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The Genome of *Rhodobacter sphaeroides* Strain 2.4.1 Encodes Functional Cobinamide Salvaging Systems of Archaeal and Bacterial Origins

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Abstract

Bacteria and archaea use distinct pathways for salvaging exogenous cobinamide (Cbi), a precursor of adenosylcobalamin (AdoCbl, coenzyme B₁₂). The bacterial pathway depends on a bifunctional enzyme with kinase and guanylyltransferase activities (CobP in aerobic AdoCbl synthesizers) to convert AdoCbi to AdoCbi-GDP via an AdoCbi-P intermediate. Archaea lack CobP, and use a different strategy for the synthesis of AdoCbi-GDP. Archaea cleave off the aminopropanol (AP) group of AdoCbi using the CbiZ AdoCbi amidohydrolase to generate adenosylcobyrinic acid (AdoCby), which is converted to AdoCbi-P by the CbiB synthetase, and to AdoCbi-GDP by the CobY guanylyltransferase. We report phylogenetic, *in vivo*, and *in vitro* evidence that the genome of *Rhodobacter sphaeroides* encodes functional enzymes for Cbi salvaging systems of both bacterial and archaeal origin. Products of the reactions were identified by high performance liquid chromatography, UV-visible spectroscopy, and bioassay. The *cbiZ* genes of several bacteria and archaea restored Cbi salvaging in a strain of *Salmonella enterica* unable to salvage Cbi. Phylogenetic data led us to conclude that CbiZ is an enzyme of archaeal origin that was horizontally transferred to bacteria. Reasons why some bacteria may contain both types of Cbi salvaging system are discussed.

INTRODUCTION

Adenosylcobalamin (AdoCbl, coenzyme B₁₂) is a complex cobalt-containing cyclic tetrapyrrole coenzyme with both upper and lower axial ligands coordinating the central cobalt atom. *De novo* synthesis of AdoCbl requires a great deal of genetic information (≥25 genes) that can be found in bacteria and archaea [reviewed in (Bentley *et al.*, 2002; Escalante-Semerena, 2007; Roessner and Scott, 2006)]. The AdoCbl biosynthetic pathway has been best studied in *Pseudomonas denitrificans* and *Salmonella enterica* serovar Typhimurium LT2 (hereafter serovar Typhimurium) (Blanche *et al.*, 1995; Escalante-Semerena, 2007). In contrast, studies of AdoCbl biosynthesis in archaea have been limited (Brindley *et al.*, 2003; Thomas and Escalante-Semerena, 2000; Woodson *et al.*, 2003a; Woodson *et al.*, 2003b; Woodson and Escalante-Semerena, 2004, 2006; Zayas *et al.*, 2006).

Many organisms salvage incomplete corrinoids (e.g., cobinamide, Cbi, a stable AdoCbl precursor) from their environments. Although Cbi is not an intermediate of the *de novo* synthesis pathway (Brushaber *et al.*, 1998; Thomas *et al.*, 2000), it can be converted into one via a process known as Cbi salvaging.

Bacteria and archaea use different pathways to salvage Cbi (Fig. 1). In bacteria, Cbi salvaging starts with the attachment of 5'-deoxyadenosine (the upper ligand) to the corrin

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ring yielding adenosylcobinamide (AdoCbi) (Fonseca and Escalante-Semerena, 2000; Fonseca *et al.*, 2002). The latter is then phosphorylated to AdoCbi-phosphate (AdoCbi-P), a true *de novo* intermediate that can be ultimately converted to AdoCbl (Brushaber *et al.*, 1998). The phosphorylation of AdoCbi is catalyzed by a bifunctional NTP:AdoCbi kinase (EC 2.7.7.62), GTP:AdoCbi-P guanylyltransferase (EC 2.7.1.156) enzyme (CobP in *P. denitrificans*; CobU in *S. enterica*), which is conserved among AdoCbl-producing bacteria (Blanche *et al.*, 1991; O'Toole and Escalante-Semerena, 1995).

Previous work from our laboratory showed that archaea lack the bifunctional NTP:AdoCbi kinase, GTP:AdoCbi-P guanylyltransferase enzyme, and use an alternative pathway to salvage Cbi (Fig. 1) (Thomas and Escalante-Semerena, 2000; Woodson *et al.*, 2003a). In the archaeal pathway AdoCbi is converted to adenosylcobyrinic acid (AdoCby) by an AdoCbi amidohydrolase enzyme called CbiZ or CbiS, when CbiZ is fused to an α -ribazole-phosphate phosphatase (CobZ, EC 3.1.3.73) domain (Woodson *et al.*, 2003b; Woodson and Escalante-Semerena, 2004, 2006; Zayas *et al.*, 2006).

The conversion of AdoCby to AdoCbl proceeds via the same biochemical reactions found in bacteria, except that a monofunctional guanylyltransferase enzyme named CobY converts AdoCbi-P to AdoCbi-GDP (Thomas and Escalante-Semerena, 2000; Woodson and Escalante-Semerena, 2006).

Putative orthologs of *cbiZ* are present in approximately 78% of the available archaeal genomes (36/46 sequenced genomes), none of which contain an ortholog of *cobP*. In contrast, only 38 of the 570 (7%) bacterial genomes sequenced to date encode predicted *cbiZ* orthologs. All the bacterial genomes predicted to encode CbiZ-like proteins also synthesize proteins orthologous to CobP (Markowitz *et al.*, 2006).

In this paper, we examine the phylogenetic distribution of CbiZ in archaea and bacteria, and report evidence that supports the conclusion that CbiZ is an archaeal function that was horizontally transferred to bacteria. We also demonstrate, using an *in vivo* complementation system that predicted *cbiZ* genes from several archaea and bacteria encode functional Cbi amidohydrolases. Finally, we show that the genome of the photosynthetic α -proteobacterium *Rhodospirillum rubrum* (Mackenzie *et al.*, 2007) encodes functional CobP and CbiZ enzymes. The function of the CbiZ and CobP proteins was demonstrated *in vivo* and *in vitro*. This is the first demonstration of AdoCbi amidohydrolase (CbiZ) activity in any bacterium. These findings raised two questions: i) why do some bacteria contain two apparently redundant Cbi salvaging pathways? and ii) how is Cbi salvaged in organisms that have both systems?

RESULTS

cbiZ orthologs are broadly distributed among archaea and bacteria

We retrieved the sequences of 93 putative CbiZ orthologs, including 38 proteins from 36 archaea (out of a total 46 available archaeal genomes), and 55 proteins from 38 bacteria (out of a total 570 available bacterial genomes). For a full list of organisms and predicted *cbiZ* alleles, see Table S1 in the supplemental information.

The phylogenetic relationships among *cbiZ* genes were inferred by maximum parsimony analysis (Fig. 2A) using the DNAPARS application of PHYLIP version 3.66 (Felsenstein, 2005). For comparison, 16S rRNA gene sequences from these 74 species were also examined (Fig 2B).

Most archaea contained only a single *cbiZ* allele, but two methanogenic archaea (*Methanocorpusculum labreanum* and *Methanospirillum hungatei*) contained two copies each. Most of the bacteria we analyzed also contained a single *cbiZ* allele, but *Pelobacter propionicus* and *Bacillus* sp. B14905 contained two each, and the three species of *Dehalococcoides* contained between three and eight copies of *cbiZ*, some of which were identical in amino acid sequence, suggesting that some of these copies arose by gene duplications. In most instances, multiple *cbiZ* alleles in a single organism clade together, but the two *cbiZ* genes of *M. hungatei* were distantly related. According to the 16S phylogeny, *M. hungatei* is most closely related to *M. labreanum*, *Methanoculleus marisnigri*, and *Methanoregula boonei* (Fig. 2B). One of the *cbiZ* alleles of *M. hungatei* (locus tag Mhun0271), however, clades with *cbiZ* genes from the more distantly related methanogens *Methanococcoides burtonii*, *Methanobrevibacter smithii*, and *Methanosphaera stadtmanae* (Fig. 2A). Mhun0271 is flanked by predicted transposases in the *M. hungatei* genome (Markowitz *et al.*, 2006).

Notably, the archaeal and bacterial *cbiZ* alleles do not cluster by domain. For example, a well-supported clade (top of Fig. 2A) includes *cbiZ* alleles from six methanogenic archaea as well as bacteria from several different taxa. Similarly, the *cbiZ* genes of the actinobacteria (including *S. coelicolor* and *M. avium*) and α -proteobacteria (including *Paracoccus denitrificans* and *Rhodospirillum rubrum*) appeared to be more closely related to the *cbiZ* genes of the extremely halophilic archaea than to any other bacterial clades.

Diverse *cbiZ* alleles complement Cbi salvaging mutant strains of serovar Typhimurium

To determine whether the putative *cbiZ* genes of representative archaeal and bacterial species encoded functional Cbi amidohydrolases, we performed complementation experiments with serovar Typhimurium strains defective in Cbi salvaging. The serovar Typhimurium strains used in these experiments lacked MetE, the Cbl-independent methionine synthase, and therefore depended on the Cbl-dependent methionine synthase (MetH) to make methionine (Jeter *et al.*, 1984). To block *de novo* synthesis of the corrin ring we grew all strains under oxic conditions. Under such conditions, in medium devoid of methionine, growth depended on corrinoid salvaging.

Genes encoding putative CbiZ proteins from different sources were cloned into the plasmid pBAD24, placing the *cbiZ* genes under control of the arabinose-inducible P_{BAD} promoter (Guzman *et al.*, 1995). Plasmids were constructed containing predicted *cbiZ* alleles from the bacteria *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Bacillus halodurans*, *Streptomyces coelicolor*, and *Dehalococcoides etheneogenes*, and from the archaea *Methanosarcina mazei*, *Methanocaldococcus jannaschii*, *Ferroplasma acidarmanus*, *Pyrococcus furiosus*, and *Aeropyrum pernix* (Fig. S2, supplemental information). Plasmids harboring putative *cbiZ* alleles were transformed into serovar Typhimurium strain JE8312 ($\Delta cobU \Delta ycfN$ /pCOBY38 *cobY*⁺). This strain lacked both CobU and YcfN, and was therefore unable to salvage Cbi (O'Toole and Escalante-Semerena, 1995; Otte *et al.*, 2007), hence it was a Cbl auxotroph. Strain JE8312 also carried a plasmid encoding CobY, the AdoCbi-P guanylyltransferase from *Methanosarcina mazei* (Thomas and Escalante-Semerena, 2000), which allowed the cell to synthesize AdoCbl from Cby (Fig. 1). Plasmid pBAD24 (Guzman *et al.*, 1995) was used as negative control. Resulting strains were grown aerobically in medium supplemented with (CN)₂Cby, (CN)₂Cbi, or CNCbl, either with or without the addition of the inducer (*i.e.*, 2.5 mM arabinose) (Fig. 3).

In the absence of arabinose induction, the *cbiZ* genes from *R. sphaeroides*, *B. halodurans*, *M. jannaschii*, *F. acidarmanus*, and *P. furiosus* supported growth of strain JE8312 on Cbi, indicating that plasmid-encoded *cbiZ* genes directed the synthesis of enzymes that converted Cbi to Cby, which was ultimately converted to Cbl and allowed growth (Fig. 3, black

tracings). Complementation in the absence of induction indicated that residual transcription generated enough protein to support growth and indicated that sufficient activity was associated with the CbiZ enzymes even when the latter were present at low level. In some cases (i.e., *R. rubrum*, *M. mazei*), addition of at least 2.5 mM arabinose was needed to support growth on Cbi, but this high concentration of inducer had a deleterious effect on Cbi or Cby salvaging when the cell carried plasmids expressing *cbiZ* genes from *R. sphaeroides* or *B. halodurans* (Fig. 3, gray tracings). Cbi salvaging was not observed when the *cbiZ* genes from *S. coelicolor* or *D. etheneogenes* or the *cbiS* gene from *A. pernix* were present in strain JE8312, regardless of the concentration of arabinose (data not shown).

Cbi salvaging in serovar Typhimurium strains lacking the native Cbi salvaging system required AdoCbi-P synthetase (CbiB) function and a functional CbiZ enzyme

It was important to determine whether the *cbiZ* genes we cloned encoded proteins with the expected Cbi amidohydrolase, or whether they had a new Cbi kinase activity. To distinguish between these possibilities we tested whether CbiZ-dependent Cbi salvaging required CbiB function as observed in the archaea *M. mazei* and *Halobacterium* sp. NRC-1 (Woodson *et al.*, 2003b; Woodson and Escalante-Semerena, 2004). For this purpose we used a serovar Typhimurium strain with a mutation in the gene encoding the L-Thr O-3-phosphate decarboxylase (CobD) enzyme (Brushaber *et al.*, 1998; Grabau and Roth, 1992). As predicted by the pathway shown in figure 1, inactivation of *cobD* prevents salvaging of Cbi, because AdoCby is not converted to AdoCbi-P in the absence of aminopropanol-phosphate (AP-P). The pathway in figure 1 also indicates that the effect of the absence of CobD is correctible by the addition of aminopropanol (AP) to the medium (Brushaber *et al.*, 1998; Grabau and Roth, 1992).

Plasmids harboring putative *cbiZ* genes were transformed into serovar Typhimurium strain JE7864 (*cbiP cobD cobU/pCOBY38 cobY⁺*). In addition to lacking CobD, this strain lacked CobU to prevent Cbi salvaging by direct phosphorylation. The presence of plasmid pCOBY38 allowed strain JE7864 to synthesize Cbl from Cby, but not from Cbi. Plasmid pBAD24 was used as negative control in these experiments.

Derivatives of strain JE7864 carrying plasmids encoding *cbiZ* genes from different organisms were grown aerobically in medium supplemented with (CN)₂Cbi, (CN)₂Cbi plus AP, or CNCbl, either with or without the addition of 2.5 mM arabinose to induce expression of *cbiZ* (Fig. 4). None of the *cbiZ* alleles restored Cbi-dependent growth of strain JE7864 in the absence of exogenous AP.

In the presence of exogenous AP without arabinose induction, Cbi-dependent growth was restored by the presence of *cbiZ* alleles from *R. sphaeroides*, *B. halodurans*, *M. jannaschii*, *F. acidarmanus*, and *P. furiosus*. At high levels of inducer (2.5 mM arabinose), growth was restored by *cbiZ* genes from *R. sphaeroides*, *R. rubrum*, *M. mazei*, *B. halodurans*, *M. jannaschii*, *F. acidarmanus*, and *P. furiosus*; the onset of growth was delayed in the presence of *cbiZ* from *B. halodurans* and *R. sphaeroides*. Cbi-dependent growth was not observed at any concentration of inducer when the *cbiZ* genes from *S. coelicolor* or *D. etheneogenes* or the *cbiS* gene from *A. pernix* were present in strain JE7864 (data not shown).

Protein extracts enriched for *R. sphaeroides* CbiZ hydrolyze corrinoids *in vitro*

To confirm the *in vivo* results reported above, and to compare the activity of a bacterial CbiZ to the reported activities of archaeal CbiZ enzymes (Woodson and Escalante-Semerena, 2004, 2006), *R. sphaeroides* CbiZ was over-produced using serovar Typhimurium strain JE10789 (*cobU ycfN/pCbiZ_{RS}*), induced with 1.5 mM arabinose. Unlike at higher arabinose

concentrations (e.g. 2.5 mM, see Fig. 3), at this level of induction, pCbiZ_{RS} does not have a deleterious effect on Cbi or Cby salvaging (data not shown).

Corrinoids were incubated with protein extract enriched with CbiZ, and amidohydrolase activity was monitored by HPLC. The identity of Cby generated by this system was confirmed by comparison of the retention time (5.2 min) and the UV-vis spectrum of authentic Cby (Fig. S2, supplemental information) (Woodson and Escalante-Semerena, 2006), and by bioassay of the HPLC-purified product (data not shown). The highest specific activity was observed with AdoCbi as a substrate (880 ± 63 pmol Cby $\text{mg}^{-1} \text{min}^{-1}$). Specific activities of the enzyme when (CN)₂Cbi or CNCbl was used as substrate were 120 ± 16 and 15 ± 2 pmol Cby $\text{mg}^{-1} \text{min}^{-1}$, respectively. No AdoCbl hydrolysis was detectable. Substantially higher specific activities have been reported for some purified archaeal CbiZ proteins (Woodson and Escalante-Semerena, 2004, 2006), but direct comparisons to the CbiZ-enriched protein extract used here are difficult.

The *cobP* gene of *R. sphaeroides* complemented the AdoCbi kinase and AdoCbi-P guanylyltransferase defects of a serovar Typhimurium *cobU* strain

We selected *Rhodobacter sphaeroides* 2.4.1 as a model organism to study in more detail the corrinoid salvaging functions of a bacterium containing both the bacterial and archaeal Cbi salvaging systems. Bacteria that synthesize the NTP:AdoCbi kinase and GTP:AdoCbi-P guanylyltransferase (CobP/CobU) enzyme can efficiently salvage Cbi (Blanche *et al.*, 1991; O'Toole and Escalante-Semerena, 1995). So, why would a bacterium that synthesizes CobP (or CobU) need CbiZ? One possible explanation may be that the former might lack the Cbi kinase activity needed to salvage Cbi. To test this possibility, plasmids pCobP_{RS} (encoding *R. sphaeroides* *cobP*) and pCobU_{Se} (encoding serovar Typhimurium *cobU*) were transformed into serovar Typhimurium strain JE0824 (*cbiP cobU*), and the resulting strains were tested for their ability to salvage either Cby or Cbi. *R. sphaeroides* *cobP* restored growth of JE0824 on both Cby and Cbi (Fig. 5), indicating that CobP from *R. sphaeroides* had kinase and guanylyltransferase activities.

R. sphaeroides* CobP converts AdoCbi into AdoCbi-GDP *in vitro

To further confirm the *in vivo* results reported above, *R. sphaeroides* CobP and serovar Typhimurium CobU were over-produced using plasmids pCobP_{RS} and pCobU_{Se}. To eliminate background activity plasmids were introduced into serovar Typhimurium strain JE8268 (*cobU ycfN*), which lacked Cbi kinase and Cbi-P guanylyltransferase activities.

To test the conversion of AdoCbi to AdoCbi-GDP, AdoCbi was incubated with GTP and protein extract enriched with either CobP or CobU; synthesis of AdoCbi-GDP was monitored by HPLC. Extracts enriched with either CobU or CobP accumulated a corrinoid that eluted 6.5 min post-injection, a time that was identical to that of authentic Cbi-GDP (Fig. S3, supplemental information). The UV-vis spectrum of the compound eluting at 6.5 min was identical to the reported spectrum of Cbi-GDP (Chowdhury *et al.*, 2001). Under these conditions, the *R. sphaeroides* CobP-enriched cell-free extract accumulated 43 ± 3 pmol Cbi-GDP $\text{min}^{-1} \text{mg}^{-1}$ protein, and the serovar Typhimurium CobU-enriched extract accumulated 536 ± 32 pmol Cbi-GDP $\text{min}^{-1} \text{mg}^{-1}$ protein.

To test AdoCbi kinase activity alone, AdoCbi was incubated with ATP and cell-free extract enriched with either CobP or CobU, and Cbi-P accumulation was monitored by HPLC. Extracts enriched with either CobU or CobP accumulated a corrinoid that eluted 7.8 min post-injection, a time that was identical to that of authentic Cbi-P (data not shown). The *R. sphaeroides* CobP-enriched extract accumulated 5.7 ± 3 pmol Cbi-P $\text{min}^{-1} \text{mg}^{-1}$ protein.

The serovar Typhimurium CobU-enriched extract accumulated 74 ± 5 pmol Cbi-P min⁻¹ mg⁻¹ total protein.

To test for AdoCbi-P guanylyltransferase activity, AdoCbi-P was incubated with GTP and cell-free extract enriched with either CobP or CobU, and Cbi-GDP accumulation was monitored by HPLC. The *R. sphaeroides* CobP-enriched extract accumulated 116 ± 14 pmol Cbi-GDP min⁻¹ mg⁻¹ protein. The serovar Typhimurium CobU-enriched extract accumulated 360 ± 117 pmol Cbi-GDP min⁻¹ mg⁻¹ total protein.

The ratio of AdoCbi-P guanylyltransferase activity to AdoCbi kinase activity was 20 for the *R. sphaeroides* CobP-enriched extract, as compared to 5 for the serovar Typhimurium CobU-enriched extract. AdoCbi-P guanylyltransferase activity to AdoCbi kinase activity ratios of 14 and 2 have been reported for purified *Pseudomonas denitrificans* CobP and serovar Typhimurium CobU enzymes, respectively (Blanche *et al.*, 1991; O'Toole and Escalante-Semerena, 1995).

DISCUSSION

The distribution of CbiZ among prokaryotes suggests a history of horizontal gene transfer

The simplest interpretation of the CbiZ phylogenetic data is that the CbiZ enzyme originated among the Archaea, and that at some points the *cbiZ* gene was transferred to different bacterial lineages. In most cases, only a subset of each bacterial taxon contains *cbiZ*. For example, among the actinobacteria *S. coelicolor*, *Thermobifida fusca*, *Saccharopolyspora erythraea*, *Salinospora tropica*, *Salinospora arenicola*, *M. avium*, and *M. avium paratuberculosis* all appear to encode CbiZ proteins, but none of the other 48 available actinobacterial genomes do, including 15 other strains of *Mycobacteria* (Markowitz *et al.*, 2006).

Most of the known archaeal genomes contain at least one *cbiZ* ortholog. The relationships among archaeal CbiZ enzymes roughly parallel the 16S rRNA phylogeny (Fig. 2B), with a few exceptions, such as the CbiZ protein synthesized by the crenarchaeon *Ferroplasma acidarmanus*. CbiZ_{Fa} clusters among CbiZ proteins from the euryarchaeal *Pyrobaculum* species (Fig. 2A), again, suggestive of horizontal gene transfer. It is interesting to note that among the archaea which do not encode a CbiZ homolog, some (*e.g.* *Sulfolobus acidocaldarius*, *Pyrobaculum arsenaticum*; see Table S2, supplemental information) have close relatives with CbiZ (Fig. 2, Table S1, supplemental information), and one (*Methanothermobacter thermoautotrophicus*) is predicted to encode a full set of AdoCbl synthetic enzymes (Thomas and Escalante-Semerena, 2000). Whether or not archaea that lack CbiZ can salvage Cbi from their environment remains an open question.

The group of bacteria whose genomes encode CbiZ include pathogens such as *Leptospira interrogans* (Nascimento *et al.*, 2004), *Porphyromonas gingivalis* (Nelson *et al.*, 2003), *Ochrobacterium anthropi* (Song *et al.*, 2007; Zuo *et al.*, 2008), *M. avium* and *M. avium paratuberculosis* (Li *et al.*, 2005), and organisms of environmental relevance such as a group of thermophilic and alkaliphilic *Bacillus* and *Geobacillus* species (Takami *et al.*, 2000; Takami *et al.*, 2004), the antibiotic-producing actinomycete *S. coelicolor* (Bentley *et al.*, 2002), and the tetrachloroethene-reducing *Dehalococcoides* species (Kube *et al.*, 2005; Seshadri *et al.*, 2005). The role of Cbl and Cbi salvaging in the metabolism of these organisms has not been investigated.

Why is the *cbiZ* gene often associated with Cbl transport genes in bacteria?

The *cbiZ* genes of many bacteria are found in genetic loci close to genes encoding homologs of components of the corrinoid transport (Btu) system (Borths *et al.*, 2005; Cherezov *et al.*,

2006; Hvorup *et al.*, 2007; Kandt *et al.*, 2006; Karpowich *et al.*, 2003). For example, in *R. sphaeroides*, *cbiZ* is the last gene in an apparent five-gene operon also encoding homologs of *btuB*, *btuF*, *btuC*, and *btuD*. The *cbiZ* genes of *R. rubrum*, *Chlorobium tepidum*, *Chlorobium limicola*, *Porphyromonas gingivalis*, *Prosthecochloris aestuarii*, *Pelobacter propinicus*, and some of the *Dehalococcoides* *cbiZ* alleles are also found in close proximity to apparent *btu* operons. Even more strikingly, nearly all of the *cbiZ* genes of *Bacillus* or *Geobacillus* species are fused to genes for *btuD*, and the *cbiZ* gene of the propionate-degrading sulfate reducer *Syntrophobacter fumaroxidans* (Harmsen *et al.*, 1998) is fused to a homolog of *btuF*. We hypothesize that these associations reflect a strategy for the assembly of nucleotide loops with specific lower ligands.

CbiZ genes vary in their level of activity in a heterologous expression system

The ability of *cbiZ* genes to restore Cbi salvaging in serovar Typhimurium strains unable to convert Cbi into Cbl fell into four categories depending on the level of induction (Figs. 3, 4): 1) complementation both in the presence and absence of inducer, 2) complementation only in the absence of inducer, 3) complementation only in the presence of inducer, and 4) unable to complement at any concentration of inducer. These results suggest that, while the majority of genes annotated as *cbiZ* do indeed encode Cbi amidohydrolases, these enzymes vary in their properties. It is difficult to determine whether variation in complementation is due to differences in enzyme activity or to differences in expression levels. It is striking, however, that the *cbiZ* alleles from the hyperthermophilic archaea *M. jannaschii* and *P. furiosus*, which have optimal growth temperatures of 85 and 100°C, respectively (Bult *et al.*, 1996; Jones *et al.*, 1983) are active in serovar Typhimurium at 37°C even in the absence of inducer. It is also notable that the CbiZ protein of *R. sphaeroides* and the BtuD-CbiZ fusion protein of *B. halodurans* inhibit the conversion of Cby to Cbl when induced, suggesting that these enzymes either hydrolyze intermediates of the Cbl biosynthesis pathway (i.e., AdoCbi-P or AdoCbi-GDP), or interfere with the function of the native AdoCbl biosynthetic machinery in unknown ways. Further work is needed to examine the properties of these enzymes, and to understand how the deleterious effects of these enzymes are prevented in the organisms that synthesize them.

It is unknown whether the three *cbiZ* alleles whose activity was not detectable *in vivo* (*S. coelicolor*, *D. etheneogenes* DET0242, and *A. pernix*) are pseudogenes, are genes encoding enzymes with functions other than Cbi amidohydrolases, or are simply genes that do not function or are not expressed in serovar Typhimurium. We note that the lack of activity of *D. etheneogenes* DET0242 CbiZ may be due to the absence of a conserved asparagine residue (Fig. S1). However, the *S. coelicolor* and *A. pernix* CbiZ sequences appear unremarkable. Further studies, either with purified enzymes or in the natural host species, are necessary to determine the functional characteristics of these enzymes.

Why would any organism have dual Cbi salvaging systems?

One of the most intriguing unanswered questions in the B₁₂ field is why cobamides with different lower ligands are commonly found in nature (Renz, 1999). It is known that some cobamide-dependent enzymes are sensitive to the identity of the lower ligand (Barker *et al.*, 1960; Lengyel *et al.*, 1960). Retention of CbiZ in an organism that already synthesizes a bifunctional CobP (CobU)-like enzyme (e.g., *R. sphaeroides*) may be a response to a strong positive selection exerted by the existence in the cell of an essential Ado-cobamide-dependent enzyme that requires a specific lower ligand to function. In these organisms, the CbiZ amidohydrolase activity would ensure the recycling of the valuable corrin ring and its conversion to the specific cobamide the organism needs as a coenzyme. We speculate that organisms where such positive selection is strongest would fuse CbiZ to the Btu transport

system to ensure that every corrinoid molecule entering the cell is stripped of its nucleotide loop. Experiments are currently underway to test this hypotheses.

Other questions that need to be addressed

In a bacterium with two Cbi salvaging pathways, is one preferentially used over the other? And if so, under what conditions? Mutational and physiological analyses of *R. sphaeroides* are needed to determine the exact pathway of Cbi salvaging in this organism. The fusion of CbiZ enzymes with proteins of the Btu corrinoid-specific transport system is also intriguing, hence it would be important to determine whether CbiZ proteins that are not fused interact with Btu proteins. Ultimately, structural analyses of CbiZ proteins are needed to provide a context for the mechanistic analysis of the CbiZ enzyme.

EXPERIMENTAL PROCEDURES

Phylogenetic analysis

Sequences of predicted CbiZ proteins (members of pfam01955 (Finn *et al.*, 2006)) were retrieved from the Integrated Microbial Genomes database (Markowitz *et al.*, 2006), whereas 16S rRNA genes from each organism encoding a CbiZ ortholog were obtained from the GreenGenes database (DeSantis *et al.*, 2006). Since CbiZ domains can be fused to other domains, CbiZ sequences were trimmed to remove non-homologous sequence fused to the CbiZ core. 16S DNA sequences and trimmed CbiZ protein sequences were aligned using ClustalX 2.0 (Thompson *et al.*, 1997); the CbiZ protein alignment was then converted into a DNA alignment. Maximum parsimony trees were generated for each dataset with PHYLIP version 3.66 (Felsenstein, 2005).

Bacterial strains and growth conditions

All strains and plasmids used in this study are listed in Table S3 in the supplemental information. *E. coli* strains were grown at 37°C in lysogenic broth (LB, Difco) (Bertani, 1951, 2004). All *Salmonella enterica* sv. Typhimurium LT2 strains used in these studies were derived from strain TR6583 (*metE205 ara-9*). Serovar Typhimurium strains were grown at 37°C in LB, nutrient broth (NB, Difco), or no-carbon essential (NCE) minimal medium (Berkowitz *et al.*, 1968) containing MgSO₄ (1 mM), glycerol (22 mM), and trace minerals (Balch and Wolfe, 1976). For growth curves of serovar Typhimurium, starter cultures were grown aerobically overnight in NB containing appropriate antibiotics and used to inoculate fresh medium (5% v/v inoculum). Growth curves were obtained using an ELx808 Ultra Microplate Reader (Bio-Tek Instruments) in a total volume of 200 µl per well. When present in the medium, ampicillin was at 100 µg ml⁻¹ and kanamycin was at 50 µg ml⁻¹. When added, corrinoids were at 15 nM, and they were in their cyano form. Adenosylated corrins and (CN)₂Cbi-GDP were generated as described (Thomas and Escalante-Semerena, 2000; Thomas *et al.*, 2000). Dicyanocobyric acid [(CN)₂Cby] was a gift from Paul Renz (Universität-Hohenheim, Stuttgart, Germany). All other chemicals were purchased from Sigma.

Genetic and molecular techniques

DNA manipulations were performed using described methods (Ausubel, 1989). Restriction and modification enzymes were purchased from Fermentas (Ontario, Canada) or Promega (Madison, WI) and used according to the manufacturer's instructions. All DNA manipulations were performed in *E. coli* DH5α (Raleigh *et al.*, 1989; Woodcock *et al.*, 1989). Plasmid DNA was isolated using the Wizard Plus SV Plasmid Miniprep kit (Promega). PCR products were purified with the QiaQuick PCR purification kit (Qiagen). DNA sequencing reactions were performed using non-radioactive BigDye® protocols (ABI

PRISM; Applied Biosystems) and resolved at the Biotechnology Center of the University of Wisconsin-Madison. All primers used in this study are listed in Table S4 in the supplemental information.

Construction of bacterial and archaeal *cbiZ* plasmids

The identity of all of the *cbiZ* genes whose clone is described below was confirmed by sequencing with primers pBAD5' and pBAD3'.

pCbiZ_{Rs}—The *Rhodobacter sphaeroides* *cbiZ* gene coding sequence plus 3 bp of 5' and 15 bp of 3' sequence was amplified using primers Rs_cbiZ_EcoRI_5' and Rs_cbiZ_XbaI_3', and the resulting product was cloned into the *EcoRI* and *XbaI* sites of plasmid pBAD24 (Guzman *et al.*, 1995) to yield plasmid pCbiZ_{Rs}.

pCbiZ_{Rr}—The *Rhodospirillum rubrum* *cbiZ* coding sequence was amplified using primers Rr_cbiZ_EcoRI_5' and Rr_cbiZ_PstI_3', and the resulting product was cloned into the *EcoRI* and *PstI* sites of plasmid pBAD24 to yield plasmid pCbiZ_{Rr}.

pCbiZ_{Bh}—The *Bacillus halodurans* *btuD-cbiZ* coding sequence was amplified using primers Bh_btuD_EcoRI_5' and Bh_btuD_PstI_3', and the resulting product was cloned into the *EcoRI* and *PstI* sites of plasmid pBAD24 to yield plasmid pCbiZ_{Bh}.

pCbiZ_{Sc}—The *Streptomyces coelicolor* *cbiZ* coding sequence was amplified using primers Sc_cbiZ_NcoI_5' and Sc_cbiZ_HindIII_3', and the resulting product was cloned into the *NcoI* and *HindIII* sites of plasmid pBAD24 to yield plasmid pCbiZ_{Sc}.

pCbiZ_{De}—The *Dehalococcoides etheneogenes* *cbiZ* coding sequence (locus tag DET0242) was amplified using the primer pairs De_0242_KpnI_5' and De_0242_XbaI_3' and the resulting product was cloned into the *KpnI* and *XbaI* sites of plasmid pBAD24 to yield plasmid pCbiZ_{De}.

pCbiZ_{Mm}—The *Methanosarcina mazei* *cbiZ* coding sequence was amplified using primers Mm_cbiZ_NcoI_5' and Mm_cbiZ_SphI_3', and the resulting product was cloned into the *NcoI* and *SphI* sites of plasmid pBAD24 to yield plasmid pCbiZ_{Mm}.

pCbiZ_{Pf}—The *Pyrococcus furiosus* *cbiZ* coding sequence was amplified using primers Pf_cbiZ_KpnI_5' and Pf_cbiZ_HindIII_3', and the resulting product was cloned into the *KpnI* and *HindIII* sites of plasmid pBAD24 to yield plasmid pCbiZ_{Pf}.

pCbiZ_{Mj}—The *Methanocaldococcus jannaschii* *cbiZ* coding sequence was amplified using primers Mj_cbiZ_NcoI_5' and Mj_cbiZ_HindIII_3', and the resulting product was cloned into the *NcoI* and *HindIII* sites of plasmid pBAD24 to yield plasmid pCbiZ_{Mj}.

pCbiZ_{Fa}—The *Ferroplasma acidarmanus* *cbiZ* coding sequence was amplified using primers Fa_cbiZ_KpnI_5' and Fa_cbiZ_HindIII_3', and the resulting product was cloned into the *KpnI* and *HindIII* sites of plasmid pBAD24 to yield plasmid pCbiZ_{Fa}.

pCbiS_{Ap}—The *Aeropyrum pernix* *cbiS* coding sequence was amplified using primers Ap_cbiS_EcoRI_5' and Ap_cbiS_XbaI_3', and the resulting product was cloned into the *EcoRI* and *XbaI* sites of plasmid pBAD24 to yield plasmid pCbiS_{Ap}.

Construction of a plasmid carrying the *cobP* gene of *R. sphaeroides*

The *R. sphaeroides cobP* coding sequence plus 205 bp of 5' and 15 bp of 3' sequence was amplified using primers Rs_cobP_EcoRI_5' and Rs_cobP_XbaI_3', and the resulting product was cloned into the *EcoRI* and *XbaI* sites of plasmid pBAD24 to yield plasmid pCobP_{Rs}. The identity of the insert was confirmed by sequencing with primers pBAD5' and pBAD3' (see Table S2).

Preparation of cell-free extracts enriched in CbiZ_{Rs}, CobP_{Rs}, and CobU_{Se}

The wild-type alleles of the *R. sphaeroides cbiZ* and *cobP* genes and the serovar Typhimurium *cobU* genes were over-expressed using plasmids pCbiZ_{Rs}, pCobP_{Rs}, and pCobU_{Se}, respectively, in serovar Typhimurium strain JE8268 ($\Delta cobU \Delta ycfN$). Over-production strains were grown overnight in 5 ml of LB broth containing ampicillin, sub-cultured into 400 ml of fresh medium containing ampicillin and 1.5 mM arabinose, and incubated at 37°C with shaking for 6 h. Cells were harvested by centrifugation at 4°C (15 min at 5,000 × g) in a Beckman-Coulter Avanti J-25I centrifuge. Cells were resuspended in 4 ml of HEPES buffer (50 mM, pH 7.5) containing NaCl (100 mM) and dithiothreitol (DTT, 5 mM). Cells were broken by sonication (5 min) with a Fisher Scientific Sonic Dismembrator 550 working at half duty. Cell lysates were clarified by centrifugation (30 min at 18,000 × g at 4°C). Soluble proteins were dialyzed against two liters of the re-suspension buffer in a Slidalyzer (Pierce) cassette (MWCO 10,000) with two buffer changes, with 10% (v/v) glycerol added to the buffer for the final dialysis step. Protein extracts were flash frozen in liquid N₂ and stored at -80°C until used. As a negative control, the same procedure was also used to prepare protein extract from cells containing the control plasmid pBAD24. Extracts were examined by SDS-PAGE (Laemmli, 1970) and Coomassie Blue staining (Sasse, 1991), but over-expressed proteins were produced at too low an abundance to be seen (data not shown).

Assay of corrinoid amidohydrolase (CbiZ) activity

Corrinoid amidohydrolase activity assays were performed as described with slight modifications (Woodson and Escalante-Semerena, 2004). Briefly, assays were performed in 200 µl volumes containing 50 µg of protein extract, HEPES buffer (50 mM, pH 7.5), dithiothreitol (5 mM), and corrinoid (30 µM). Reactions were incubated at 30°C for 15 min in the dark and then heat-inactivated at 80°C for 20 min. Precipitated protein was removed by centrifugation at room temperature (5 min @ 16,100 × g). KCN was added to a final concentration of 0.1 M, corrinoids were converted to their cyano forms under strong light for 10 min. Samples containing cyano-corrinoids were filtered using 0.2 µm Spin-X columns (Corning) before they were resolved by HPLC (see below).

Assay of NTP:adenosylcobinamide kinase/GTP:adenosylcobinamide-phosphate guanylyltransferase activity

NTP:adenosylcobinamide kinase/GTP:adenosylcobinamide-phosphate guanylyltransferase activity assays were performed as described with slight modifications (Thomas *et al.*, 2000). Briefly, assays were performed in 200 µl volumes containing 25 or 50 µg of protein extract, Tris-HCl (50 mM, pH 8.5), Tris(2-carboxyethyl)-phosphine hydrochloride (1.25 mM, TCEP-HCl), MgCl₂ (10 mM), glycerol (680 mM), NaCl (0.1 M), GTP (100 µM), and AdoCbi (30 µM). Reactions were incubated at 30°C for 30 min in the dark followed by heat inactivation at 80°C for 20 min. Precipitated protein was removed by centrifugation at room temperature (5 min @ 16,100 × g). KCN was added to a final concentration of 0.1 M, corrinoids were converted to their cyano forms under strong light for 10 min, samples were filtered using 0.2 µm Spin-X columns (Corning), and corrinoids were resolved by HPLC (see below).

HPLC analysis

Corrinoids were separated by HPLC using a modification of the System I of Blanche *et al.* (Blanche *et al.*, 1990). Authentic (CN)₂Cby, dicyanocobinamide [(CN)₂Cbi], (CN)₂Cbi-GDP, and cyanocobalamin (CNCbl) were used as standards. Corrinoids were resolved using a Beckman Coulter System Gold® 126 HPLC system equipped with a Beckman Coulter System Gold® 508 autosampler and a 150 × 4.6 mm Alltima HP C18 AQ column (Alltech). Corrinoids were detected using a photodiode array detector that acquired data in the 200 – 600 nm range. The column was equilibrated at 1 ml min⁻¹ with 77% solvent A [potassium phosphate (0.1 M, pH 6.5), KCN (10 mM)] and 23% solvent B [potassium phosphate (50 mM, pH 8), KCN (5 mM), acetonitrile (50% v/v)]. The column was developed with a 43.2 min linear gradient to 47% A/53% B. A second linear gradient developed the column to 100% B over 5 min, and after 5 min, a third linear gradient returned the column to 23% B over 5 min.

The area under peaks containing CN-corrinoids was integrated and CN-corrinoids were quantified by comparison to a standard curve of authentic (CN)₂Cbi subjected to the HPLC protocol described above. The detection limit was 1 pmol of (CN)₂Cbi.

The identity of corrinoids was established by bioassay using serovar Typhimurium strains TR6583 (*cob*⁺), JE8126 (*cbiB*), and JE8312 (*cobU ycfN/pCOBY38 cobY*⁺) as indicator strains. A 2-μl sample of HPLC-purified corrinoid was spotted onto a 0.7% (w/v) agar overlay containing cells of the indicator strain on NCE glycerol minimal medium. As controls, 5 pmol of authentic (CN)₂Cby, (CN)₂Cbi, and CNCbl were also spotted onto the overlays. Plates were incubated aerobically at 37°C for 24 h. Under aerobic conditions, *de novo* corrin ring biosynthesis is blocked in serovar Typhimurium, making growth dependent on exogenously provided incomplete (e.g., Cbi, Cby) or complete corrinoids (e.g., Cbl) (Jeter *et al.*, 1984). Growth of strain TR6583 indicated the presence of a corrinoid in the extract. In strain JE8126 (*cbiB cobU*⁺), AdoCbi-P synthesis is blocked, preventing growth on Cby. However, strain JE8126 would grow if Cbi were present in the sample. In contrast, the genetic background of strain JE8312 (*cobU ycfN/pCOBY38 cobY*⁺) would support growth on Cbi, but would support growth on Cby if a functional CbiZ enzyme were made by the cell. In the absence of CbiZ, any growth of strain JE8312 indicated the presence of a corrinoid beyond Cbi in the extract, e.g., Cbl.

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Abbreviations

AP	1-amino-2-propanol
Cby	cobyric acid
AdoCby	adenosylcobyric acid
Cbi	cobinamide
AdoCbi	adenosylcobinamide
AdoCbi-P	adenosylcobinamide-phosphate

AdoCbi-GDP	adenosylcobinamide-guanosine diphosphate
Cbl	cobalamin
AdoCbl	adenosylcobalamin

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Figure 1. Abbreviated view of the cobinamide salvaging pathways in bacteria and archaea
 Corrin ring-containing intermediates are in bold text. The letter **A** indicates the *de novo* corrin ring biosynthesis pathway. Abbreviations: AP, 1-amino-2-propanol; Cby, cobyrinic acid; AdoCby, adenosylcobyrinic acid; Cbi, cobinamide; AdoCbi, adenosylcobinamide; AdoCbl, adenosylcobalamin; CbiB, adenosylcobinamide-phosphate synthetase; CobD, threonine-phosphate decarboxylase; CobU and CobP, NTP:adenosylcobinamide kinase, GTP:adenosylcobinamide-phosphate guanylyltransferase; CobY, GTP:adenosylcobinamide-phosphate guanylyltransferase; CbiZ, adenosylcobinamide amidohydrolase, CobA, ATP:corrinoid adenosyltransferase. Functional groups are indicated as follows: Me, methyl; Ac, acetamide; Pr, propionamide.

Figure 2. Phylogenetic distribution of *chiZ* orthologs among archaea and bacteria

A. Phylogenetic distribution of *chiZ* homologs. Maximum parsimony phylogeny of *chiZ* homologs, labeled with the species of origin for each gene. Genes from archaeal species are indicated with grey boxes. Nodes with bootstrap support greater than 70% are labeled. Genes whose function have been directly examined in this or previous studies (Woodson and Escalante-Semerena, 2004, 2006) are indicated with an asterisk.

B. 16S rRNA phylogeny of organisms whose genomes contain *chiZ* homologs. Maximum parsimony phylogeny of organisms whose genomes contain homologs of *chiZ*, inferred from the 16S rRNA gene. Nodes with bootstrap support greater than 70% are indicated. Organisms whose *chiZ* homologs have been directly examined in this or previous studies (Woodson and Escalante-Semerena, 2004, 2006) are indicated with an asterisk.

Figure 3. Complementation of Cbi salvaging in serovar Typhimurium by *cbiZ* alleles from bacteria and archaea

Corrinoid dependent aerobic growth of serovar Typhimurium JE8312 (*metE205 ara-9 cob1315* ($\Delta cobU$) *ycfN112* ($\Delta ycfN$)/pCOBY38 [*cobY⁺ kan⁺*]) derivatives in NCE containing glycerol (22 mM), MgSO₄ (1 mM), trace minerals, ampicillin (100 $\mu\text{g } \mu\text{l}^{-1}$), and kanamycin (50 $\mu\text{g } \mu\text{l}^{-1}$). Optical density at 650 nm was measured for 24 h at 37°C. Corrinoids were added at 15 nM. Arabinose (2.5 mM) was added as indicated. Plasmids used were: vector, pBAD24; *R. sphaeroides cbiZ*, pCbiZ_{Rs}; *R. rubrum cbiZ*, pCbiZ_{Rr}; *B. halodurans btuD-cbiZ*, pCbiZ_{Bh}; *M. mazei cbiZ*, pCbiZ_{Mm}; *M. jannaschii cbiZ*, pCbiZ_{Mj}; *P. furiosus cbiZ*, pCbiZ_{Pf}; *F. acidarmanus cbiZ*, pCbiZ_{Fa}. Growth curves were obtained using an ELx808 Ultra Microplate reader (Bio-Tek Instruments). Each growth curve was performed in triplicate.

Figure 4. CbiZ-dependent Cbi salvaging in serovar Typhimurium requires CbiB function

Corrinoid dependent aerobic growth of serovar Typhimurium JE7864 (*metE205 ara-9 cbiP236::Tn10d(tet^r) cobD1302::Tn10d(cat⁺) cobU330/pCOBY38 [cobY⁺ kan⁺]) derivatives in NCE containing glycerol (22 mM), MgSO₄ (1 mM), trace minerals, ampicillin (100 µg µl⁻¹), and kanamycin (50 µg µl⁻¹). Optical density at 650 nm was measured for 24 h at 37°C; corrinoids were added at 15 nM. Arabinose (2.5 mM) was added as indicated. 1-Amino-2-propanol (AP, 10 mM) was added as indicated. Plasmids used were: vector, pBAD24; *R. sphaeroides* *cbiZ*, pCbiZ_{RS}; *R. rubrum* *cbiZ*, pCbiZ_{Rr}; *B. halodurans* *btuD-cbiZ*, pCbiZ_{Bh}; *M. mazei* *cbiZ*, pCbiZ_{Mm}; *M. jannaschii* *cbiZ*, pCbiZ_{Mj}; *P. furiosus* *cbiZ*, pCbiZ_{Pf}; *F. acidarmanus* *cbiZ*, pCbiZ_{Fa}. Growth curves were obtained using an ELx808 Ultra Microplate reader (Bio-Tek Instruments). Each growth curve was performed in triplicate.*

Figure 5. *R. sphaeroides* *cobP* complements both the cobinamide kinase and cobinamide-phosphate guanylyltransferase defects of an serovar Typhimurium *cobU* mutant
 Corrinoid dependent aerobic growth of serovar Typhimurium JE0824 (*metE205 ara-9 cbiP236::Tn10d(tet⁺) cobU330*) derivatives at 37°C in NCE medium containing glycerol (22 mM), MgSO₄ (1 mM), and ampicillin (100 µg µl⁻¹). When added, corrinoids were present at 15 nM. Plasmids used were: vector, pBAD24; *R. sphaeroides* *cobP*, pCobP_{Rs}; serovar Typhimurium *cobU*, pCobU_{Se}. Error bars represent one standard deviation. Growth curves were obtained using an ELx808 Ultra Microplate reader (Bio-Tek Instruments). Each growth curve was performed in triplicate.