinoids were extracted with methanol from cell pellets, then cyanated and desalted as described (6). An Agilent 1200 series HPLC system equipped with a UV-diode array detector was used to analyze the extracted corrinoids. An Agilent Zorbax SB-Aq reverse phase column (5 µm, 4.6 x 150 mm) was used at a flow rate of 1 ml min<sup>-1</sup> at 30 °C. Chromatograms were recorded at 365, 525, and 550 nm. Mobile phases used were 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B). Samples were analyzed by the following method: 25% solvent B over 2 min, followed by a linear gradient of 25 to 34% solvent B over 11 min, 34 to 100% solvent B over 3 min, and 100 to 25% solvent B over 0.5 min.

**Biochemical characterization of *G. sulfurreducens* BzaF:** His-tagged *G. sulfurreducens* PCA BzaF was expressed in *E. coli* BL21(DE3) containing a plasmid encoding the *suf* operon for *in vivo* [4Fe-4S] reconstitution (7). A 15 ml culture of this strain was grown overnight in LB medium containing kanamycin (40 mg/L) and chloramphenicol (25 mg/L). 1.9 L of M9 minimal media containing 0.7% glucose, 40 mg/L kanamycin and 25 mg/L chloramphenicol was

inoculated with this culture. The culture was incubated at 37 °C with shaking (180 rpm) until the O.D.<sub>600</sub> reached 0.50 to 0.55. The culture was then incubated at 4 °C without shaking for 2 h. Subsequently, 50 mg of ferrous ammonium sulfate and 50 mg of cysteine were added, followed by induction of the culture with 70 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was incubated at 15 °C with shaking (50 rpm) for 18-20 h. The cultures were then incubated at 4 °C for 3 h without shaking. The cells were harvested and stored in liquid nitrogen overnight before enzyme purification. All subsequent steps were performed in an anaerobic chamber. The cell pellets were thawed at room temperature and suspended in lysis buffer (100 mM Tris-HCl, pH 7.5) in the presence of 2 mM DTT, lysozyme (0.2 mg/ml) and benzonase (100 units). This mixture was then cooled in an ice bath for 2 h. The cell suspension was sonicated and centrifuged to obtain the cell-free extract. The enzyme was purified using standard Ni-NTA chromatography. The column was first equilibrated with the lysis buffer. The cell-free extract was then passed over the column, followed by 8-9 column volumes of wash buffer (100 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 2 mM DTT, pH 7.5). The enzyme was eluted with 100 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, 2 mM DTT, pH 7.5. The purified enzyme was buffer exchanged into 100 mM potassium phosphate, 30% glycerol, 2 mM DTT, pH 7.5 using an Econo-Pac 10DG desalting column (Bio-Rad) and the purified enzyme was stored in liquid nitrogen. The purified protein contains a [4Fe-4S] cluster as verified by UV-Vis spectroscopy (8).

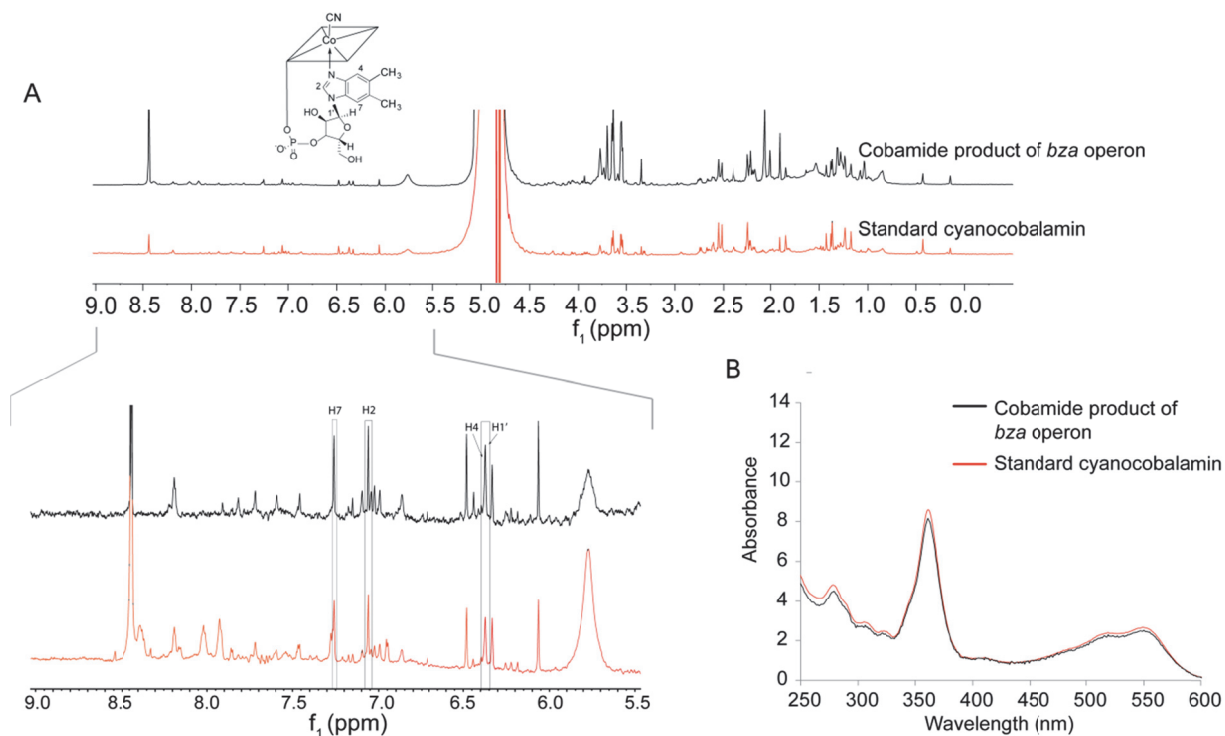


**Figure S1. *E. limosum* genes adjacent to cobalamin riboswitches.** Additional cobalamin riboswitches in the *E. limosum* KIST612 genome. Most of the cobalamin riboswitches are associated with genes involved in cobalamin biosynthesis, transport or regulation, as indicated by the color scheme. Arrows indicate open reading frames (ORFs); ORF numbers are shown below and gene names are above, as annotated in the Joint Genomic Institute (JGI) database. Riboswitch #7 is located in an orientation not predicted to regulate the expression of any ORFs.



**Figure S2. An expanded view of the phylogenetic tree shown in Fig. 2B.** The tree was constructed from genes annotated as *thiC* in the entire Pfam seed alignment (113 sequences) and all genes annotated as *thiC* from genomes that had multiple *thiC* hits (200 sequences). The putative BzaA/BzaB, BzaF and ThiC translated gene sequences appear in distinct clades. Experimentally verified functions for BzaA,

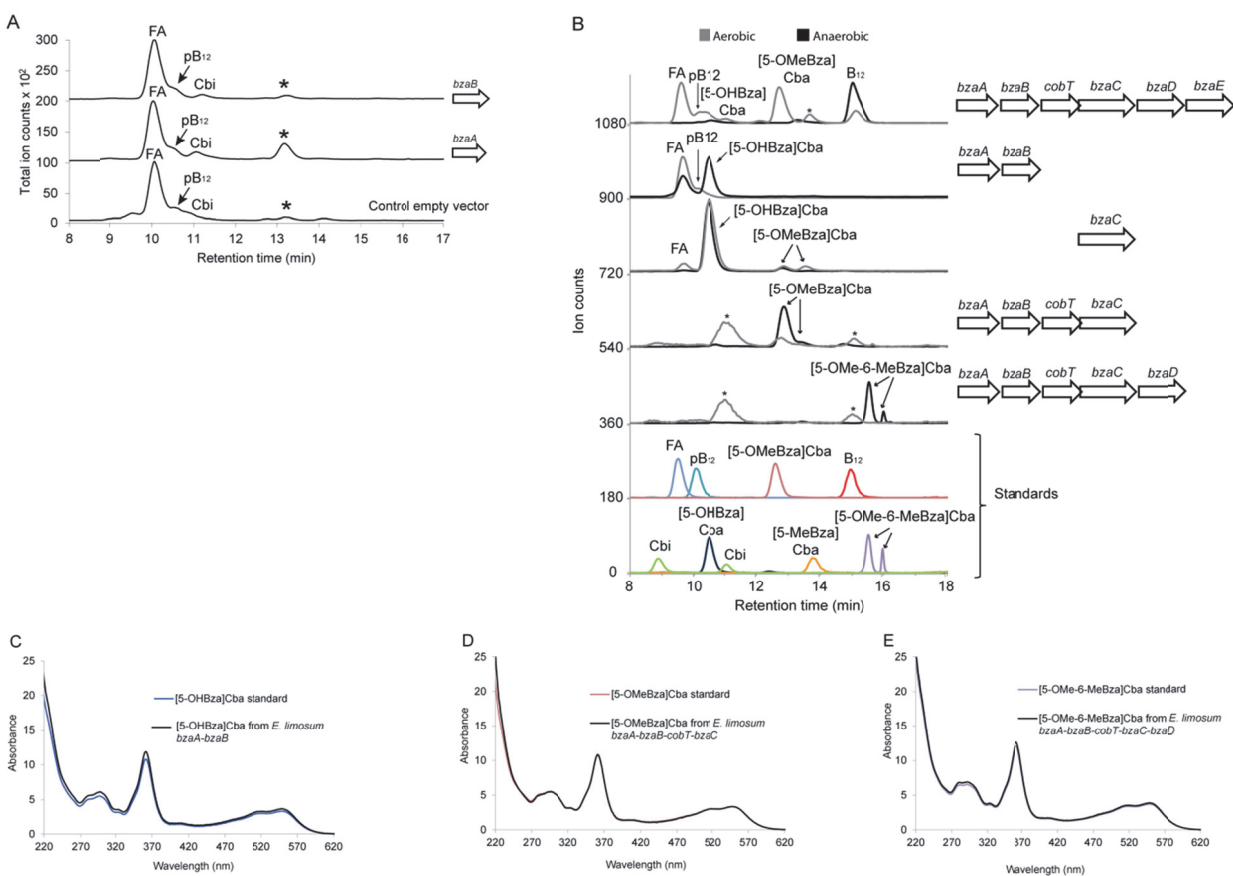
BzaB and BzaF homologs and for ThiC homologs are indicated by \* and #, respectively (corresponding to boldface in Fig. 2B).



**Figure S3. Chemical analysis of the cobamide produced by *E. coli* expressing the *bza* operon.**

A. 1-D <sup>1</sup>H NMR analysis of the HPLC-purified cobamide product of the *bza* operon (black) compared to a 1-D <sup>1</sup>H NMR of standard cyanocobalamin purified under the same HPLC conditions (red). The proton peaks of the lower ligand DMB are shown in the expanded view.

B. UV-Vis spectrum of the cobamide product of the *bza* operon (black) is identical to that of standard cyanocobalamin (red).



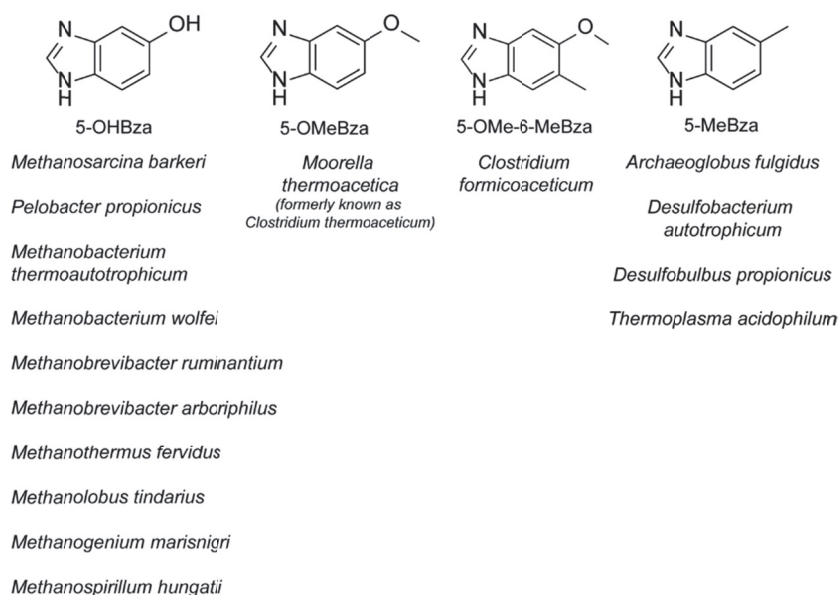
**Figure S4. Expression of *E. limosum* *bza* genes under aerobic and anaerobic growth conditions**

A. LC-MS analysis of the corrinooids extracted from *E. coli*  $\Delta metE$  strains containing the empty vector or expressing the individual *bzaA* and *bzaB* genes. No benzimidazolyl cobamides are detectable in these corrinooid extracts. The asterisk (\*) indicates an unknown corrinooid with the same mass as FA but with a different retention time.

B. Oxygen sensitivity of the *E. limosum* DMB biosynthesis pathway. *E. coli* strains expressing the indicated genes from the *bza* operon were cultured anaerobically (black traces) or aerobically (gray traces) in minimal media containing Cbi. LC-MS analysis of corrinooid extracts from these cultures is shown. In the strain containing the complete *bza* operon, the aerobic culture contained the benzimidazolyl cobamides [5-OHBza]Cba, [5-OMeBza]Cba and B<sub>12</sub> at low levels, whereas the anaerobic culture contained predominantly B<sub>12</sub>, indicating that some steps in the pathway are sensitive to oxygen. The first step in the pathway is oxygen-sensitive, as evidenced by the absence of [5-OHBza]Cba in the aerobically grown culture containing the *bzaA-bzaB* construct. The strain expressing only *bzaC* was supplemented with 5-OHBza; in the extract from this culture, [5-OMeBza]Cba was produced from 5-OMeBza synthesized by the cells under both anaerobic and aerobic conditions, indicating that 5-OMeBza biosynthesis can be performed in the presence of oxygen (in this culture, [5-OHBza]Cba was also formed from the added 5-OHBza). A small amount of [5-OMeBza]Cba was formed in the aerobically grown culture containing the *bzaA-bzaB-cobT-bzaC* construct, suggesting that the prior intermediate, 5-OHBza, could be formed at low levels. The culture containing the *bzaA-bzaB-cobT-bzaC-bzaD* construct produced [5-OMe-6-MeBza]Cba only under anaerobic conditions. An *E. coli*  $\Delta metE$  background was used for the

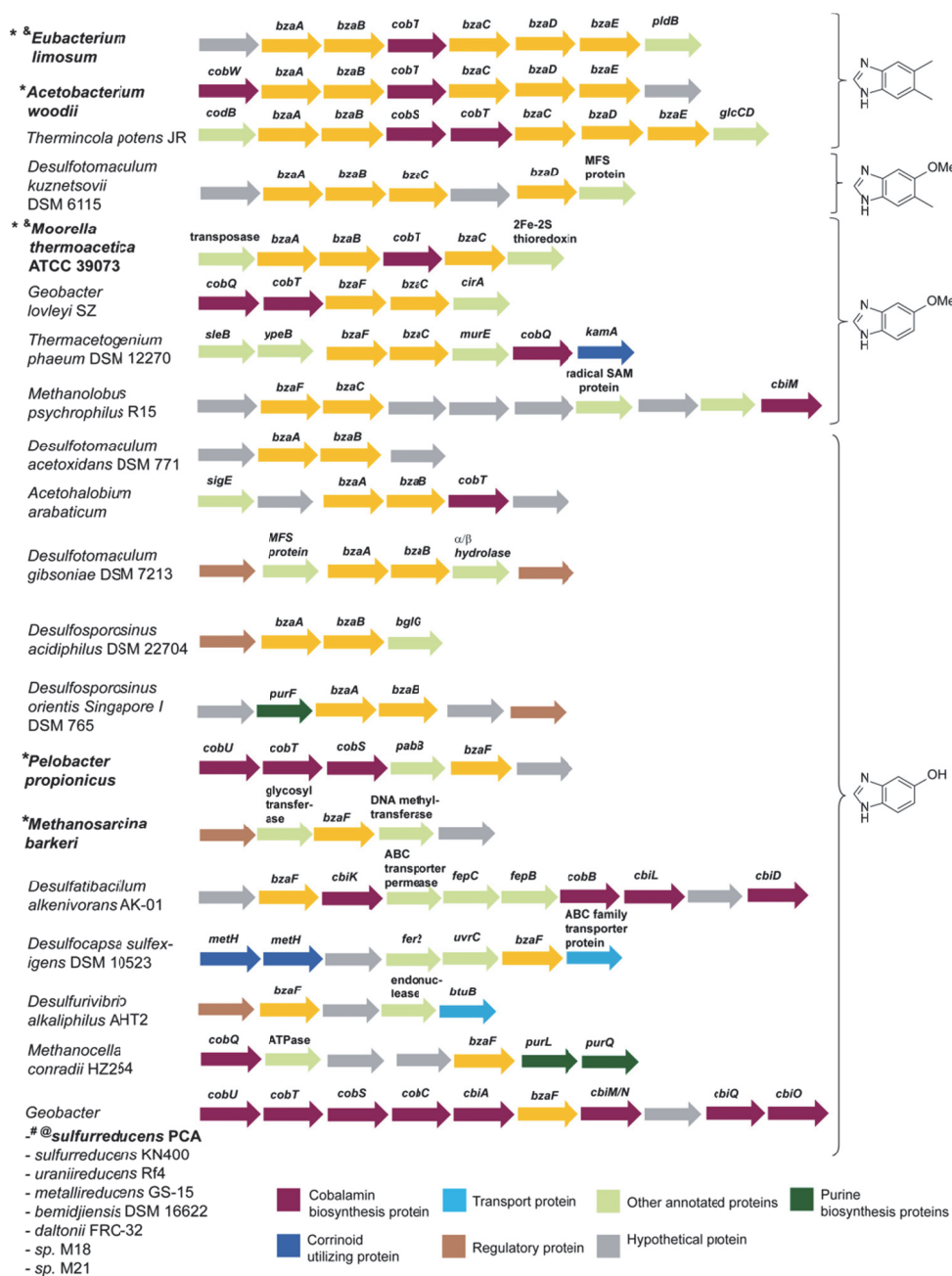
plasmids containing *bzaA-bzaB* and *bzaC*, whereas the remaining constructs were in a  $\Delta metE \Delta cobT$  background. The peaks labeled with an asterisk (\*) are unknown corrinoids.

C, D, E. The UV-Vis spectra of [5-OHBza]Cba, [5-OMeBza]Cba and [5-OMe-6-MeBza]Cba respectively, produced by *E. coli* expressing *bza* gene combinations, are indistinguishable from those of the spectra of the corresponding standards.



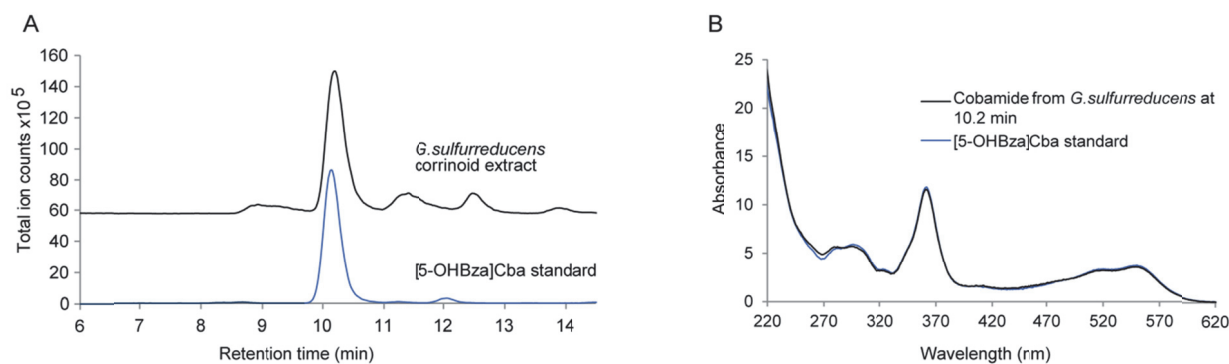
**Figure S5. Benzimidazole lower ligands in anaerobic bacteria and archaea.** The structures and abbreviations of the lower ligands and the names of the organisms reported to produce them are shown (9-13).





**Figure S6. Expanded list of the putative *bza* genes in bacteria and archaea.**

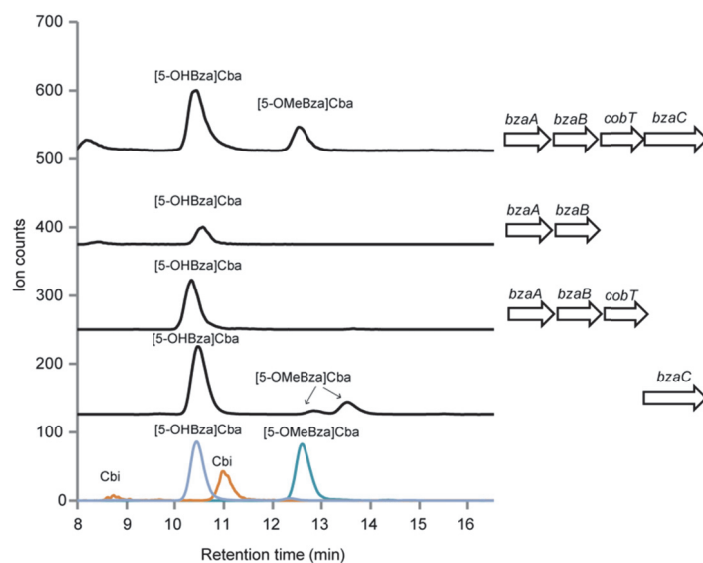
Genetic loci containing predicted *bza* genes adjacent to cobamide biosynthesis or metabolism genes were identified in the JGI database. Based on the subset of *bza* genes present, we have predicted the benzimidazole lower ligand that these organisms synthesize. The predictions are validated in the organisms for which experimental data for cobamide production are available (bold face) from this (#) or previous studies (\*), or by heterologous expression studies performed on the *bza* genes (&) or biochemical characterization of the enzymes (@) in this study.



**Figure S7. *Geobacter sulfurreducens* produces [5-OHBza]Cba.**

A: LC-MS/MS analysis of corrinoids extracted from *G. sulfurreducens*. The major peak co-elutes with a standard of [5-OHBza]Cba.

B: The UV-Vis spectrum of the major cobamide produced by *G. sulfurreducens* (10.2 min in panel A, black) is overlaid with that of a standard of [5-OHBza]Cba (blue).



**Figure S8. Expression of the *Moorella thermoacetica* *bza* operon in *E. coli* directs 5-OMeBza biosynthesis.**

LC-MS/MS analysis of corrinoid extracts from anaerobically grown *E. coli* expressing homologs of the *bza* genes from *M. thermoacetica* is shown. The complete operon, *bzaA-bzaB-cobT-bzaC*, directs the formation of [5-OMeBza]Cba, the previously reported cobamide of *M. thermoacetica*. [5-OHBza]Cba production in this strain is likely due to the attachment of the intermediate 5-OHBza to added Cbi. The strain expressing only *bzaC* was cultured with 5-OHBza. The plasmids containing only *bzaA-bzaB* or *bzaC* were expressed in a  $\Delta metE$  background, whereas the remaining constructs were expressed in a  $\Delta metE \Delta cobT$  background.

## Supplemental Tables

**Table S1. Percent identity matrix table of BzaA, BzaB, BzaF and ThiC proteins.** The BzaA, BzaB, BzaF and ThiC sequences are from the organisms *E. limosum* (*E. l.*), *A. woodii* (*A. w.*), *M. thermoacetica* (*M. t.*), *G. sulfurreducens* (*G. s.*), *P. propionicus* (*P. p.*) and *M. barkeri* (*M. b.*). The data were generated using the pairwise alignment tool in BioEdit.

|                   | <i>E. l.</i><br>BzaA | <i>E. l.</i><br>BzaB | <i>E. l.</i><br>ThiC | <i>A. w.</i><br>BzaA | <i>A. w.</i><br>BzaB | <i>A. w.</i><br>ThiC | <i>M. t.</i><br>BzaA | <i>M. t.</i><br>BzaB | <i>M. t.</i><br>ThiC | <i>G. s.</i><br>BzaF | <i>G. s.</i><br>ThiC | <i>P.p.</i><br>BzaF | <i>P.p.</i><br>ThiC | <i>M. b.</i><br>BzaF | <i>M. b.</i><br>ThiC |
|-------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|---------------------|---------------------|----------------------|----------------------|
| <i>E. l.</i> BzaA | 100                  | 46                   | 41                   | 75                   | 44                   | 40                   | 61                   | 48                   | 50                   | 43                   | 47                   | 42                  | 48                  | 45                   | 37                   |
| <i>E. l.</i> BzaB |                      | 100                  | 37                   | 44                   | 76                   | 40                   | 45                   | 62                   | 50                   | 43                   | 45                   | 44                  | 46                  | 47                   | 36                   |
| <i>E. l.</i> ThiC |                      |                      | 100                  | 39                   | 35                   | 62                   | 42                   | 41                   | 50                   | 37                   | 50                   | 37                  | 66                  | 40                   | 39                   |
| <i>A. w.</i> BzaA |                      |                      |                      | 100                  | 43                   | 39                   | 59                   | 48                   | 49                   | 43                   | 46                   | 43                  | 47                  | 45                   | 37                   |
| <i>A. w.</i> BzaB |                      |                      |                      |                      | 100                  | 38                   | 43                   | 59                   | 48                   | 43                   | 44                   | 44                  | 44                  | 45                   | 37                   |
| <i>A. w.</i> ThiC |                      |                      |                      |                      |                      | 100                  | 42                   | 43                   | 51                   | 39                   | 48                   | 39                  | 49                  | 40                   | 38                   |
| <i>M. t.</i> BzaA |                      |                      |                      |                      |                      |                      | 100                  | 54                   | 56                   | 46                   | 50                   | 44                  | 50                  | 44                   | 36                   |
| <i>M. t.</i> BzaB |                      |                      |                      |                      |                      |                      |                      | 100                  | 59                   | 48                   | 50                   | 47                  | 51                  | 50                   | 41                   |
| <i>M. t.</i> ThiC |                      |                      |                      |                      |                      |                      |                      |                      | 100                  | 51                   | 61                   | 49                  | 60                  | 51                   | 44                   |
| <i>G. s.</i> BzaF |                      |                      |                      |                      |                      |                      |                      |                      |                      | 100                  | 45                   | 87                  | 45                  | 55                   | 35                   |
| <i>G. s.</i> ThiC |                      |                      |                      |                      |                      |                      |                      |                      |                      |                      | 100                  | 45                  | 85                  | 47                   | 44                   |
| <i>P. p.</i> BzaF |                      |                      |                      |                      |                      |                      |                      |                      |                      |                      |                      | 100                 | 44                  | 56                   | 35                   |
| <i>P. p.</i> ThiC |                      |                      |                      |                      |                      |                      |                      |                      |                      |                      |                      |                     | 100                 | 50                   | 43                   |
| <i>M. b.</i> BzaF |                      |                      |                      |                      |                      |                      |                      |                      |                      |                      |                      |                     |                     | 100                  | 40                   |
| <i>M. b.</i> ThiC |                      |                      |                      |                      |                      |                      |                      |                      |                      |                      |                      |                     |                     |                      | 100                  |

Table S2. Primers used in this study

| Name  | Sequence  | Constructs   |
|-------|---|--|
| P604F | CACCTCGAGATCTATCGATGCATGCTAGGAGGATTTAAAAACC<br>ATGACTTTTGTGG        | <i>E. limsum</i><br>i. <i>bzaA-bzaB-cobT-bzaC-bzaD-bzaE</i> (operon);<br>ii. <i>bzaA</i> ;<br>iii. <i>bzaA-bzaB</i> ;<br>iv. <i>bzaA-bzaB-cobT</i> ;<br>v. <i>bzaA-bzaB-cobT-bzaC</i> ;<br>vi. <i>bzaA-bzaB-cobT-bzaC-bzaD</i> |
| P605R | GCTTGAATTCGAGCTCCCGGGTACCGTCTTGCTCCTTACTCT<br>GCTCT                 | <i>E. limsum bzaA</i>  |
| P606F | CACCTCGAGATCTATCGATGCATGCGCAGAGTAAGGAGGACA<br>AGACAATG              | <i>E. limsum bzaB</i>  |
| P607R | GCTTGAATTCGAGCTCCCGGGTACCCGGATCAGGCGATACCGA<br>AAAA                 | <i>E. limsum</i><br>i. <i>bzaB</i> ;<br>ii. <i>bzaA-bzaB</i>   |
| P615F | CACCTCGAGATCTATCGATGCATGCCAGTGGGAGGGAGAATA<br>GTTGAAAC              | <i>M. thermoacetica</i><br>i. <i>bzaA-bzaB-cobT-bzaC</i> (operon);<br>ii. <i>bzaA</i> ;<br>iii. <i>bzaA-bzaB</i> ;<br>iv. <i>bzaA-bzaB-cobT</i>  |
| P616R | GCTTGAATTCGAGCTCCCGGGTACCCGGATCCTCCAGCCGGTAT<br>TTC                 | <i>M. thermoacetica</i><br>i. <i>bzaA-bzaB-cobT-bzaC</i> (operon);<br>ii. <i>bzaC</i>  |
| P619R | GCTTGAATTCGAGCTCCCGGGTACCTTATGCGGTGATTTGTCA<br>ATTTCAGC             | <i>E. limsum bzaA-bzaB-cobT-bzaC-bzaD-bzaE</i> (operon)  |
| P639F | CACCTCGAGATCTATCGATGCATGCCAGCCAGACATACATGTT<br>AGGGG                | <i>M. thermoacetica bzaB</i>   |
| P640R | GCTTGAATTCGAGCTCCCGGGTACCCCTAACATGTATGTCT<br>GGCTG                  | <i>M. thermoacetica bzaA</i>   |
| P641R | GCTTGAATTCGAGCTCCCGGGTACCTCAGTTCATCCCGAAATA<br>ACGGG                | <i>M. thermoacetica</i><br>i. <i>bzaB</i> ;<br>ii. <i>bzaA-bzaB</i>  |
| P649R | GCTTGAATTCGAGCTCCCGGGTACCCCTGGTTTCATACCGCAT<br>ACTCC                | <i>M. thermoacetica bzaA-bzaB-cobT</i>   |
| P752F | CACCTCGAGATCTATCGATGCATGCGAAATCTGTAATAATCAA<br>ATAACGAGGTAATTATCATG | <i>E. limsum cobT</i>  |
| P753R | GCTTGAATTCGAGCTCCCGGGTACCTTAAGCGAGGCCATTGGT<br>GAC                  | <i>E. limsum</i><br>i. <i>cobT</i> ;<br>ii. <i>bzaA-bzaB-cobT</i>  |
| P761F | CACCTCGAGATCTATCGATGCATGCCAGGGAGTATGCGGTATG<br>AAACC                | <i>M. thermoacetica bzaC</i>   |
| P762R | GCTTGAATTCGAGCTCCCGGGTACCCGCTCCAGTCTTTGTTATT<br>AGGCG               | <i>E. limsum bzaA-bzaB-cobT-bzaC-bzaD</i>  |
| P764F | CACCTCGAGATCTATCGATGCATGCGAAGACGCAGGAGTCACC<br>AATG                 | <i>E. limsum bzaC</i>  |
| P765R | GCTTGAATTCGAGCTCCCGGGTACCTTCAACTCCGTTACTCC<br>AAAAGC                | <i>E. limsum</i><br>i. <i>bzaC</i> ;<br>ii. <i>bzaA-bzaB-cobT-bzaC</i>   |

**Table S3. Strains and plasmids used in this study**

| Name   | Genotype   | Source                     |
|--|--|----------------------------|
| <b>Strains:</b>                                    |  |                            |
| <i>E. coli</i> MG1655                              | Wild type parent   | G. Walker                  |
| MET1104  | <i>E. coli</i> $\Delta$ metE::Kan <sup>R</sup>   | This study                 |
| MET1160  | <i>E. coli</i> $\Delta$ purM::Kan <sup>R</sup>   | This study                 |
| MET1162  | <i>E. coli</i> $\Delta$ purK::Kan <sup>R</sup>   | This study                 |
| MET1164  | <i>E. coli</i> $\Delta$ metE (FRT) $\Delta$ cobT::Kan <sup>R</sup>                     | This study                 |
| MET1161  | <i>E. coli</i> $\Delta$ metE (FRT) $\Delta$ cobT (FRT) $\Delta$ purM::Kan <sup>R</sup> | This study                 |
| MET1163  | <i>E. coli</i> $\Delta$ metE (FRT) $\Delta$ cobT (FRT) $\Delta$ purK::Kan <sup>R</sup> | This study                 |
| <i>E. coli</i> BL21(DE3)                           | Overexpression strain  | C. Kinsland                |
| <i>Salmonella enterica</i> serovar Typhimurium LT2 | Wild type  | J. Roth                    |
| <i>Eubacterium limosum</i> ATCC 10825              | Wild type  | ATCC                       |
| <i>Moorella thermoacetica</i> ATCC 39073           | Wild type  | ATCC                       |
| <i>Geobacter sulfurreducens</i> PCA                | Wild type  | D. Lovley                  |
| <b>Plasmids:</b>                                   |  |                            |
| pTH1227  | Empty vector   | Cheng et al. <sup>14</sup> |
| pKM077   | pTH1227 with <i>E. limosum</i> bza operon  | This study                 |
| pKM065   | pTH1227 with <i>E. limosum</i> bzaA  | This study                 |
| pKM067   | pTH1227 with <i>E. limosum</i> bzaB  | This study                 |
| pKM069   | pTH1227 with <i>E. limosum</i> bzaA-bzaB   | This study                 |
| pAH024   | pTH1227 with <i>E. limosum</i> cobT  | This study                 |
| pAH025   | pTH1227 with <i>E. limosum</i> bzaA-bzaB-cobT-bzaC                                     | This study                 |
| pAH026   | pTH1227 with <i>E. limosum</i> bzaA-bzaB-cobT-bzaC-bzaD                                | This study                 |
| pAH027   | pTH1227 with <i>E. limosum</i> bzaC  | This study                 |
| pKM076   | pTH1227 with <i>M. thermoacetica</i> bza operon  | This study                 |
| pKM080   | pTH1227 with <i>M. thermoacetica</i> bzaA  | This study                 |
| pKM082   | pTH1227 with <i>M. thermoacetica</i> bzaB  | This study                 |
| pKM084   | pTH1227 with <i>M. thermoacetica</i> bzaA-bzaB   | This study                 |
| pAH033   | pTH1227 with <i>M. thermoacetica</i> bzaA-bzaB-cobT                                    | This study                 |
| pAH034   | pTH1227 with <i>M. thermoacetica</i> bzaC  | This study                 |

## References

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