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***Salmonella enterica* synthesizes 5,6-dimethylbenzimidazolyl-(DMB)- α -riboside. Why some Firmicutes do not require the canonical DMB activation system to synthesize adenosylcobalamin**

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Abstract

5,6-Dimethylbenzimidazolyl-(DMB)- α -ribotide, [α -ribose-5'-phosphate (α -RP)] is an intermediate in the biosynthesis of adenosylcobalamin (AdoCbl) in many prokaryotes. In such microbes, α -RP is synthesized by nicotinate mononucleotide (NaMN):DMB phosphoribosyltransferases (CobT in *S. enterica*), in a reaction that is considered to be the canonical step for the activation of the base of the nucleotide present in adenosylcobamides. Some Firmicutes lack CobT-type enzymes, but have a two-protein system comprised of a transporter (*i.e.*, CblT) and a kinase (*i.e.*, CblS) that can salvage exogenous α -ribose (α -R) from the environment using CblT to take up α -R, followed by α -R phosphorylation by CblS. We report that *Geobacillus kaustophilus* CblT and CblS proteins restore α -RP synthesis in *S. enterica* lacking the CobT enzyme. We also show that a *S. enterica cobT* strain that synthesizes *GkCblS* ectopically makes only AdoCbl, even under growth conditions where the synthesis of pseudoCbl is favored. Our results indicate that *S. enterica* synthesizes α -R, a metabolite that had not been detected in this bacterium, and that *GkCblS* has a strong preference for DMB-ribose over adenine-ribose as substrate. We propose that in some Firmicutes DMB is activated to α -RP via α -R using an as-yet-unknown route to convert DMB to α -R, and CblS to convert α -R to α -RP.

Graphical Abstract

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Introduction

Cobamides (Cbas) are cobalt containing cyclic tetrapyrroles. In these structurally complex molecules the pyrrolic N atoms of the ring (a.k.a. the corrin ring) chelate the cobalt ion. In addition to these equatorial ligands, the cobalt ion interacts with two axial ligands (a.k.a. upper, lower ligands). The lower ligand of cobamides is tethered to the ring through a structure known as the nucleotide loop (Fig. 1A). Cobamides differ from each other by the nature of the base of the nucleotide loop, which can range from purines, purine analogues, or phenolics (Chan & Escalante-Semerena, 2011). The best-studied cobamide is cobalamin (Cbl), which contains 5,6-dimethylbenzimidazole (DMB) as its lower ligand base. In its coenzymic form, the upper ligand of Cbl is 5'-deoxyadenosine (AdoCbl, Fig. 1A).

Salmonella enterica sv Typhimurium strain LT2 (hereafter *S. enterica*) synthesizes the corrin ring *de novo* only under anoxic conditions (Jeter & Roth, 1987, Escalante-Semerena & Roth, 1987); for a review see (Escalante-Semerena & Warren, 2008). This bacterium incorporates different ligand bases as a function of oxygen in the environment. Under microaerophilic conditions, *S. enterica* synthesizes AdoCbl, but under anoxic conditions *S. enterica* incorporates adenine or 2-methyladenine into the final product of the pathway (Keck & Renz, 2000). The cobamide containing adenine as the lower ligand base is known as pseudoCbl (Friedmann & Fyfe, 1969).

Notably, under normoxic conditions and in the presence of precursors including a complete corrin ring *S. enterica* can assemble the nucleotide loop via the branch of the pathway known as the nucleotide loop assembly (NLA) pathway. (Escalante-Semerena, 2007). Under normoxic conditions *S. enterica* cannot synthesize the corrin ring, therefore the synthesis of AdoCba can only occur if the cell has complete corrin ring precursors in its environment. Under such conditions five enzymes are needed to assemble the nucleotide loop starting with cobinamide (Fig. 1): CobA, adenosylates cobinamide to yield AdoCbi; CobU activates AdoCbi at the expense of GTP to yield AdoCbi-GDP; CobT activates the lower ligand base at the expense of nicotinate mononucleotide (NaMN) to yield α -base-RP (where 'base' could DMB, adenine, etc); CobS joins the activated ring and base to yield AdoCba-P; and the last enzyme, CobC, dephosphorylates the molecule yielding the final product, AdoCba.

We are interested in the synthesis and activation of DMB in this bacterium (Fig. 1A, shaded, upper right). The biosynthesis of DMB has been described in other organisms. For example, in *Eubacterium limosum*, DMB synthesis proceeds through a five-enzyme pathway starting from 5'-aminoimidazole ribotide (AIR), the central branch point of thiamine and purine biosynthesis (Hazra *et al.*, 2015) by an enzyme homologous to the HMP-P synthase (ThiC) (Martinez-Gomez & Downs, 2008, Martinez-Gomez *et al.*, 2009). In *Rhodobacter sphaeroides* and other organisms, DMB synthesis proceeds through a single-enzyme conversion of reduced flavin mononucleotide (Gray & Escalante-Semerena, 2007, Taga *et al.*, 2007, Campbell *et al.*, 2006). To date, neither mechanism has been identified in *S. enterica*, although a report in the literature suggests that riboflavin may be a precursor to DMB in this bacterium (Keck *et al.*, 1998).

In *S. enterica*, DMB is activated in one step to its ribotide form (Fig. 1B). This reaction is catalyzed by the nicotinate mononucleotide (NaMN):DMB phosphoribosyltransferase CobT enzyme (Trzebiatowski *et al.*, 1994), which transfers the ribosyl-5'-phosphate moiety from NaMN to DMB, releasing the activated α -ribotide, α -ribazole-phosphate (hereafter α -RP) and nicotinate (Fig. 1A). CobT-like enzymes are wide spread amongst AdoCba producers (Rodionov *et al.*, 2003).

The *S. enterica* CobT enzyme can activate a wide variety of substrates including adenine (Cheong *et al.*, 2001). Homologues of the *Sc*CobT enzyme in the acetogenic bacterium *Sporomusa ovata* can also activate phenolic bases such as *p*-cresol and phenol (Chan & Escalante-Semerena, 2011, Chan *et al.*, 2014).

The lack of substrate specificity of the *S. enterica* CobT enzyme poses a problem for the studies of DMB biosynthesis in this bacterium. Notably, to date true DMB auxotrophs of *S. enterica* have not been isolated. *S. enterica cobT* strains require exogenous DMB to grow, but this requirement is not due to a block in DMB biosynthesis but to the presence of an alternative enzyme (Trzebiatowski *et al.*, 1994). The NAD⁺-dependent CobB sirtuin can substitute for CobT in the activation of DMB (Tsang & Escalante-Semerena, 1998), further complicating the analysis of DMB biosynthesis in this bacterium.

In *Listeria innocua*, we identified a pair of proteins that are responsible for the transport and conversion of the DMB riboside (α -R) to its α -ribotide (α -RP) (Gray & Escalante-

Semerena, 2010). The above-mentioned *L. innocua* proteins are known as *LinCblT* (a transporter), and *LinCblS* (a kinase). Notably, the *LinCblTS* proteins showed specificity for α -R, but it required α -R to be provided exogenously (Gray & Escalante-Semerena, 2010). Here, we report the identification of homologues of *LinCblT* and *LinCblS* in *Geobacillus kaustophilus*, which support Cbl-dependent growth of a *S. enterica* *cobT* strain without the addition of α -R, opening the doors for the use of genetic approaches for the analysis of DMB synthesis, and its activation to α -R.

Results

Geobacillus kaustophilus CblT and CblS restore cobamide-dependent methionine synthesis in S. enterica in the absence of exogenous α -R. Efforts aimed at validating the function of CblS and CblT homologues in Firmicutes led us to a pair of proteins from the deep-sea thermophile *Geobacillus kaustophilus*, namely GK2255 (hereafter *GkCblS*) and GK2256 (hereafter *GkCblT*). We used Clustal Omega (Sievers *et al.*, 2011) to compare the sequence of these proteins. Using this approach we determined that the *GkCblT* and *GkCblS* proteins were 45% and 51% similar, respectively, to the *L. innocua* counterparts (Fig. S1B and S1C). In *G. kaustophilus*, the *cblS* and *cblT* genes are located within the *cob* operon of *G. kaustophilus* (Fig. S1A), suggesting that in this bacterium, the CblS and CblT proteins participate in AdoCbl biosynthesis.

Assessing the functionality of *G. kaustophilus* CblTS proteins in *S. enterica*

To determine whether or not *GkCblS* and *GkCblT* were functional, the *G. kaustophilus* *cblT*⁺ and *cblS*⁺ alleles were placed under the control of the arabinose-inducible P_{araBAD} promoter of plasmid pBAD24 (Guzman *et al.*, 1995). The resulting plasmid (p*GkCblTS2*) was transformed into JE12939 (*cobT cobB*), which lacked the two enzymes (CobT, CobB) known to activate lower ligand bases into their corresponding α -ribotides (Trzebiatowski *et al.*, 1994, Tsang & Escalante-Semerena, 1998). Strain JE12939 also lacked the cobamide-independent methionine synthase MetE enzyme, resulting in a methionine auxotrophy that, under normoxic conditions, could be corrected by cobamides or methionine (Jeter *et al.*, 1984). In the absence of methionine, the strain uses the cobamide-dependent methionine synthase MetH enzyme to methylate homocysteine yielding methionine (Jeter *et al.*, 1984); the *metE cobT cobB/pGkCblTS2* strain is hereafter referred to as JE17830. A negative control strain (JE17827) had the same genetic background but carried the empty cloning vector pBAD24. A positive control strain (JE17828) harboring a plasmid encoding the wild-type CobT protein was also constructed.

The above-mentioned strains were used in experiments aimed at assessing α -RP synthesis *in vivo* and whether *GkCblT*, *GkCblS* or both were needed. For this purpose, strains were grown under normoxic conditions at 37°C. In all experiments described below, minimal NCE medium was supplemented with (CN)₂Cbi as a precursor of AdoCbl. We supplemented the culture medium with (CN)₂Cbi because under normoxic conditions *S. enterica* does not synthesize the corrin ring (Jeter & Roth, 1987, Jeter *et al.*, 1984), due to the lack of activation of the adenosylcobinamide-phosphate (AdoCbi-P) biosynthetic genes

(Escalante-Semerena & Roth, 1987). In *S. enterica*, the conversion of (CN)₂Cbi into AdoCbl requires the synthesis of α -RP by *SeCobT*.

i) GkCbITS compensate for the absence of CobT in the presence of high concentrations of cobinamide and the absence of exogenous α -R—

In the first experiment, the medium was supplemented with (CN)₂Cbi (5 nM) but not with α -R. Under these conditions strain JE17827 carrying the cloning vector failed to grow (Fig. 2A, triangles) and required the addition of CNCbl to grow (Fig. 2A, diamonds), the strain carrying plasmids encoding *cobT*⁺ (Fig. 2A, squares) or *cbITS*⁺ (Fig. 2A, circles) exhibited robust growth.

ii) At low concentrations of cobinamide GkCbITS compensate for the absence of CobT only if DMB or α -RP is provided—

It has been reported that the activity of the AdoCbl-5'-P synthase CobS enzyme of *S. enterica* increases when the enzyme is saturated with either AdoCbi-GDP or α -RP (Zayas & Escalante-Semerena, 2007). With this in mind, we decreased the concentration of (CN)₂Cbi to determine whether the addition of DMB would stimulate α -R synthesis, hence growth. Strain JE17830 was grown as above, except that the concentration of cobinamide in the medium was reduced to 0.25 nM. (a 200-fold reduction). At this cobinamide concentration, strain JE17830 failed to grow (Fig. 2B, solid circles). In contrast, the addition of DMB (200 μ M) or α -R (300 nM) restored growth (Fig. 2B, open circles, black triangles, respectively). These data suggested that DMB synthesis or scavenging was the limiting factor in the synthesis of α -RP in strain JE17830 under the conditions tested.

iii) GkCbIT is not required for the conversion of DMB to α -R—These of the experiments described in section ii) above did not inform on whether or not CblT function (α -R transporter) was needed for the conversion of DMB to α -R. To address the latter question, a strain carrying a plasmid encoding *GkCbIS* under P_{araBAD} control was constructed and grown under limited concentration of cobinamide (0.25 nM) with or without α -R or DMB supplementation, or with excess cobinamide (5nM). As shown in figure 2C, when the concentration of cobinamide was limiting and neither DMB nor α -R was present in the medium, strain JE21364 (*metE ara-9 cobT cobB/pGkCbIS*) did not grow (Fig. 2C, squares). Strain JE21364 failed to respond to the presence of DMB (Fig. 2C, diamonds), but did respond to the presence of α -R (Fig. 2C, solid triangles). In addition, when the concentration of cobinamide was increased 200 fold (to 0.5 nM) without α -R supplementation, the strain grew fast and to high density with a minimal lag time (Fig. 2C, open triangles). Our interpretation of these is discussed below.

***G. kaustophilus* CblS is an α -ribazole kinase**

The similarity of *GkCbIS* to *L. innocua* CblS (Fig. S1) suggested that *GkCbIS* had α -R kinase activity. To investigate this possibility, *G. kaustophilus cblS*⁺ was cloned into vector pTEV5 (Rocco *et al.*, 2008) and over-produced as a hexahistidine-tagged fusion protein in strain JE13607 (*E. coli* BL21(λ DE3) *cobT762::kan*⁺). The protein was purified by nickel-affinity chromatography; the His₆ tag was not removed. Elution of the tagged protein from

the column resulted in highly enriched His₆-*GkCblS* around approximately 28.5 kDa (Fig. 3, lane 6), consistent with the expected molecular mass of *GkCblS* (25.5 kDa).

The purified protein was tested for α -R kinase and NaMN::DMB phosphoribosyltransferase (CobT) activity. To test for α -R kinase activity, protein (3 μ g) was added to a reaction mixture containing HEPES (50 mM, pH 7.4), MgCl₂ (5 mM), KCl (750 mM), TCEP (5 mM), ATP (0.5 mM), and α -R (0.25 mM). The reaction was incubated for 3 h at 37 °C. As a negative control, a reaction mixture devoid of protein was incubated under the same conditions. The products of the reactions were separated by RP-HPLC on a C18 column as described under *Experimental procedures*. The addition of *GkCblS* resulted in the loss of the α -R peak (Fig. 4A, 10.2 min) and formation of a new peak (Fig. 4B, 8.9 min). To verify the identity of this peak, DMB and NaMN were incubated with purified *SeCobT* to generate α -RP, and the reaction products were separated by RP-HPLC using the same conditions. Addition of *SeCobT* resulted in the loss of the DMB peak (Fig. 4C, 11 min) and the formation of the expected product α -RP peak (Fig. 4D, 8.5 min). The UV-spectra and retention times were similar to those observed in Fig. 4B, indicating that the product of *GkCblS* was α -RP. An observed shift in the retention time for α -RP (8.5 min to 8.9 min) between the *SeCobT* and the α -R kinase results was likely due to a difference in the pH of the CobT reaction mixture (10 vs 7.4). The UV-vis spectrum of the compound eluting 8.5 min post injection was similar to the spectrum of the compound eluting 8.9 min post injection. In a parallel experiment, DMB and NaMN were incubated with *GkCblS*, but no α -RP formation was detected (data not shown).

S. enterica* does not produce pseudoCbl in the presence of *GkCblS

To determine what lower ligand *GkCblS* activated when produced in *S. enterica*, corrinoids were extracted from cells grown under different conditions. Deproteinized extracts obtained as described under *Experimental procedures* were resolved by RP-HPLC and fractionated. Two indicator strains were used to establish the presence of cobamides in deproteinized extracts of strains of interest. The first indicator strain was JE8214 (*metE cobS*), a strain lacking the Cbl-independent methionine synthase (MetE) and the AdoCbl-5'-P synthase (CobS) enzymes. The absence of MetE demanded that the methylation homocysteine to methionine occurred via the Cba-dependent MetH enzyme; the absence of CobS in strain JE8214 blocked adenosylcobamide biosynthesis. The presence of *metE* and *cobS* null alleles in strain JE8214 rendered the strain auxotrophic for cobamides or methionine. To determine whether methionine or cobamides were present in deproteinized extracts, we used a second indicator strain, namely strain JE12403. In addition to the *metE* and *cobS* null alleles, strain JE12403 also carried a *btuB* null allele. The BtuB protein is responsible for the transport of corrinoids (including cobamides) across the *S. enterica* outer membrane. Unlike strain JE8214, strain JE12403 grew only if methionine was present in the deproteinized extract being tested. A soft agar layer seeded with one of these strains was poured over a minimal medium plate lacking corrinoids. Samples of fractions obtained after RP-HPLC analysis of deproteinized extracts were spotted onto the hardened soft agar layer. A fraction was determined to contain a complete cobamide if it supported growth of strain JE8214 but not JE12403. The presence of cobamides in a fraction of interest was confirmed by UV-vis spectroscopy and mass spectrometry.

As a control, a mixture of authentic pseudoCbl, (CN)₂Cbi, and CNCbl at a known concentration was resolved, fractionated, and assayed (Fig. 5A). Bioassay of the fractions collected indicated the presence of complete cobamides in fractions eluting between 11.5 min and 18.5 min post injection. The strongest signals were detected at 12 and 17 min. UV-vis spectra and mass spectrometry confirmed the presence of pseudoCbl and Cbl, respectively. The peak observed at 14 min in the HPLC trace was cobinamide (denoted by (CN)₂Cbi in Fig. 5).

RP-HPLC analysis of deproteinized extracts of strain JE17828 (p*SeCobT*^{WT}) grown on minimal medium supplemented with (CN)₂Cbi (300 nM) but no DMB, showed the presence of complete cobamides in fractions collected between 11.5 min and 13 min as well as between fractions collected between 16 min and 17.5 min (Fig. 5C). The strongest signal was observed with compounds eluting 12 min post injection. When strain JE17830 (p*GkCblTS*^{WT}) was grown under similar conditions, complete cobamides were detected in fractions collected between 14.5 and 18 min, with the strongest signal obtained with compounds eluting 17 min post injection (Fig. 5B). These data suggested that strain JE17830 did not synthesize pseudoCbl, an idea that was supported by the lack of signal in the UV-vis spectra (Fig. 5B) or the mass spectrometry data (data not shown).

To try and guide cobamide biosynthesis towards pseudoCbl, adenine (200 μM) was added to the culture medium already containing (CN)₂Cbi (300 nM). Deproteinized extracts of strain JE17828 contained complete cobamides with the strongest signal observed at 12.5 min post injection (Fig. 5E). Extracts of strain JE17830 grown under the same conditions contained complete cobamides between 14.5 min and 18 min, with the strongest signal observed at 17 min (Fig. 5D). These data supported the conclusion that JE17830 did not produce pseudoCbl under the growth conditions used. For comparison, a strain with the same genetic background but carrying a plasmid encoding *SeCobT*^{WT} made pseudoCbl (Fig. 5E).

To determine whether or not the addition of DMB would change the cobamide made by strains JE17828 (p*SeCobT*) and JE17830 (p*GkCblTS*), DMB (200 μM) was added to medium containing (CN)₂Cbi (300 nM). RP-HPLC analysis of deproteinized extracts of strain JE17828 showed a shift, with cobamides detected between 12.5 min and 18.5 min, with the largest peak at 17 min (Fig. 5G). That is, strain JE17828 made Cbl only, with no detectable evidence of pseudoCbl production. In contrast, extracts of strain JE17830 contained complete cobamides that were detected between 14.5 min and 18 min post injection, with the strongest growth response observed when material eluted at 17 min was tested (Fig. 5F). Notably, no detectable increase was observed in the bioassay of any of the fractions of extracts of strain JE17830. Close analysis of the chromatogram at 17 min revealed a signal in extracts of the strain grown in DMB-supplemented medium, but not in extracts of the same strain grown in adenine-supplemented medium or in extracts of the strain grown in medium devoid of base supplements. We note that a signal detected in all prepared samples around 16 min (denoted by X in Fig. 6) was not a cobamide on the basis of the UV-vis spectrum and bioassay results (Fig. S2), did not support growth (data not shown) and was not further identified.

Discussion

Here we report that the CblS and CblT proteins from the Firmicute *G. kaustophilus* can substitute for the CobT enzyme in the synthesis of the intermediate α -RP of the AdoCba biosynthetic pathway in *S. enterica*. One interesting finding of this work was that when *GkCblS* substituted for *SeCobT*, the the product of the pathway was AdoCbl. Below we discuss our interpretation of the results and the implications of the findings.

What is the physiological role of the *GkCblTS* proteins in Firmicutes?

Prior to this work, it was accepted that in cobamide producers DMB activation to α -RP was catalyzed by a DMB:NaMN phosphoribosyltransferase, *e.g.*, the CobT enzyme of *S. enterica* (Trzebiatowski & Escalante-Semerena, 1997). From previous work we learned that some Firmicutes (*e.g.*, *L. innocua*) possessed what appeared to be an α -R salvaging system comprised of an α -R transporter (CblT) and an α -R kinase (CblS) (Gray & Escalante-Semerena, 2010). The identification of the CblTS system in *L. innocua* left one question unanswered. Since the *L. innocua* genome does not encode a CobT homologue, is *L. innocua* an obligate α -R salvaging bacterium? If the answer were yes, then one wonders how this bacterium survives in an environment where it needs to synthesize AdoCbl, but the environment is devoid of α -R. The results reported herein recapitulate the *L. innocua* conundrum in a *S. enterica* *cobT* strain that can synthesize CblTS. Growth analyses of such a strain showed that *S. enterica* has an as-yet-unknown mechanism to synthesize α -R. We propose that Firmicutes also synthesize α -R endogenously, and that the CblTS proteins serve the dual purpose of being a system that generates α -RP from exogenous or endogenously synthesized α -R.

S. enterica synthesizes α -R, a previously unknown capability of this bacterium

The *in vivo* analyses of *GkCblTS* functions revealed an unexpected metabolic capability of *S. enterica*, which is that *S. enterica* can convert DMB into its riboside, α -R. Results shown in figure 2, support the following conclusions: i) In the presence of high amounts of cobinamide, an AdoCbl precursor, *GkCblTS* can compensate for the lack of *SeCobT* during the synthesis of AdoCbl without any addition to the medium, meaning that the α -R needed by the *GkCblS* kinase to make α -RP must be endogenously synthesized (Fig. 2A); ii) under the same growth conditions (*i.e.*, high exogenous cobinamide levels), the *GkCblT* protein is dispensable (Fig. 2C, open triangles), supporting the idea that *GkCblS* is necessary and sufficient for the phosphorylation of α -R; iii) that *GkCblT* may be able to transport DMB (compare open circles in Fig. 2B to diamonds in Fig. 2C); iv) that *S. enterica* can acquire α -R in the absence of *GkCblT* (Fig. 2C, solid triangles). Since *S. enterica* does not make a CblT homologue, these results suggest that α -R is transported into *S. enterica* via non-specific means. Although it is unclear whether DMB was endogenously synthesized or salvaged in the experiments performed in these studies, the results strongly support the conclusion that *S. enterica* can convert DMB to α -R. To the best of our knowledge, this is the first example of such a conversion in any organism.

GkCblS has kinase activity, and appears to prefer α -R over α -adenosine as substrate

Given the primary sequence similarity between *GkCblS* and *LinCblS* (Fig. S1), it was not unexpected to find that *GkCblS* had α -R kinase activity. What was surprising, however, was the strong preference of the enzyme for α -R over α -adenosine. Results of guided biosynthesis experiments with *GkCblS* as the sole means to activate DMB or adenine failed to yield pseudoCbl, even though the cultures were grown in the presence of excess adenine and no exogenous DMB. These results suggest that *GkCblS* had a strong preference for α -R over α -adenosine (Fig. 5D). When the control experiment was performed with *SeCobT*, the cell made mostly pseudoCbl (Fig. 5E), consistent with our knowledge that the *SeCobT* enzyme can use either DMB or adenine as substrate. The conclusion that *GkCblS* prefers α -R over α -adenosine as substrate is further supported by results of *in vivo* (Fig. 2) and *in vitro* experiments (Fig. 4).

An opportunity to identify the *S. enterica* enzyme that converts DMB to α -R, and to investigate the putative DMB biosynthetic pathway in this bacterium

An understanding of if and how *S. enterica* makes DMB has remained an enigma since the discovery of cobamide biosynthesis in this bacterium was reported over three decades ago (Jeter *et al.*, 1984). The lack of a system that would use α -R but not α -adenosine as substrate made it difficult to isolate *bona fide* DMB auxotrophs of *S. enterica*. Because strain JE21634 (*met ara-9 cobT cobB/pGkCblS*) only makes AdoCbl, this genetic background offers an opportunity for the use of *in vivo* genetic approaches to investigate the putative DMB biosynthetic pathway in this bacterium, and the conversion of DMB to α -R. To our knowledge, the existence of these enzymes has not been reported in *S. enterica* or any other cobamide producer.

At present, it is unclear whether *S. enterica* synthesizes DMB *de novo*. The claim that this bacterium synthesizes DMB is weakened by the fact that genes encoding enzymes responsible for its synthesis have not been identified. It is possible that *S. enterica* and other organisms synthesize DMB via an as-yet uncharacterized pathway distinct from the five-enzyme, anoxic pathway found in *Eubacterium limosum* (Hazra *et al.*, 2015) and the single enzyme, oxygen-dependent pathway identified in *Rhodobacter sphaeroides* and *Sinorhizobium meliloti* (Gray & Escalante-Semerena, 2007, Campbell *et al.*, 2006, Taga *et al.*, 2007). The absence of *bona fide* *S. enterica* DMB auxotrophs suggests that either that this bacterium does not have a dedicated pathway for DMB synthesis, that the pathway is also needed for other metabolic purposes, or that one or more of the functions involved in the putative pathway is essential. The isolation of DMB auxotrophs probably has been complicated by the broad distribution of DMB in the environment and culture media commonly used in our laboratories (Crofts *et al.*, 2014).

An expanded model for the synthesis of α -RP in bacteria

Collectively, our data support the conclusion that *S. enterica* can synthesize α -R but cannot phosphorylate it to α -RP (Figs. 2, 5). It appears, however, that some Firmicutes have evolved an α -R kinase that can phosphorylate α -R. It is unclear what the selective pressure for the evolution of such a kinase may have been in *G. kaustophilus*, since the genome of this bacterium also encodes a homologue of the *SeCobT* enzyme (locus tag *gk1793*). It is

possible that this bacterium is exposed to α -R in the marine environment it inhabits, which is not unlikely since the presence of α -R in marine environments was recently reported (Johnson *et al.*, 2016). If that were the case, then the *GkCblTS* proteins would likely be the α -R scavenging system, while the *GkCobT* enzyme would play a role in *de novo* synthesis in this bacterium. Figure 6 shows a model for the synthesis and activation of DMB in bacteria that either have the canonical DMB activation system (e.g., *CobT*) or the *CblS*-dependent pathway to convert α -R, to α -RP. We suggest that *G. kaustophilus* uses *GkCblTS* and *GkCobT* for different purposes.

Experimental procedures

Chemicals

Unless otherwise indicated, chemicals were obtained from Sigma-Aldrich. Pseudocobalamin was extracted from laboratory strains by growing *S. enterica* in medium containing 1,2-propanediol as the sole source of carbon and energy. Under such conditions, this bacterium produces only pseudoCbl (Anderson *et al.*, 2008). Corrinoids were extracted from cultures grown on 1,2-propanediol using procedures described below. Alpha-ribazole (α -R) was obtained by the method described in (Pakin *et al.*, 2005) with the modifications described in (Gray & Escalante-Semerena, 2010).

Bacterial strain construction

All strains and plasmids used are listed in table 1. Transformations were performed as described (Maniatis *et al.*, 1982) with no modifications.

Plasmid construction

All primers used in this study are listed in table 2. DNA amplification and restriction digestions were performed according to manufacturer's protocols. All restriction enzymes used were purchased from Thermo Fisher Scientific Inc., unless otherwise stated.

pGkCblTS1—The adjacent *Geobacillus kaustophilus cblT*⁺ and *cblS*⁺ coding sequences (locus tags *gk2256* and *gk2255*, respectively) were amplified from *G. kaustophilus* HTA426 genomic DNA using primers Gk_cblTS_EcoRI_5' and Gk_cblTS_HindIII_3' and the resulting PCR product was inserted into plasmid pGEM-T Easy (Promega) using the cloning strategy described by the manufacturer; the resulting plasmid was named pGkCblTS1.

pGkCblTS2—The *G. kaustophilus cblT*⁺ and *cblS*⁺ coding sequences were excised from pGkCblTS1 and subcloned into the EcoRI and HindIII sites of pBAD24 to yield plasmid pGkCblTS2. The plasmid pGkCblTS2 was used for complementation assays and corrinoid analyses.

pGkCblS1—The *G. kaustophilus cblS*⁺ coding sequence was amplified off pGkCBLTS2 using primers Gk_cblS_XbaI_RBS_5' and Gk_cblTS_HindIII_3'. The DNA fragment was cut and inserted between the XbaI and HindIII sites of pBAD33. The resulting plasmid was named pGkCblS1

pGkCblS2—This vector was constructed using the polymerase incomplete primer extension (PIPE) method (Klock *et al.*, 2008). The *G. kaustophilus cblS*⁺ coding sequence was amplified off pGkCBLs1 using the primers pTEV5_PIPE_gkCblS_5' and pTEV5_PIPE_gkCblS_3'. The vector backbone pTEV5 was amplified using the primers pTEV5_PIPE_NheI_5' and pTEV5_PIPE_XhoI_3'. Following overnight cutting with DpnI, the fragments were mixed in equal volumes and transformed into chemically competent *E. coli* DH5α cells (Maniatis *et al.*, 1982). The constructed plasmid results in the *cblS* coding sequence being inserted between the NheI and XhoI sites of pTEV5. The plasmid pGkCblS2 was used for protein overproduction.

pGkCblS3—The *G. kaustophilus cblS*⁺ coding sequence was amplified off pGkCBLs1 using primers Gk_cblS_EcoRI_5' and Gk_cblTS_HindIII_3'. The DNA fragment was cut and inserted between the EcoRI and HindIII sites of pBAD24. The plasmid pGkCblS3 was used for complementation assays.

pCOBT81—The *Salmonella enterica cobT*⁺ coding sequence was excised from pJO27 (O'Toole *et al.*, 1993) and subcloned into the SacI and HindIII sites of pBAD33 to yield plasmid pCOBT81. The plasmid pCOBT81 was used for constructing pCOBT140.

pCOBT140—The *Salmonella enterica cobT*⁺ coding sequence was amplified off pCOBT81 using primers Sal_CobT_BAD24_EcoRI_5' and Sal_CobT_BAD24_XbaI_3'. The DNA fragment was inserted between the EcoRI and XbaI sites of pBAD24. The plasmid pCOBT140 was used for complementation assays and corrinoid analyses.

Growth analyses

Strains were grown at 37 °C in no-carbon essential (NCE) minimal medium (Berkowitz *et al.*, 1968) supplemented with ribose (22 mM) as a carbon and energy source. Minimal medium also contained trace minerals, magnesium sulfate (1 mM), ampicillin (100 µg mL⁻¹), and L(+)-arabinose (500 µM). Where indicated, 5,6-dimethylbenzimidazole (DMB, 200 µM), α-ribazole (α-R, 300 nM), dicyanocobinamide [(CN)₂Cbi] (0.25 nM or 5 nM), and cyanocobalamin (CNCbl, 0.5 nM) were added. α-Ribazole was synthesized as reported (Gray & Escalante-Semerena, 2010). Growth analyses were performed in 96-well microtiter dishes, with each strain grown under indicated conditions in duplicate. Each well contained 200 µL of medium inoculated with 1% (v/v) of an overnight starter culture grown for at least 20 h on nutrient broth (Difco) to lower the level of residual nutrients in the medium. Cell density was monitored at 650 nm using a computer-controlled BioTek ELx808 absorbance plate reader (BioTek Instruments). Readings were acquired every 30 min with continuous shaking. Data were analyzed using the GraphPad Prism v4 software package (GraphPad Software).

Corrinoid extraction and analysis

Production—Strains were grown at 37 °C in NCE minimal medium supplemented with ribose (22 mM) as a carbon and energy source. Minimal medium also contained trace minerals, magnesium sulfate (1 mM), ampicillin (100 µg mL⁻¹), (CN)₂Cbi (300 nM), and L(+)-arabinose (500 µM). Where indicated, DMB or adenine (200 µM) was added. Culture

medium (100 mL) was prepared in 500-mL baffled flasks and inoculated by the addition of 1% (v/v) of an overnight culture grown for at least 20 h in nutrient broth (NB, Difco). Cultures were grown for >20 h at 37 °C with shaking (150 rpm) (New Brunswick Innova 44R refrigerated incubator shaker). The cells were then harvested by centrifugation at 5,500 × *g* for 15 min (Beckman/Coulter Avanti J25-I centrifuge, equipped with a JLA-16.250 rotor). Cell pellets were resuspended in 5 mL of 100 mM ammonium acetate solution containing potassium cyanide (10 mM) at pH 4, and frozen at –20 °C until processed.

Extraction protocol—Cobamide extraction and isolation was performed using a modification of procedures reported elsewhere (Keller *et al.*, 2014, Kittaka-Katsura *et al.*, 2002). Briefly, frozen cell suspensions were thawed and incubated at 70 °C with constant shaking (100 rpm) for 2.5 h using a ThermoScientific MaxQ 4000 shaker. Cell debris was removed by centrifugation at 43,000 × *g* for 1 h (Beckman/Coulter Avanti J25-I centrifuge, equipped with a JA-25.25 rotor). The supernatant was passed over a 0.45-μm polyethersulfone syringe filter (VWR). Amberlite XAD4 resin (Rohm and Haas) was cleaned of impurities by washing with methanol, followed by extensive washing with water prior to use. Amberlite XAD4 resin (250 mg) was used per ml of extract, and allowed to incubate overnight at 37 °C with shaking to bind corrinoids. The unbound material was removed separated from the resin by suction, and the resin was washed with 10 volumes of MilliQ water (EMD Millipore). Two volumes of methanol were added to the resin and incubated overnight to desorb corrinoids. The methanol layer was pulled off, dispensed into a tube, and concentrated under vacuum (Eppendorf Vacufuge Plus) at 60 °C for 5.5 h at max speed (1400 rpm). The pellets were resuspended in 400 μL of a 1:4 ratio of buffer B (KH₂PO₄ (100 mM), KCN (10 mM), pH 8 + 50% (v/v) acetonitrile) and buffer C (KH₂PO₄ (100 mM), KCN (10 mM), pH 6.5). Resuspended samples were passed over a Costar® Spin-X® Centrifuge Tube Filter (Corning; 0.45 μm cellulose acetate) and incubated overnight at 4 °C.

Isolation—Ten-fold dilutions of the extracts were prepared and passed over a Costar® Spin-X® centrifuge tube filter (Corning; 0.45 μm cellulose acetate). The filtrates were dispensed into Prominence 12×32mm MT-IT autosampler vials (Shimadzu) and capped with 9-mm screw caps fitted with PTFE/Silicone septa (VWR). Cobamides were resolved by RP-HPLC using a Shimadzu Prominence UFLC equipped with a Phenomenex Synergi Hydro-RP (150 × 4.6 mm) as described (Blanche *et al.*, 1990, Chan & Escalante-Semerena, 2011), with some modifications. The column was equilibrated at 20 % buffer B (see above) for 2.5 min at a flow rate of 1.0 mL min^{–1} and developed on a linear gradient to 35% buffer B over 9 min, further developed to 100% buffer B over 7 min, and operated for 5 min at 100% buffer B. Corrinoids were detected at 367 nm on a computer-controlled Shimadzu Nexera X2 SPD-30A diode array detector. Fractions (0.5 mL ea.) were collected on a computer-controlled Shimadzu FRC-10 fraction collector between 7 and 20 min post injection.

Bioassay—Fractions were tested for the presence of complete corrinoids by bioassay. Agar plates containing NCE medium were supplemented with trace minerals, ribose (22 mM), and magnesium sulfate (1 mM) and antibiotic (chloramphenicol, 5 μg/mL; or kanamycin, 25 μg/mL). A 4-mL soft agar (0.7%, w/v) overlay was seeded with 100 μL of an overnight

starter culture of indicator strains JE8214 (*cobS*) or JE12403 (*cobS btuB*) and poured over the minimal plates. Samples (2 μ L ea.) of the fractions were spotted on the overlay and plates were incubated overnight at 37 °C. A halo of growth in the soft agar layer seeded with JE8214 (*cobS*) but not in the layer seeded with JE12403 (*cobS btuB*) indicated the presence of a cobamide. The diameter of the halo was recorded and compared to the HPLC trace. Cobamide identity was analyzed by MALDI-TOF mass spectrometry.

Purification of His₆-tagged GkCblS protein

His₆-tagged GkCblS was overproduced in JE13607 (BL21 (λ DE3) *cobT762::kan^r*) (Chan & Escalante-Semerena, 2011) using plasmid pGkCblS2 (*cblS^r*). Cells were grown in 50 mL of Terrific Broth (Cold Spring Harbor Laboratory Protocols, <http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8620>) overnight at 37 °C in a 250-mL flask. The cells were harvested by centrifugation at 6,000 $\times g$ using an Avanti J-20 XPI floor centrifuge equipped with a JLA-8.1000 rotor (Beckman Coulter). Eight grams of cell paste were frozen at –80 °C until use. Cell paste was resuspended in binding buffer containing HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 50 mM), NaCl (500 mM), imidazole (10 mM), pH 7.3 @ 24 °C). The cell paste (g): buffer (mL) ratio used was 1:5. To ensure cell lysis, 15 mg of lysozyme was added to the suspension and the cell was allowed to incubate for 30 min at room temperature. The serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Acros Organics) was added at a concentration of 200 μ M. Cells were broken by sonication (3 cycles, 50% duty, 1.2-min cycle, 2 s on, 8 s off) with a 550 Sonic Dismembrator (Fisher Scientific). A salted ice bath was used to prevent enzyme inactivation due to heat. Cell debris was removed by centrifugation at 39,000 $\times g$ using an Avanti J-25I floor centrifuge with a JA-25.25 rotor (Beckman Coulter). The supernatant was passed over a 0.45- μ m polyethersulfone syringe filter (VWR) to remove any remaining debris.

The filtered cell-free extract was loaded onto 5 mL of Ni-NTA affinity resin (HisPur; ThermoFisher Scientific) column. After loading, the column was washed with four column volumes using the following buffers: binding buffer (described above); washing buffer [HEPES (50 mM, pH 7.3), NaCl (500 mM), imidazole (150 mM)]; and elution buffer [HEPES (50 mM, pH 7.3), NaCl (500 mM), imidazole (750 mM), *tris*(2-carboxyethyl)phosphine (TCEP, 5 mM), glycerol (5%, v/v)]. The final elutant was concentrated using an Amicon-15 concentrator (MWCO 10 kDa; Millipore) for 13 min at 3,220 $\times g$ (Eppendorf 5810). The concentrate was dialyzed against decreasing salt concentrations down to 250 mM NaCl. The first dialysis also included EDTA (0.5 mM) for nickel chelation. The final protein concentration was quantified using a microtiter plate protocol for the Bradford protein assay (Bradford, 1976) using a commercially available kit (BioRad). Purified protein was flash frozen in liquid nitrogen and stored at –80 °C until used.

Assessment of α -ribazole kinase activity

***In vitro* activity assay**—The enzymatic activity of purified His₆-GkCblS protein was assayed using a modification of the α -R kinase assay (Gray & Escalante-Semerena, 2010). Reaction mixtures (100 μ L) contained HEPES buffer (50 mM, pH 7.4), MgCl₂ (5 mM), KCl (750 mM), TCEP (5 mM), ATP (0.5 mM), α -ribazole (0.25 mM) and protein (3 μ g); α -R

was obtained as described (Gray & Escalante-Semerena, 2010). Reactions were incubated for 3 h at 37 °C in a BioRad DNA Engine Dyad ThermoCycler, and stopped by heating for 10 min at 80 °C. A sample (120 µL) of ammonium acetate (20 mM) at pH 4.5 was added to the reactions, which were then passed over a Costar® Spin-X® Centrifuge Tube Filter (Corning; 0.45 µm cellulose acetate). The filtrates were dispensed into Prominence 12×32mm MT-IT autosampler vials (Shimadzu) and capped with 9-mm screw caps fitted with PTFE/Silicone septa (VWR).

HPLC analysis—The product of the kinase assay was resolved by RP-HPLC using a modification of the described separation protocol for α-RP (Gray & Escalante-Semerena, 2010). A Shimadzu Prominence UFLC equipped with a Kinetex 5µ C18 column (150 × 4.6 mm; Phenomenex) and a SecurityGuard ULTRA guard column (C18 cartridge; Phenomenex) was equilibrated at a flow rate of 0.8 mL min⁻¹ with 3% (v/v) acetonitrile/97% (v/v) of ammonium acetate (20 mM, pH 4.5). After injection, the column was developed for 12 min to 40% (v/v) acetonitrile and then 5 min to 80% (v/v) acetonitrile. Compounds were detected at 287 nm using a computer-controlled Shimadzu Nexera X2 SPD-30A diode array detector. As a reference for the formation of α-ribazole-5'-phosphate (α-RP), retention time and spectra of the product peak were compared to the product formed as a result of the described *SeCobT* activity assay (Claas *et al.*, 2010, Chan & Escalante-Semerena, 2011) for the formation of α-RP from DMB and NaMN. Data were analyzed using the GraphPad Prism v4 software package (GraphPad Software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Late steps in AdoCbl biosynthesis

A. Structures relevant to this study. Adenosylcobalamin, AdoCbl; 5,6-dimethylbenzimidazole, DMB; α -ribazole-5'-phosphate, α -RP. B. Activation of DMB in *S. enterica* and its incorporation into AdoCbl. In *S. enterica*, DMB and other purines and purine analogues, are activated to the corresponding α -ribotide by the NaMN:DMB phosphoribosyltransferase CobT. Abbreviations: NaMN, nicotinate mononucleotide; Na, nicotinate; HO-Cbi, hydroxocobinamide; CobA, ATP:Co(I)rrinoid adenosyltransferase; CobU, NTP:Ado-cobinamide kinase/NTP:Ado-cobinamide-phosphate guanylyltransferase; CobS, AdoCbl-5' phosphate synthase; CobC, Ado-Cba-P phosphatase. Biosynthetic intermediates are generated in the following order: [1], AdoCbi; [2], AdoCbi-P; [3], AdoCbi-GDP; [4], AdoCbl-5'-P.

Figure 2. *GkCbIS* is necessary and sufficient for incorporation of endogenously generated α -R into AdoCba

Four strains were used in these experiments, all of which had the following chromosomal mutations: *metE ara-9 cobT cobB*. The difference among strains was the plasmid they harbored. JE17827 (pBAD24), JE17828 (pSeCobT), JE17830 (pGkCbITS), and JE21364 (pGkCbIS). A. Growth behavior of strains JE17827, JE17828, and JE17830 under conditions of excess cobinamide (5 nM) in the medium. B. Growth behavior of strain JE17830 under conditions of limiting cobinamide in the medium (0.25 nM). When added to the medium DMB and α -R were present at the indicated concentrations. C. Growth behavior of strain JE21364 under limiting cobinamide conditions (0.25 nM). When added to the medium, DMB and α -R were present at the indicated concentrations. These results are

representative of six biological replicates each one of which was performed in technical duplicates.

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Figure 3. Purified His₆-GkCblS

Lanes, left to right: 1, Molecular mass markers; 2, crude cell-free extract; 3, column flow through; 4, bind buffer eluate (10 mM imidazole); 5, wash buffer eluate (150 mM imidazole); 6, elution buffer eluate [imidazole (750 mM), TCEP (5 mM), glycerol (5%, v/v)]. Molecular mass of His₆-GkCblS (28.5 kDa) calculated from its primary sequence.

Figure 4. *GkCblS* converts α -R to α -RP at the expense of ATP

In vitro assay conditions and the RP-HPLC protocol are described in the *Experimental procedures* section. The addition of purified *GkCblS* to a reaction mixture containing α -R (A) and ATP gave rise to a peak at 8.9 min (B). The UV-spectrum of the compound generating the signal is shown in the inset. The identity of this peak was confirmed to be α -RP by using the *SeCobT* phosphoribosyltransferase reaction as a control. *SeCobT* synthesizes α -RP from DMB and NaMN (Claas *et al.*, 2010, Trzebiatowski & Escalante-Semerena, 1997, Trzebiatowski *et al.*, 1994). The retention time of *SeCobT*-derived α -RP (panel D) was the same as that of the *GkCblS* reaction product (B). The UV-spectra of each product are shown in the insets. Compound elution was monitored at 287 nm. Chromatograms do not show the peaks for donor nucleotides, which eluted between 2 and 3 min post injection.

Figure 5. RP-HPLC Analysis of corrinoid extracts

RP-HPLC. Corrinoid extracts from JE12939 derivatives were separated by RP-HPLC as described in the *Experimental procedures* section; their elution was monitored at 367 nm. DMB or adenine was added as indicated to determine whether biosynthesis could be directed. Traces were normalized for comparison purposes. Normalization was achieved by considering the peak with the highest absorbance as 100%. Dashed lines indicate fractions at 12 and 17 min post injection. Expected retention times for pseudoCbl, CNCbl, and (CN)₂Cbl are indicated below the traces. Peak indicated by “X” is not a corrinoid as indicated by its UV-vis spectrum and bioassay results (Fig. S2); its identity was not established. As a control, a 0.5 μ M solution of authentic pseudoCbl, (CN)₂Cbl, and CNCbl was analyzed under the same conditions (Fig. 6A). **UV-vis spectra.** Spectra were obtained from the RP-HPLC chromatogram at the elution time identified by the dashed line. UV-vis spectra shown in row A were obtained with authentic compounds. The first column reflects the UV-vis spectrum collected at 12 min and the second column reflects the spectrum at 17 min. Peaks at 367 nm and 525 nm were indicative of a corrinoid. **cobS bioassay.** Bioassays were performed as described in the *Experimental procedures* section. These fractions are representative of the highest peak for pseudoCbl and CNCbl elution, respectively, as confirmed by mass spectrometry. Growth indicates the presence of a complete cobamide. Abbreviations: NG, no growth; +, halo diameter < 5 mm; ++, diameter between 5 mm and 10 mm; +++, halo diameter > 10 mm. The following strains were used: Panel A, none; panels B, D, F, JE17830; panels C, G, E, JE17828.

Figure 6. Expanded view of α -RP biosynthesis via direct DMB activation or α -R scavenging

The presence of the CobT-dependent synthesis of α -RP and the CbITS proteins in a single organism (e.g., *G. kaustophilus*) may be an indicator of the presence of α -R in the environment. As shown in this study, *S. enterica* can convert DMB to α -R via an unknown mechanism. Other bacteria, like *G. kaustophilus* may also be able to convert DMB to α -R, thus expanding the role of the CbITS system from salvaging to *de novo* synthesis. In *S. enterica*, it has been proposed that DMB is derived from FMN, but the enzymes involved have not been identified. Abbreviations: FMN, flavin mononucleotide; CobU, NTP:Ado-cobinamide kinase and NTP:Ado-cobinamide-phosphate guanylyltransferase; CobS, AdoCbl-phosphate synthase; CobC, Ado-Cba-P phosphatase.

Table 1
Strains and plasmids used in this study

Unless otherwise stated, strains and plasmids were constructed during the course of this work

Strains	Antibiotic resistance	Relevant genotype	Reference or source
<i>E. coli</i> strains¹			
DH5α/F'			
JE13607	Km ^R	BL21 (λDE3) <i>cobT762::kan⁺</i>	(Chan & Escalante-Semerena, 2011)
<i>S. enterica</i> strains²			
TR6583		<i>metE205 ara-9</i>	K. Sanderson via J. Roth
JE7088		<i>metE2702 ara-9</i>	Lab collection
JE8214	Cm ^R	<i>metE205 ara-9 cobS1312::cat</i>	Lab collection
JE12403	Km ^R	<i>metE205 ara-9 cobS1313 btuB7::MudJ³</i>	Lab collection
JE12939		<i>metE2702 ara-9 cobT1380 cobB1375</i>	Lab collection
JE17827	Ap ^R	<i>metE2702 ara-9 cobT1380 cobB1375/pBAD24</i>	
JE17828	Ap ^R	<i>metE2702 ara-9 cobT1380 cobB1375/pCOBT140</i>	
JE17830	Ap ^R	<i>metE2702 ara-9 cobT1380 cobB1375/pGkCblTS2</i>	
JE21364	Ap ^R	<i>metE2702 ara-9 cobT1380 cobB1375/pGkCblS3</i>	
Plasmids			
pGEM-T Easy	Ap ^R	TA cloning vector	Promega
pT7-5	Ap ^R	Overexpression vector	(Tabor & Richardson, 1992)
pBAD24	Ap ^R	Cloning vector with P _{araBAD} arabinose-inducible promoter	(Guzman <i>et al.</i> , 1995)
pBAD33	Cm ^R	Cloning vector with P _{araBAD} arabinose-inducible promoter	(Guzman <i>et al.</i> , 1995)
pTEV5	Ap ^R	TEV protease-cleavable His ₆ tag overexpression vector	(Rocco <i>et al.</i> , 2008)
pJO27	Ap ^R	<i>S. enterica cobT⁺</i> in pT7-5	(Trzebiatowski <i>et al.</i> , 1994)
pGkCblTS1	Ap ^R	<i>G. kaustophilus cblT⁺ cblS⁺</i> in pGEM-T Easy	
pGkCblTS2	Ap ^R	<i>G. kaustophilus cblT⁺ cblS⁺</i> in pBAD24	
pGkCblS1	Cm ^R	<i>G. kaustophilus cblS⁺</i> in pBAD33	
pGkCblS2	Ap ^R	<i>G. kaustophilus cblS⁺</i> translational fusion to His ₆ tag for protein purification in pTEV5	
pGkCblS3	Ap ^R	<i>G. kaustophilus cblS⁺</i> in pBAD24	
pCOBT81	Cm ^R	<i>S. enterica cobT⁺</i> in pBAD33	
pCOBT140	Ap ^R	<i>S. enterica cobT⁺</i> in pBAD24	

¹ All *E. coli* strains used in this study were derivatives of *E. coli* K-12

² All *S. enterica* strains used in this study were derivatives of *S. enterica* sv Typhimurium strain LT2

³ MudJ is an abbreviation of MudIII1734 (Castilho *et al.*, 1984)

Ap^R, Ampicillin resistance; Cm^R, chloramphenicol resistance; Km^R, kanamycin resistance

Table 2

Oligonucleotides used in this study.

Primer	Sequence (5' → 3')*
Gk_cblTS_EcoRI_5'	AGAATTCCACCATGAACCGCCGCTTGGCTT
Gk_cblTS_HindIII_3'	ATGAAGCTTTTACCACTCGACGTGCAGTG
Gk_cblS_XbaI_RBS_5'	GAGGCGTCTAGAGGAGGCACCATGCGTGATGTGCTTTTCC
pTEV5_PIPE_gkCblS_5'	AACCTGTATTTTCAGGGCATGCGTGATGTGCTTTTCC
pTEV5_PIPE_gkCblS_3'	AGCTCGAGAATTCCATGGTTACCACTCGACGTGCAG
pTEV5_PIPE_NheI_5'	GCCCTGAAAATACAGGTTTTCACTAGTTG
pTEV5_PIPE_XhoI_3'	CCATGGAATTCTCGAGCTCCCG
Gk_CblS_EcoRI_5'	GAGGCGGAATTCCACCATGCGTGATGTGCTTTTCC
Sal_CobT_BAD24_EcoRI_5'	CAGGAATTCGAGACATCTTATGCAGACACTACACGC
Sal_CobT_BAD24_XbaI_3'	CAGTCTAGACGATGGGTCGGGTATGTTGC

* Restriction sites introduced for cloning are underlined.