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Cobamide Structure Depends on Both Lower Ligand Availability and CobT Substrate Specificity

Terence S. Crofts,[1] Erica C. Seth,[1] Amrita B. Hazra,[1] and Michiko E. Taga[1,*]
[1]Department of Plant and Microbial Biology, University of California, Berkeley, Berkeley, CA 94720, USA
*Correspondence: taga@berkeley.edu
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SUMMARY

Cobamides are members of the vitamin B_{12} family of cofactors that function in a variety of metabolic processes and are synthesized only by prokaryotes. Cobamides produced by different organisms vary in the structure of the lower axial ligand. Here we explore the molecular factors that control specificity in the incorporation of lower ligand bases into cobamides. We find that the *cobT* gene product, which activates lower ligand bases for attachment, limits the range of lower ligand bases that can be incorporated by bacteria. Furthermore, we demonstrate that the substrate specificity of CobT can be predictably altered by changing two active site residues. These results demonstrate that sequence variations in *cobT* homologs contribute to cobamide structural diversity. This analysis could open new routes to engineering specific cobamide production and understanding cobamide-dependent processes.

INTRODUCTION

Many environmentally and industrially important microbial processes such as methanogenesis, acetogenesis, and reductive dechlorination are dependent on corrinoids, a class of cofactors produced solely by a subset of prokaryotes (Roth et al., 1996; Banerjee and Ragsdale, 2003). Corrinoids are modified tetrapyrroles that contain a centrally bound cobalt atom (Roth et al., 1996). Corrinoids that contain an upper and lower ligand are termed cobamides. Cobalamin **1**, also known as vitamin B_{12} (Figure 1A), is the best-studied cobamide, and is an essential micronutrient for most animals including humans (Roth et al., 1996). Whereas humans are thought to have a specific requirement for cobalamin **1**, 16 different cobamides with structural variability in the lower ligand (Figure 1B) have been described (Renz, 1999). Most microbes described to date produce only one to two different cobamides when grown in pure culture (Kräutler et al., 1988; Stupperich et al., 1989, 1990; Keck and Renz, 2000). Although the factors that limit the range of cobamides produced by an organism remain unclear, previous studies have shown that different cobamides may not be functionally equivalent as cofactors for cobamide-dependent enzymes (Ford et al., 1955; Barker et al., 1960a, 1960b; Chan and Escalante-Semerena, 2011; Yi et al., 2012; Mok and Taga, 2013).

The de novo biosynthesis of a cobamide requires approximately 30 gene products (Warren et al., 2002). The attachment of the lower ligand base to the cobamide precursor GDP-cobinamide (GDP-Cbi **10**) is the last step in cobamide biosynthesis. This process has been studied most extensively in *Salmonella enterica* (Roth et al., 1996). Prior to attachment, the lower ligand base is activated by the CobT enzyme by the transfer of a phosphoribose moiety from nicotinate mononucleotide or a related compound to form an α-glycosidic linkage (Figure 1C) (Friedmann, 1965; Friedmann and Harris, 1965; Cameron et al., 1991; Trzebiatowski and Escalante-Semerena, 1997). The phosphoribosylated base is subsequently attached to GDP-Cbi **10** by CobS, and the phosphate group is removed by CobC to form the cobamide (Figure 1C) (Roth et al., 1996; Maggio-Hall and Escalante-Semerena, 1999; Zayas and Escalante-Semerena, 2007). Homologs of *cobT* are present in nearly all bacterial genomes that contain the complete corrinoid biosynthesis pathway (Rodionov et al., 2003). However, in organisms that produce cobamides by an aerobic pathway, the *cobT* homolog is termed *cobU* (Roth et al., 1996). Two additional *cobT* homologs, termed *arsA* and *arsB*, have been described in the bacterium *Sporomusa ovata*. These genes together encode a heterodimeric enzyme responsible for the activation of phenolic lower ligand bases (Chan and Escalante-Semerena, 2011).

In this work, we investigate the molecular factors that limit the range of cobamides that can be produced by a single bacterial species. Two hypotheses are tested to address this question. First, the range of lower ligands that can be attached to form cobamides may be limited by the substrate specificity of the CobT enzyme. Alternatively, the cobamides produced by an organism may be limited solely by the availability of potential lower ligands. Evidence in favor of the first hypothesis comes from our previous observations of corrinoid biosynthesis in the *Sinorhizobium meliloti bluB* mutant. *Si. meliloti bluB* is unable to synthesize 5,6-dimethylbenzimidazole (DMB, the lower ligand of cobalamin **1**) and instead produces the incomplete corrinoid GDP-Cbi **10** rather than incorporating intracellular adenine (Ade) to produce pseudo-B_{12} **6** (adeninyl cobamide, [Ade]Cba) (Campbell et al., 2006). Similarly, *Lactobacillus reuteri* does not produce cobalamin **1** when DMB is provided and instead produces only [Ade]Cba **6** (Santos et al., 2007). The second hypothesis is supported by previous studies demonstrating the ability of several bacteria to attach a variety of exogenously provided lower ligands (Ford et al., 1955; Perlman and Barrett, 1958; Kamikubo and Matsuura,

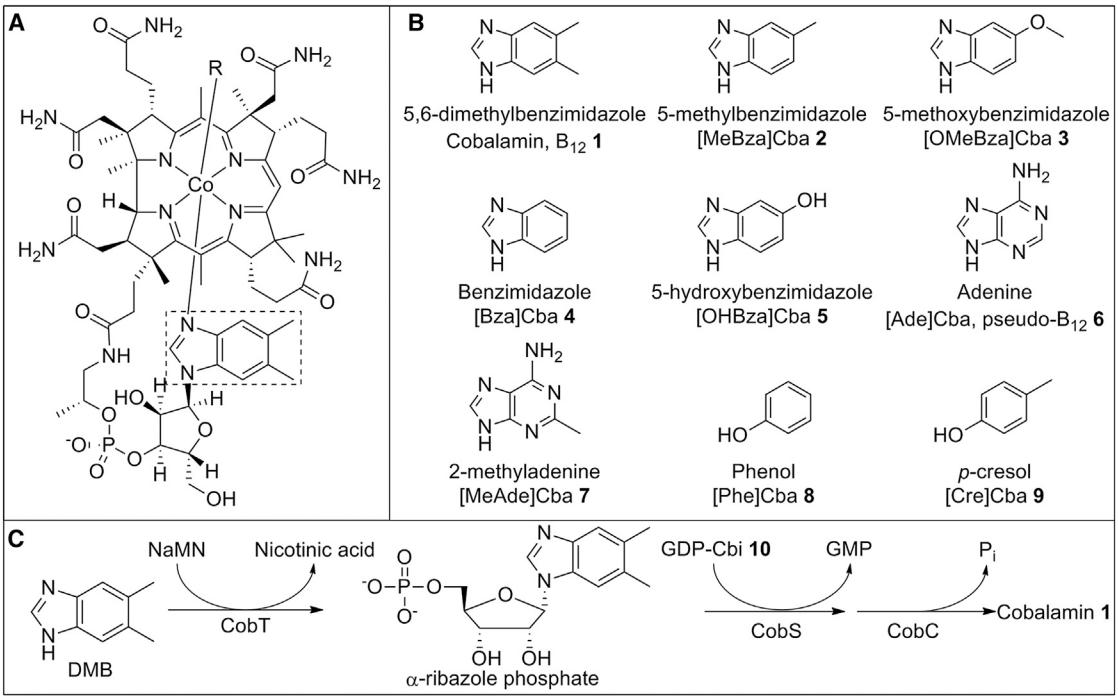

Figure 1. Structures of Cobamides and Lower Ligands

(A) Structure of cobalamin. The upper ligand, R, is a methyl or 5′-deoxyadenosyl group in the cofactor forms and a cyano group in the vitamin form. The lower ligand, DMB, is indicated by the box.

(B) Structures of cobamide lower ligands. The name of each compound and the abbreviation for the corresponding cobamide are given below each structure.

(C) The lower ligand attachment pathway. The pathway for the activation and attachment of DMB to form cobalamin is shown with the names of the *Sa. enterica* enzymes given. CobT catalyzes the activation of DMB by the attachment of a phosphoribose moiety derived from nicotinate mononucleotide to form α-ribazole phosphate. The CobS and CobC enzymes catalyze the attachment of the activated base to the cobamide precursor GDP-cobinamide (GDP-Cbi **10**) and dephosphorylation of the product to form cobalamin, respectively. NaMN, nicotinic acid mononucleotide; GDP, guanosine diphosphate; GMP, guanosine monophosphate; P_i, inorganic phosphate.

See also Figure S1.

1969; Trzebiatowski and Escalante-Semerena, 1997; Maggio-Hall and Escalante-Semerena, 1999). For example, *Sa. enterica* produces [Ade]Cba **6** and 2-methyladenylcobamide **7** ([MeAde]Cba), and *Sp. ovata* produces phenolylcobamide **8** ([Phe]Cba) and *p*-cresolylcobamide **9** ([Cre]Cba) when cultured without a lower ligand base, but both can produce benzimidazolyl cobamides when benzimidazole bases are provided (Stupperich and Eisinger, 1989; Stupperich et al., 1989, 1990; Keck and Renz, 2000; Anderson et al., 2008; Newmister et al., 2012; Mok and Taga, 2013). In addition, X-ray crystallography studies of *Sa. enterica* CobT and *Sp. ovata* ArsAB have demonstrated the ability to bind a variety of lower ligand bases in their active sites (Cheong et al., 1999, 2001, 2002; Newmister et al., 2012). Currently, the literature as a whole does not support one hypothesis over the other.

Here we test these hypotheses by examining the ability of five phylogenetically diverse bacteria (Figure S2A available online) to attach a variety of exogenously supplied lower ligand bases. In addition to *Si. meliloti*, *L. reuteri*, and *Sa. enterica*, which are discussed above, we have investigated lower ligand attachment in *Veillonella parvula*, a sequenced relative of *Sp. ovata* (Gronow et al., 2010), and found that *V. parvula* also produces [Cre]Cba **9**. Using *Si. meliloti* as a genetic host, we examine the role of *cobT* homologs in determining the range of lower ligands that can be attached. We also include *Dehalococcoides mccartyi* in this study, which we previously found is capable of attaching some of the benzimidazoles investigated here, despite its inability to synthesize a corrinoid or lower ligand base de novo (Yi et al., 2012). Our results show that the range of lower ligands that can be attached is controlled by both the availability of lower ligand bases and the substrate specificity of CobT.

RESULTS

Guided Biosynthesis in Four Bacteria Shows that a Limited Set of Lower Ligand Bases Can Be Incorporated into Cobamides

The bacteria *Si. meliloti*, *Sa. enterica*, *L. reuteri*, and *V. parvula* were chosen as model organisms to investigate the range of lower ligands that can be attached to form cobamides. The cobamides produced by *Si. meliloti*, *Sa. enterica*, and *L. reuteri* have previously been identified as cobalamin **1**, [Ade]Cba **6** and [MeAde]Cba **7**, and [Ade]Cba **6**, respectively (Keck and Renz, 2000; Campbell et al., 2006; Santos et al., 2007). To confirm that these cobamides are synthesized by our laboratory strains, corrinoids were extracted and analyzed from cultures of each organism. High-performance liquid chromatography (HPLC) (Figure 2) and liquid chromatography-tandem mass

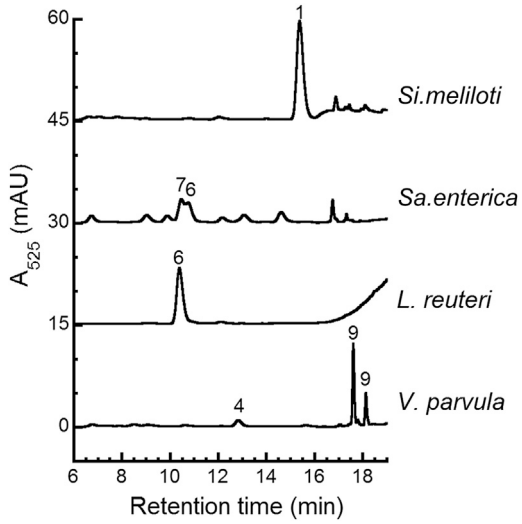

Figure 2. Native Cobamides Produced by Bacteria in This Study
HPLC analysis of corrinoid extracts from cultures of the indicated bacteria is shown. Numbers correspond to the compounds shown in Figure 1. The identity of each numbered peak was determined by LC-MS/MS.

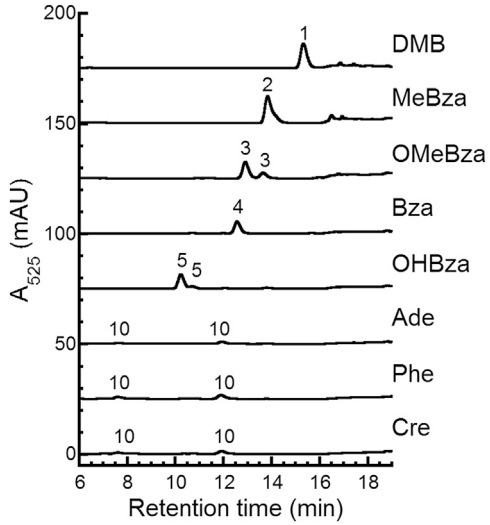

Figure 3. Corrinoids Produced by Guided Biosynthesis in *Si. meliloti bluB*
HPLC analysis of corrinoid extracts of an *Si. meliloti bluB* mutant cultured in the presence of the indicated lower ligand base is shown. Numbered peaks were verified by LC-MS/MS.

spectrometry with multiple reaction monitoring (LC-MS/MS) (data not shown) demonstrated that the expected cobamides were present.

Sp. ovata is known to produce phenolyl cobamides due to the activity of the ArsAB enzyme (Stupperich and Eisinger, 1989; Stupperich et al., 1989; Chan and Escalante-Semerena, 2011). As the genome sequence of *Sp. ovata* was not available at the time, we tested whether *V. parvula*, a sequenced (Gronow et al., 2010) relative of *Sp. ovata*, might also produce phenolyl cobamides. HPLC analysis of corrinoid extracts from *V. parvula* shows that this bacterium produces a pair of corrinoid species that coelute with [Cre]Cba **9** extracted from *Sp. ovata* cultures (Figure 2). LC-MS/MS analysis confirmed these to be [Cre]Cba **9** by comparison of both retention time and *m/z* (data not shown). Surprisingly, an additional corrinoid in the extract from *V. parvula* was identified as benzimidazolylcobamide **4** ([Bza]Cba) by LC-MS/MS and comparison to a standard prepared in *Sa. enterica*. As such, *V. parvula* is the only organism known to synthesize both phenolyl and benzimidazolyl cobamides.

We next examined the ability of these organisms to incorporate lower ligand bases provided in the culture medium, a process known as guided biosynthesis. Each of the lower ligand bases shown in Figure 1B (excluding 2-methyladenine) was tested for incorporation into cobamides in each bacterium by culturing in media containing the lower ligand base followed by corrinoid extraction and analysis. A representative HPLC analysis of corrinoids extracted from *Si. meliloti* is shown in Figure 3. An *Si. meliloti bluB* mutant was used for the guided biosynthesis experiment because this strain does not produce the lower ligand DMB (Campbell et al., 2006; Taga et al., 2007). HPLC (Figure 3) and LC-MS/MS analysis (data not shown) demonstrated that *Si. meliloti bluB* attached each of the five benzimidazoles to form the corresponding benzimidazolyl cobamides. However, no cobamides were detected when Ade, Phe, or Cre was provided. LC-MS/MS analysis revealed that the only corrinoids present in these extracts were mono- and dicyano forms of GDP-Cbi **10**, a cobamide precursor lacking a lower ligand that we previously detected in cultures of *Si. meliloti bluB* (Campbell et al., 2006). The lower ligand incorporation profiles for *Sa. enterica*, *L. reuteri*, and *V. parvula* were also analyzed. The results of these experiments, as well as previously published results for the bacterium *Dc. mccartyi* (Yi et al., 2012), are summarized in Table 1. *Sa. enterica* is capable of incorporating adenine in addition to each of the five benzimidazoles, but was not capable of incorporating the phenolic compounds, in agreement with previous findings (Cheong et al., 2001; Chan and Escalante-Semerena, 2011). *L. reuteri* could incorporate only adenine, whereas *V. parvula* incorporated all of the compounds tested with the exception of adenine (Table 1). The failure of *L. reuteri* to incorporate other lower ligand bases does not appear to be due to an inability of the molecules to enter the cell, as we detected an average of 105 ± 2 pmol/OD_{600} of free DMB from the cell-pellet fraction of three *L. reuteri* cultures grown with 5 μmol DMB. Together, these results indicate that, with the exception of *L. reuteri*, each bacterium can incorporate multiple lower ligands to form cobamides, although only *Sa. enterica* and *V. parvula* were capable of incorporating multiple classes of lower ligands.

The *Si. meliloti cobU* Mutant Can Be Complemented by Heterologous Expression of *cobT* Homologs

The results of the guided biosynthesis experiments suggest that the range of lower ligands that can be attached by a bacterium is not controlled solely by the availability of different lower ligand bases in the environment. Next, we examined the influence of *cobT* on lower ligand selectivity, as *cobT* acts first in the lower ligand attachment pathway (Roth et al., 1996). Specifically, we tested whether expression of *cobT* homologs from different bacteria could influence the range of lower ligands

Table 1. Cobamides Produced by Guided Biosynthesis in Five Bacteria

	Lower Ligand Base Provided							
	DMB	MeBza	OMeBza	Bza	OHBza	Ade	Phe	Cre
Si. meliloti	+	+	+	+	+	–	–	–
Sa. enterica	+	+	+	+	+	+	–	–
L. reuteri	–	–	–	–	–	+	–	–
V. parvula	+	+	+	+	+	–	+	+
Dc. mccartyi[a]	+	+	+	+	ND	ND	ND	ND

+, corrinoid detectable by HPLC; –, corrinoid peak not detectable by HPLC or LC-MS/MS; ND, not determined. See also Figure S3 and Table S1.
[a]Data reported by Yi et al. (2012) based on cultures supplemented with cobinamide and a lower ligand base.

that can be attached. *cobT* homologs were expressed in an *Si. meliloti* strain with a mutation in *cobU* (the *cobT* homolog in *Si. meliloti*). The *cobU* mutant constructed for this study was unable to grow on LB medium without cobalamin **1** supplementation due to the cobalamin **1** requirement of the ribonucleotide reductase enzyme (Campbell et al., 2006; Taga and Walker, 2010). This cobalamin **1** auxotrophy of the *cobU* strain and the *bluB cobU* double mutant strain could be rescued by the addition of cobalamin **1** to the growth medium or by expression of the *Si. meliloti cobU* gene on a plasmid. To test whether *cobT* homologs from other bacteria could function in *Si. meliloti*, *cobT* homologs from *Si. meliloti*, *Dc. mccartyi*, *Sa. enterica*, *L. reuteri*, and *V. parvula* were expressed on a plasmid in an *Si. meliloti bluB cobU* strain in the presence of DMB. These *Si. meliloti* strains are indicated hereafter by the shorthand *Sm* $cobT_{Dm}^{+}$, in which two-letter abbreviations for each organism are used (*Si. meliloti*, *Sm*; *Dc. mccartyi*, *Dm*; *Sa. enterica*, *Se*; *L. reuteri*, *Lr*; *V. parvula*, *Vp*). All of the *Si. meliloti* strains expressing *cobT* homologs from *Dc. mccartyi*, *Sa. enterica*, and *L. reuteri* were viable and produced cobalamin **1** when DMB was provided, indicating that these *cobT* homologs were functional. The ability of *Sm* $cobT_{Lr}^{+}$ to grow in the presence of DMB is considered later.

An examination of the *V. parvula* genome revealed the presence of three different *cobT* homologs, Vpar_1456, Vpar_1457, and Vpar_1602, whereas a single *cobT* homolog was present in each of the other bacterial genomes we examined (except *Dc. mccartyi*, which contains two *cobT* genes with identical sequences) (Seshadri et al., 2005; Gronow et al., 2010). However, only one of the three *cobT* homologs from *V. parvula*, Vpar_1602, complemented the cobalamin **1** auxotrophy of the *Si. meliloti bluB cobU* strain. Vpar_1456 and Vpar_1457 are located adjacent to one another, similar to the recently identified *Sp. ovata cobT* homologs *arsA* and *arsB* (Chan and Escalante-Semerena, 2011). To test whether Vpar_1456 and Vpar_1457, like *Sp. ovata arsA* and *arsB*, function when expressed together, both genes were cloned on a single plasmid. Coexpression of these genes rescued the cobalamin **1** auxotrophy of the *bluB cobU* strain in media containing DMB. Based on the function of these genes as described below, and because the predicted protein sequences share 46.7% and 32.6% identity with *Sp. ovata arsA* and *arsB*, we refer to Vpar_1457 and Vpar_1456 as *arsA* and *arsB*, respectively. The degree of relatedness among the *cobT* homologs examined in this study is shown in the pairwise identity matrix in Table S1 and the phylogenetic analysis in Figure S2A.

Expression of *cobT* Homologs Alters the Lower Ligand Attachment Profile of *Si. meliloti*

To test whether the expression of *cobT* from other bacteria could alter the range of lower ligand bases that can be incorporated into cobamides, guided biosynthesis assays were performed in the *Si. meliloti bluB cobU* strains expressing each *cobT* homolog. A representative HPLC analysis of this assay performed with *Sm* $cobT_{Se}^{+}$ and *Sm* $arsAB_{Vp}^{+}$ is shown in Figure 4. *Sm* $cobT_{Se}^{+}$ was found to incorporate the five benzimidazoles as well as adenine, but was unable to attach phenol or cresol (Figure 4A), whereas *Sm* $arsAB_{Vp}^{+}$ could attach all of the bases provided except adenine (Figure 4B). These results match the results of the guided biosynthesis study for *Sa. enterica* and *V. parvula*, respectively (Table 1), and demonstrate that these *cobT* homologs are sufficient to specify the range of lower ligands that can be attached.

In analyzing the results above, we observed differences in the amount of incorporation of each substrate, as variable levels of GDP-Cbi **10** were present in the corrinoid extracts (Figure 4). We reasoned that these differences likely reflect both the overall degree of complementation of the heterologously expressed *cobT* genes as well as differences in the ability of CobT homologs to activate or attach each lower ligand. The amount of each cobamide produced by guided biosynthesis as a percentage of the total extracted corrinoids was used as an indication of the level of incorporation of each lower ligand (Table 2). We found that the lower ligand bases that could be incorporated by each bacterium (Table 1) mirrored the lower ligand incorporation profile of *Si. meliloti* expressing their respective *cobT* homologs (Table 2). An exception to this trend was observed in *L. reuteri*. The inability of *L. reuteri* to incorporate benzimidazoles was not transferred to *Sm* $cobT_{Lr}^{+}$, because the *Sm* $cobT_{Lr}^{+}$ strain was able to attach all five benzimidazoles such that GDP-Cbi **10** made up less than half of the total corrinoids in each culture (Table 2). Although adenine was the only lower ligand incorporated by *L. reuteri*, it appears to be incorporated less efficiently than the benzimidazoles in *Sm* $cobT_{Lr}^{+}$ (Table 2). Interestingly, both *Sm* $cobT_{Se}^{+}$ and *Sm* $cobT_{Lr}^{+}$ were capable of producing [Ade]Cba **6** without the exogenous addition of adenine, indicating that intracellular adenine pools are available for use as lower ligands in *Si. meliloti* as has previously been observed in *Sa. enterica* (Anderson et al., 2008). This is illustrated in Figure 4A, where [Ade]Cba **6** was detected in all corrinoid extracts from *Sm* $cobT_{Se}^{+}$.

The two *cobT* homologs of *V. parvula* exhibited distinct lower ligand incorporation profiles when expressed individually in *Si. meliloti*. Whereas both *Sm* $cobT_{Vp}^{+}$ and *Sm* $arsAB_{Vp}^{+}$ were

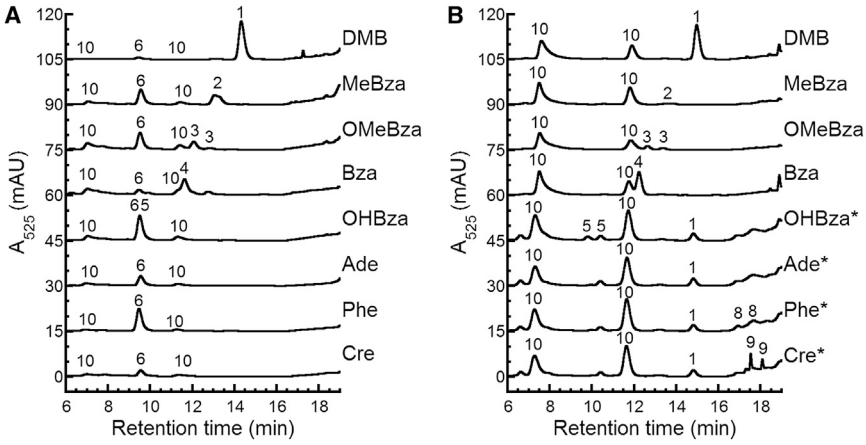

Figure 4. Guided Biosynthesis in *Si. meliloti bluB cobU* Expressing *cobT* Homologs

HPLC analysis of corrinoids extracted from (A) *Sm* $cobT_{Se}^{+}$ and (B) *Sm* $arsAB_{Vp}^{+}$ containing the indicated lower ligand bases. The identities of the numbered peaks were verified by LC-MS/MS. Asterisks indicate the addition of 0.1 μM cobalamin **1** to cultures to support growth.

capable of incorporating all of the benzimidazole lower ligands, *Sm* $arsAB_{Vp}^{+}$ was the only strain that also incorporated the phenolic substrates *p*-cresol and phenol (Figure 4B; Table 2). *Sm* $cobT_{Vp}^{+}$ also incorporated adenine, although to a lesser extent than either *Sm* $cobT_{Se}^{+}$ or *Sm* $cobT_{Lr}^{+}$, even though adenine incorporation was not observed in *V. parvula* (Table 1). Together, these results indicate that the range of lower ligands that can be incorporated into cobamides in a given organism is heavily influenced by *cobT*.

Site-Directed Mutagenesis of *Sa. enterica cobT* Alters Lower Ligand Attachment Specificity

The results presented above demonstrate that expression of *cobT* homologs from different bacteria in *Si. meliloti* allowed it to produce cobamides that wild-type *Si. meliloti* is incapable of producing. This finding led us to investigate the sequence variations among *cobT* homologs that may contribute to the observed differences in substrate specificity. A multiple sequence alignment of the CobT homologs revealed that the residues at positions equivalent to Ser80 and Gln88 in *Sa. enterica* CobT covary with the ability to attach adenine (Figure 5A). CobT enzymes with Ser and Glu at these positions allow the incorporation of adenine, whereas those that do not incorporate adenine have Phe/Tyr/Trp and Met, respectively (Figure 5A). These residues were previously implicated in the stabilization of adenine in the active site of *Sa. enterica* CobT by interacting with the amino group (N10) and the N3 ring nitrogen of adenine (Cheong et al., 2001).

To test the hypothesis that residues Ser80 and Gln88 of *Sa. enterica* CobT influence the ability to activate adenine, we performed site-directed mutagenesis on *Sa. enterica cobT* to change these residues to Phe and Met, respectively, which are found at equivalent positions in *Si. meliloti* CobU (Figure 5A). Plasmids containing $cobT_{Se}$ with the S80F, Q88M, or S80F/Q88M mutations were introduced into the *Si. meliloti bluB cobU* strain. These strains were cultured in the presence of a limiting amount of DMB to compare the level of attachment of DMB and intracellular adenine. HPLC analysis of corrinoid extracts from these cultures showed that *Sm* $cobT_{Se}^{+}$ produced approximately equal amounts of [Ade]Cba **6** and cobalamin **1** under these conditions (Figure 5B). *Sm* $cobT_{Se}$ S80F produced 2.7-fold less [Ade]Cba **6** than *Sm* $cobT_{Se}^{+}$ but produced a similar level of cobalamin **1**, after normalizing for culture density (Figure 5B). The Q88M mutation impacted both DMB and adenine incorporation, because 2.4-fold less [Ade]Cba **6** and 2.3-fold more cobalamin **1** was produced by *Sm* $cobT_{Se}$ Q88M compared to *Sm* $cobT_{Se}^{+}$ (Figure 5B). The effects of the S80F and Q88M mutations appeared to be additive, as the *Sm* $cobT_{Se}$ S80F/Q88M double mutant produced 3.5-fold less [Ade]Cba **6** and 2.6-fold more cobalamin than *Sm* $cobT_{Se}^{+}$ (Figure 5B). These results provide further evidence that differences in *cobT* sequence are responsible for the observed differences in lower ligand incorporation. The active site residues Ser and Gln are not the sole determinants of adenine activation, however, because *Si. meliloti* strains expressing *cobU* genes with the reciprocal mutations were unable to incorporate adenine (data not shown).

Altered Lower Ligand Specificity Results in a Loss of Viability in *Si. meliloti*

In the course of our experiments, we observed that the growth of the *Si. meliloti* strains varied according to the lower ligand base provided, and supplementation with cobalamin **1** was required for growth when certain strains were supplied with adenine or phenolic compounds (Figure 4). This result suggested that some of the cobamides produced did not support growth in *Si. meliloti*. To measure the effect of different cobamides on growth, the OD_{600} of *Sm* $cobT_{Se}^{+}$ cultures was measured following growth to stationary phase in minimal media containing each lower ligand base that could be incorporated into a cobamide. We found that DMB, 5-methylbenzimidazole (MeBza), and benzimidazole (Bza) were equivalent in supporting the growth of *Si. meliloti*, whereas cultures supplied with 5-methoxybenzimidazole (OMeBza), 5-hydroxybenzimidazole (OHBza), or adenine reached a lower final culture density (Figure 6A). This result indicates that cobalamin **1**, [MeBza]Cba **2**, and [Bza]Cba **4** support the growth of *Si. meliloti* to a greater extent than [OMeBza]Cba **3**, [OHBza]Cba **5**, or [Ade]Cba **6**.

To verify that the phenotype observed when lower ligand bases were added to cultures was due to the effect of the cobamides produced rather than differences in the efficiency of incorporation of the lower ligands, an *Si. meliloti cobD bluB* strain, which is incapable of producing corrinoids or DMB, was cultured in the presence of a representative purified cobamide from each structural class. Cultures grown with [Ade]Cba **6** or [Cre]Cba **9** led to 3.7- and 13-fold lower final culture densities, respectively, than those grown with cobalamin **1** (Figure 6B). These results demonstrate that different cobamides are not functionally equivalent in *Si. meliloti*.

Table 2. Cobamides Produced by Guided Biosynthesis in *Si. meliloti bluB cobU* Expressing *cobT* Homologs

	Lower Ligand Base Provided[a]							
	DMB	MeBza	OMeBza	Bza	OHBza	Ade	Phe	Cre
Sm $cobU_{Sm}^+$	100	100	2.0	27	4.3	0	0	0
Sm $cobT_{Dm}^+$	64	14	40	33	0.61	0	0	0
Sm $cobT_{Se}^+$	96	38	22	40	NA[b]	56	0	0
Sm $cobT_{Lr}^+$	100	76	59	75	NA	9.7[c]	0[c]	0[c]
Sm $cobT_{Vp}^+$	63	94	100	100	41	1.0	0	0
Sm $arsAB_{Vp}^+$	42	4.6	11	30	3.9[c]	0[c]	1.0[c]	11[c]

[a]Numbers represent the level of incorporation of each lower ligand base as a percentage of the total corrinoids present.
[b]NA, not available. Percentage is not available due to coelution of [OHBza]Cba and [Ade]Cba.
[c]Cultures were grown with cobalamin to achieve higher growth, but cobalamin present in the corrinoid extracts was not used in the calculations.

DISCUSSION

It has historically been recognized that different prokaryotes produce a variety of different cobamides, and that the structural differences among these cofactors are almost entirely limited to the lower ligand (Brown et al., 1955; Ford et al., 1955). Although diverse cobamides have been observed in environments such as the human intestine, bovine rumen, and contaminated groundwater, the microbes that produce cobamides have been observed to synthesize only one or two different cobamides when grown in pure culture (Brown et al., 1955; Kräutler et al., 1988; Stupperich et al., 1989, 1990; Keck and Renz, 2000; Allen and Stabler, 2008; Girard et al., 2009; Y. Men, E.C.S., S. Yi, T.S.C., R.H. Allen, M.E.T., and L. Alvarez-Cohen, unpublished data). This study addresses the molecular basis of lower ligand selectivity in a representative set of bacteria. We have identified the *cobT* gene as being responsible for limiting the range of lower ligands that can be incorporated. Specificity in CobT activity could be a mechanism of limiting the production of cobamides to those that function as cofactors for a particular organism.

Our genetic system in *Si. meliloti* allowed us to examine the influence of substrate specificity by CobT on cobamide synthesis in the absence of factors that may be unique to each organism. We found that the range of lower ligands that could be attached by the *Si. meliloti* strains expressing *cobT* homologs mirrored that of the originating bacteria in nearly all cases. The fact that multiple lower ligand bases can be incorporated by guided biosynthesis suggests that the structure of the cobamide produced by each organism is governed in part by the availability of lower ligand bases in the cell. Additionally, our results show that the range of lower ligands that can be incorporated is limited by substrate specificity in *cobT*, and that *cobT* homologs from different organisms have distinct specificity profiles. A notable exception to this pattern was *L. reuteri*, which was incapable of incorporating benzimidazoles by guided biosynthesis, whereas expression of *L. reuteri cobT* enabled the incorporation of benzimidazoles in *Si. meliloti*. It is possible that in *L. reuteri* another enzyme, such as CobS, prevents the incorporation of benzimidazoles into cobamides. This hypothesis could be addressed experimentally by expressing the *L. reuteri cobS* gene in a *Si. meliloti cobS* mutant.

Our results showed that the major differences in substrate specificity among the *cobT* homologs are in the ability to activate adenine and phenolic compounds, whereas the activation of benzimidazoles is common to all of the *cobT* homologs tested. Differences in the preferences of each CobT enzyme for each lower ligand substrate are investigated in more detail in the accompanying paper (Hazra et al., 2013) in this issue of *Chemistry & Biology*. The activation of phenolic compounds appears to be a specialized function of the *arsA* and *arsB* gene products, because none of the other CobT homologs in this or previous studies is capable of activating phenolic compounds (Cheong et al., 2001, 2002; Chan and Escalante-Semerena, 2011). This is likely due to significant differences in the reactivity required for the formation of an *O*-linked rather than an *N*-linked glycosidic bond. In addition, consistent with previous studies of *Sp. ovata* ArsAB, *V. parvula* and *Sm* $arsAB_{Vp}^+$ can produce both benzimidazolyl and phenolyl cobamides by guided biosynthesis (Chan and Escalante-Semerena, 2011; Newmister et al., 2012). Given that *V. parvula* naturally produces both [Cre]Cba **9** and [Bza]Cba **4**, and that *Sm* $arsAB_{Vp}^+$ is capable of activating benzimidazoles in addition to phenolic compounds, it is puzzling that *V. parvula* possesses an additional CobT homolog that activates only benzimidazoles (and adenine, to a limited extent). A possible explanation is that *V. parvula cobT* and *arsAB* are differentially expressed in order to produce varying ratios of [Cre]Cba **9** and [Bza]Cba **4** under different environmental conditions.

We found that the ability to activate adenine for the production of [Ade]Cba **6** in *Si. meliloti* is limited to only three of the *cobT* homologs. In the case of *Sa. enterica* and *L. reuteri*, this is consistent with their production of [Ade]Cba **6**. Our observation that *Sm* $cobT_{Vp}^+$ produces a small amount of [Ade]Cba **6** is unexpected, considering the absence of [Ade]Cba **6** in the *V. parvula* corrinoid extracts. This may be explained either by an inability to detect low levels of [Ade]Cba **6** that may be present in the *V. parvula* extract or an insufficient concentration of free adenine in *V. parvula*. The production of a relatively low level of [Ade]Cba **6** by *Sm* $cobT_{Vp}^+$ may be due to the presence of Tyr and Gln in *V. parvula* CobT at positions equivalent to residues S80 and Q88 in *Sa. enterica* CobT. Previously, it was proposed that hydrophilic residues are important for stabilizing adenine in the binding pocket and that the hydrophobic residues Y79 and M87 at analogous positions in *Sp. ovata* ArsA are important for stabilizing phenolic lower ligand bases (Cheong et al., 2001; Newmister et al., 2012). If additional CobT enzymes are found that share this pattern, it may be possible to use this analysis in the bioinformatic prediction of cobamide structures based

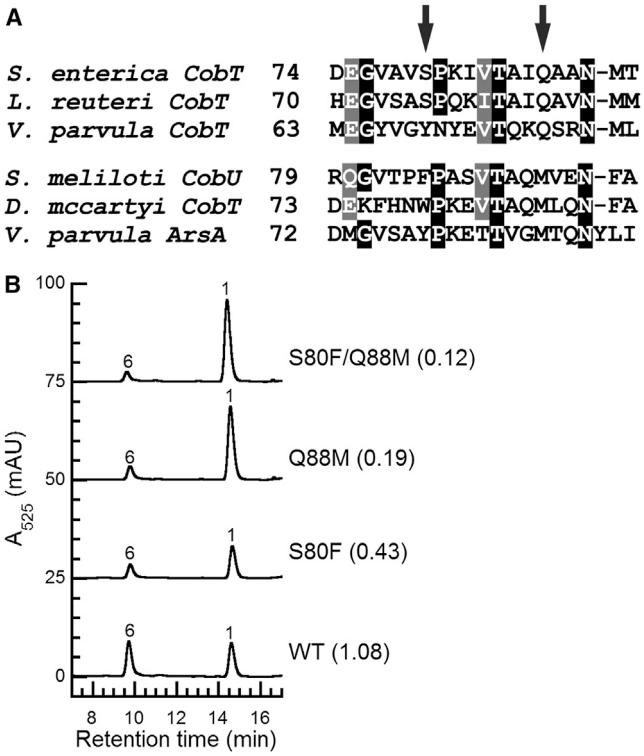

Figure 5. Site-Directed Mutagenesis of *Sa. enterica* CobT Leads to Altered Lower Ligand Incorporation

(A) Multiple sequence alignment of CobT homologs that are capable of activating adenine when expressed in *Si. meliloti* (*Sa. enterica* CobT, *L. reuteri* CobT, and *V. parvula* CobT) and those that do not activate adenine (*Si. meliloti* CobU, *Dc. mccartyi* CobT, and *V. parvula* ArsA). Only the region surrounding the two amino acid residues targeted for site-directed mutagenesis (arrows) is shown. ArsB is not included because it lacks a true active site (Newmister et al., 2012).

(B) HPLC analysis of corrinoids extracted from *Sm cobT*$_{Se}^{+}$ (WT), *Sm cobT*$_{Se}$ S80F, *Sm cobT*$_{Se}$ Q88M, and *Sm cobT*$_{Se}$ S80F/Q88M cultures grown in the presence of 0.5 μM DMB. The ratio of [Ade]Cba **6** to cobalamin **1** is shown in parentheses.

See also Figure S2.

on *cobT* sequences. Indeed, analysis of these positions in several CobT homologs reveals a co-occurrence of aromatic residues (Phe/Tyr/Trp) with Met, or H-bonding residues (Ser/Thr/Cys) with Gln or Val (Figure S2B).

We speculate that the ability to exclude certain lower ligand bases from incorporation into cobamides is a mechanism of preventing an organism from producing a cobamide that it cannot use. Based on this hypothesis, specificity in *cobT* would be particularly important for the exclusion of adenine, which is present intracellularly but is used as a lower ligand only by a subset of bacteria. This idea is supported by our observation that the production of [Ade]Cba **6** in *Sm cobT*$_{Se}^{+}$ leads to poor growth (Figure 6A). A counterexample to this case is *Sp. ovata*, which can incorporate benzimidazoles as cobamide lower ligands but is incapable of using benzimidazolyl cobamides for the metabolism of certain carbon sources (Stupperich et al., 1990; Mok and Taga, 2013). The absence of a mechanism of excluding benzimidazoles from incorporation into cobamides in *Sp. ovata* suggests that *Sp. ovata* does not produce benzimidazoles or encounter them in the environment.

The results of this study suggest that diversity in cobamide structure is achieved in nature by a combination of the biosynthesis or availability of various lower ligand bases and the molecular specificity in the lower ligand activation gene *cobT*. Because the structure of cobamides has been shown to affect cofactor function in many cases, specificity in *cobT* likely ensures that bacteria produce only cobamides that can be used for their metabolic processes. However, the reason why such diversity in cobamide structure exists in nature remains unclear. The use of lower ligand bases that also function in other cellular processes, such as purines, or are products of amino acid catabolism, such as phenolic compounds, is relatively common among bacteria (Stupperich and Eisinger, 1989; Anderson et al., 2008). It is therefore puzzling that some bacteria have dedicated pathways for the biosynthesis of benzimidazoles that, to the best of our knowledge, only function as cobamide lower ligands. Substrate specificity in CobT could be explained by the requirement of certain metabolic processes for specific cobamides (Yan et al., 2012, 2013; Yi et al., 2012; Mok and Taga, 2013). In addition, diversity in cobamide structure may be driven by a need for cobamide-producing organisms to limit the cobamide remodeling or salvaging activity of other organisms. Further biochemical and ecological studies will be necessary to explore these possibilities.

SIGNIFICANCE

Cobamides, which include vitamin B$_{12}$, are essential cofactors for many organisms, both eukaryotes and prokaryotes, in a variety of metabolic pathways, but are produced only by a subset of prokaryotes. Cobamides are distinguished by the structure of the lower ligand, and different cobamides are not necessarily functionally equivalent as cofactors. Although 16 structurally distinct cobamides have been described, organisms typically synthesize only one or two different cobamides when grown in pure culture. Here, we sought to identify the molecular factors that determine which cobamides are produced by bacteria. The final steps in cobamide biosynthesis involve activation of a lower ligand base and attachment to a cobamide precursor. We utilized guided biosynthesis assays in four bacteria and found that a distinct set of lower ligand bases is incorporated by each organism. Heterologous expression of *cobT* homologs from these bacteria and from *Dehalococcoides mccartyi* in *Sinorhizobium meliloti* demonstrated that CobT is responsible for preventing the incorporation of certain lower ligand bases. Additionally, the *Salmonella enterica cobT* gene was engineered by site-directed mutagenesis to lower its affinity for adenine relative to 5,6-dimethylbenzimidazole, the lower ligand of vitamin B$_{12}$. We also report on the potential for prediction of the cobamide biosynthetic capability of microbial communities based on metagenomic analysis of *cobT* homologs. Our results show that both the availability of lower ligand bases and the substrate specificity of CobT limit the lower ligands that can be attached to form cobamides. Finally, we found that growth is inhibited in *Si. meliloti* strains that have been engineered to synthesize alternate cobamides. Our results suggest that substrate specificity

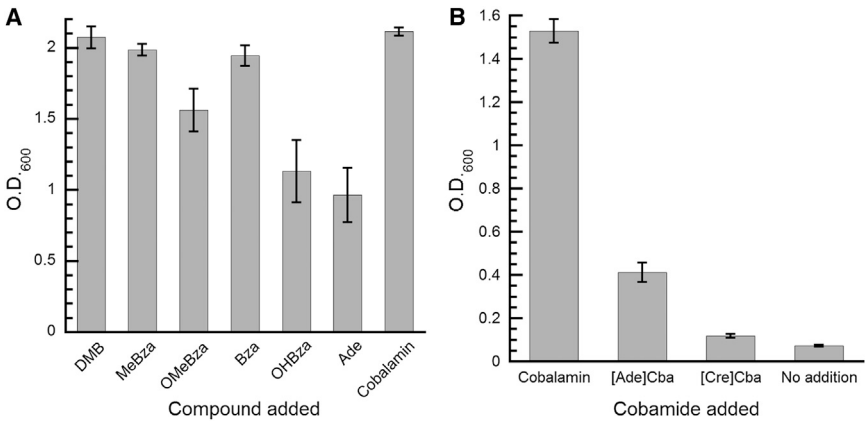

Figure 6. Biosynthesis of Nonnative Corrinoids Affects Growth of *Si. meliloti*

The OD_{600} of *Si. meliloti* cultures following growth to stationary phase was measured for (A) *Sm cobT*$_{Se}^{+}$ cultures containing 5 μM indicated compounds and (B) *Si. meliloti cobD bluB* cultures containing 1 μM indicated purified cobamides. Error bars represent the standard error of three samples.

in CobT protects bacteria from producing cobamides that do not support their metabolism. It is important to understand the molecular determinants that underlie cobamide structural diversity due to the integral role played by cobamides in several important microbial communities.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

Si. meliloti strains were grown at 30°C with aeration on LB agar plates containing 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LBMC) or in M9 sucrose medium containing 10 μg/l biotin, 10 μM cobalt chloride, and 1 mg/ml methionine (Maniatis et al., 1982). When necessary, media were supplemented with antibiotics as follows: 10 μg/ml tetracycline; 100 μg/ml spectinomycin; 50 μg/ml gentamicin; and 500 μg/ml streptomycin. Cobalamin auxotrophs of *Si. meliloti* were cultured with 10 μM cyanocobalamin when needed. For extraction of corrinoids from *Si. meliloti*, 5 ml of M9 was inoculated with a colony of *Si. meliloti* grown on LBMC and incubated for 40–50 hr. These cultures were diluted to an OD_{600} of 0.02–0.04 in 250 ml M9 and incubated for 48–60 hr.

For extraction of native corrinoids from *Sa. enterica* serovar Typhimurium strain LT2, a 500 ml culture was grown anaerobically under an N_2 atmosphere in a minimal medium with ethanolamine as a nitrogen source (Keck and Renz, 2000). For guided biosynthesis experiments, *Sa. enterica* cultures were grown aerobically in NCE medium supplemented with 1 μM dicyanocobinamide and the indicated lower ligand bases with 1,2-propanediol as the carbon source (Gray and Escalante-Semerena, 2009). *L. reuteri* DSM 20016 was cultured anaerobically in vitamin B_{12}-free assay medium (Difco) at 37°C under an atmosphere of 95% N_2 and 5% CO_2 at pH 6.3. *V. parvula* DSM 2008 was cultured anaerobically in a modified *Veillonella* medium at 37°C under 95% N_2 and 5% CO_2 at pH 7.0 in which 1.5 g/l casamino acids were substituted for individual amino acids (Lopes and Cruz, 1976). For all guided biosynthesis experiments, the media were supplemented with 5 μM lower ligand base unless otherwise noted. OD_{600} measurements were recorded using a BioTek Synergy 2 multiwell plate reader. Corrinoids were purified for feeding experiments from cultures of *Sa. enterica* or *Sp. ovata* and quantified as described (Gray and Escalante-Semerena, 2009; Yi et al., 2012).

To determine the ability of DMB to enter *L. reuteri* cells, three 10 ml cultures of anaerobic MRS medium containing 5 μmol DMB were inoculated with *L. reuteri* to an OD_{600} of 0.03. Following 24 hr of growth, cells were harvested by centrifugation at 9,000 × *g* and washed in 1 ml of 0.85% saline, and the cell pellet was resuspended in 1 ml methanol. The lysate was applied to a Sep-Pak C_{18} cartridge (Waters), and the cartridge was washed with 6 ml of 20% methanol and eluted with 2 ml of 80% methanol. The extract was dried under vacuum and resuspended in 0.25 ml deionized H_2O. The DMB in the extract was quantified by HPLC as described below.

Genetic and Molecular Techniques

To construct the *Si. meliloti cobU*::Spc^R strain, a 1.3 kb genomic fragment containing the *Si. meliloti cobU* gene and flanking region was amplified by PCR and cloned into pUC19 (Yanisch-Perron et al., 1985) at the EcoRI and PstI restriction sites. An Spc^R fragment obtained by BamHI digestion of pHP45Ω (Fellay et al., 1987) was cloned into a BglII site located within the *cobU* open reading frame (ORF). The EcoRI-PstI fragment containing *cobU*::Spc^R was transferred to the suicide plasmid pK19 *mob sacB* (Schäfer et al., 1994). The resulting plasmid was introduced onto the *Si. meliloti* chromosome by triparental mating (Leigh et al., 1985). Screening for the *cobU* gene replacement and loss of the pK19 *mob sacB* plasmid were performed as described (Schäfer et al., 1994; Taga and Walker, 2010). Double mutant strains were constructed by M12 phage transduction (Finan et al., 1984).

For expression of *cobT* homologs in *Si. meliloti*, the *Sa. enterica trp* promoter sequence was introduced at the BglII and EcoRI restriction sites in the vector pMP220 to produce pMP220+P_{trp}. To construct plasmids expressing *cobT* homologs from *Si. meliloti*, *Sa. enterica*, and *Dc. mccartyi*, genomic regions including the *cobT* ORF and 20 bp of upstream sequence were amplified by PCR from purified genomic DNA (Chen and Kuo, 1993) and cloned into pMP220+P_{trp} at the XbaI and PstI restriction sites. To avoid potential codon usage incompatibility, the *L. reuteri cobT* ORF was synthesized (GeneArt) to contain codons optimized for expression in *Si. meliloti* (Puigbò et al., 2007, 2008) and fused with the 25 bp sequence directly upstream of the *Si. meliloti cobU* ORF (Figure S3) and cloned into pMP220+P_{trp}. A genomic sequence spanning the *V. parvula arsA* and *arsB* ORFs and 20 bp upstream of *arsA* was amplified by PCR from the *V. parvula* genome and cloned into pMP220+P_{trp}. The *Sa. enterica cobT* S80F and Q88M mutations were constructed by PCR-mediated site-directed mutagenesis (Weiner et al., 1994). All plasmids were introduced into *Si. meliloti cobU*::Spc^R *bluB::gus* Gm^R by triparental mating (Leigh et al., 1985).

Phylogenetic Analysis of CobT Sequences

CobT sequences were downloaded from PFAM (Chen et al., 2011) and edited using BioEdit (Hall, 1999). The phylogenetic tree was obtained from CIPRES (Stamatakis, 2006; Stamatakis et al., 2008; Miller et al., 2010) and visualized using MEGA5 (Tamura et al., 2011). Alignments were prepared using MUSCLE (Edgar, 2004). The identity matrix was constructed using BioEdit (Hall, 1999). More detailed methods may be found in Supplemental Experimental Procedures.

Corrinoid Extraction and Analysis

Corrinoids were extracted from cell pellets essentially as described (Yi et al., 2012). Briefly, cell pellets were resuspended in methanol with 0.1% potassium cyanide. Following cell lysis, clarification, and removal of solvent, the extract was applied to a Sep-Pak C_{18} cartridge (Waters) and eluted in 1.8 ml of 75% methanol. The sample was dried at 45°C under reduced pressure, resuspended in 0.5 ml distilled water, and filtered through a 10,000 MWCO filter (Pall). Samples were stored at −20°C prior to analysis.

An Agilent 1200 series HPLC system equipped with a UV-diode array detector was used to analyze the extracted corrinoids. Samples were injected onto an Agilent SB-Aq 4.5 × 150 mm column with 5 μm pore size at a flow rate of 1 ml/min with mobile phases of A, 0.1% formic acid in water, and B, 0.1% formic acid in methanol. The column was maintained at 30°C. Corrinoids were eluted with a gradient of 25% solvent B for 2 min, 25%–34% solvent B over 11 min, and 34%–70% solvent B over 3.5 min. LC-MS/MS analysis was performed using the above LC method on an Agilent 6410 liquid

chromatograph-triple quadrupole mass spectrometer with multiple reaction monitoring (Yi et al., 2012).

Synthesis and Purification of 5-Hydroxybenzimidazole

OHBza was synthesized from OMeBza as previously described (Renz et al., 1993) with some modifications and purified (see Supplemental Experimental Procedures).

ACCESSION NUMBERS

The GenBank accession numbers for the *V. parvula* arsA and arsB sequences are KF615856 and KF615857, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.08.006.

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