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ArsAB, a Novel Enzyme from *Sporomusa ovata* Activates Phenolic Bases for Adenosylcobamide Biosynthesis

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Summary

In the homoacetogenic bacterium *Sporomusa ovata*, phenol and *p*-cresol are converted into α-ribotides, which are incorporated into biologically active cobamides (Cbas) whose lower ligand bases do not form axial coordination bonds with the cobalt ion of the corrin ring. Here we report the identity of two *S. ovata* genes that encode an enzyme that transfers the phosphoribosyl group of nicotinate mononucleotide (NaMN) to phenol or *p*-cresol, yielding α-*O*-glycosidic ribotides. The alluded genes were named *arsA* and *arsB* (for alpha-ribotide synthesis), *arsA* and *arsB* were isolated from a genomic DNA library of *S. ovata*. A positive selection strategy using an *Escherichia coli* strain devoid of NaMN:5,6-dimethylbenzimidazole (DMB) phosphoribosyltransferase (CobT) activity was used to isolate a fragment of *S. ovata* DNA that contained *arsA* and *arsB*, whose nucleotide sequences overlapped by 8 bp. *So*ArsAB was isolated to homogeneity, shown to be functional as a heterodimer, and to have highest activity at pH 9. *So*ArsAB also activated DMB to its α-*N*-glycosidic ribotide. Previously characterized CobT-like enzymes activate DMB but do not activate phenolics. NMR spectroscopy was used to confirm the incorporation of phenol into the cobamide, and mass spectrometry was used to identity of the *So*ArsAB products.

Introduction

Cobamides (Cbas) are cobalt containing modified tetrapyrroles mainly involved in enzyme-catalyzed carbon skeleton rearrangements, methyl-group transfers, and reductive dehalogenation (Halpern, 1985; Kräutler *et al.*, 2003; Marsh, 1999). The biosynthesis of cobamides is complex and is only performed by some bacteria and archaea (Warren *et al.*, 2002). Cobamides have an upper (*Co*β) ligand and a lower (*Co*α) ligand base (Fig. 1). Some cobamides have a 5'-deoxyadenosyl (Ado) group as the *Co*β ligand that participates in radical chemistry reactions [e.g., methylmalonyl-CoA mutase (EC 5.4.99.2), ethanolamine ammonia-lyase (EC 4.3.1.7), diol dehydratase (EC 4.2.1.28), etc]. When cobamides serve as transient methyl carriers, the upper ligand is the methyl group being transferred [*e.g.*, Cbl-dependent methionine synthase (EC 2.1.1.13), tetrahydromethanopterin *S*-methyltransferase (EC 2.1.1.86), etc]. The chemical nature of the lower (*Co*α) ligand, however, varies depending on the organism (Renz, 1999). For example, *Salmonella enterica* sv Typhimurium LT2 (hereafter *S. enterica*) synthesizes three AdoCbas that differ by their lower ligand base. One AdoCba contains 5,6-dimethylbenzimidazole (DMB) (a.k.a. adenosylcobalamin, AdoCbl, $AdoB_{12}$, CoB_{12}), another one contains adenine (a.k.a. Ado-pseudoCbl, Ado-pseudoB_{12}, pseudo-CoB_{12}), and a third one contains 2-methyladenine (Ado-factor A) (Johnson and Escalante-Semerena, 1992; Keck and Renz, 2000). In other

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organisms, cobamides may contain substituted benzimidazoles, purine analogs, or phenolic compounds (*i.e.*, phenol, *p*-cresol) (Renz, 1999) (Fig. 1).

Lower ligand bases need to be activated before they can enter biosynthetic pathways that incorporate them into AdoCbas. The general scheme for the conversion of the base to its activated α-ribotide is shown below in equation #1.

$$\text{Base} + \text{nicotinate mononucleotide (NaMN)} + \text{Enzyme} * \alpha\text{−base−ribotide−5'−P} + \text{nicotinate (Na)} \quad (1)$$

The activation of the lower ligand base of cobamides has been studied in *S. enterica*, *Pseudomonas denitrificans*, *Clostridium sticklandii* and *Propionibacterium shermanii* (Cheong *et al.*, 1999; Friedmann and Harris, 1965; Friedmann and Fyfe, 1969; Trzebiatowski *et al.*, 1994; Trzebiatowski and Escalante-Semerena, 1997).

Figure 2 shows a scheme for the late steps of AdoCbl biosynthesis in *S. enterica*. In this bacterium the NaMN:5,6-dimethylbenzimidazole (DMB) phosphoribosyltransferase (CobT, EC 2.4.2.21) enzyme activates DMB yielding α-DMB-ribotide (a.k.a. α-ribazole-5'-P), the co-substrate for the AdoCbl-5'-P synthase (CobS, EC 2.7.8.26) that catalyzes the penultimate step of the pathway (Maggio-Hall and Escalante-Semerena, 1999; Zayas and Escalante-Semerena, 2007). Notably, *Se*CobT cannot activate phenolic bases such as phenol or *p*-cresol (Cheong *et al.*, 2001). Figure 2 also depicts the pathway used by *S. enterica* to salvage exogenous cobinamide; this information is relevant to some of the experiments performed in this work.

Cobamides containing phenolic bases comprise about 16% of the total cobamides isolated from gut microbiome of humans (Allen and Stabler, 2008). Prior to this work, the identity of genes encoding enzymes responsible for the activation of phenolics during AdoCba biosynthesis was unknown, and the enzymes had not been studied.

Phenolyl- and *p*-cresolyl-Cba were first isolated from the Gram-negative anaerobe *Sporomusa ovata* (Stupperich *et al.*, 1988; Stupperich and Eisinger, 1989b), a member of the *Veillonellaceae* family of the Firmicutes. Phenolyl- and *p*-cresolyl-Cba have two distinctive features. First, an α-*O*-glycosidic bond links the base to ribose, rather than the α-*N*-glycosidic bond observed in cobamides containing purines, purine analogs, and derivatives of benzimidazole. And second, neither phenol nor *p*-cresol can establish a coordination bond with the Co ion of the ring. The absence of a coordination bond between the Co ion and the lower ligand base limits the use of phenolyl- and *p*-cresolyl-Cba to enzymes that use cobamide coenzymes in their base-off conformation (Stupperich and Eisinger, 1989a). Enzymes using the cobamide coenzymes in their base-on conformation require coordination of the Co ion by the lower ligand base.

S. enterica is a good system to investigate the use of phenolyl- and *p*-cresolyl-Cba in vivo, because base-off and base-on cobamide-dependent enzymes are present in this bacterium, and conditions that require their activities for growth are known (Jeter *et al.*, 1984; Roof and Roth, 1988). For example, the cobalamin-dependent methionine synthase (MetH) enzyme requires a base-off form of the cofactor for function (Drennan *et al.*, 1994), whereas ethanolamine ammonia-lyase (EutBC) uses AdoCbl in its base-on form (Abend *et al.*, 1999; Shibata *et al.*, 1999; Shibata *et al.*, 2010).

The limiting factor for the in vivo analysis has been the availability of cobamides unable to form the base-on conformation, such as phenolyl- and *p*-cresolyl-Cba. To circumvent this limitation we sought to identify the genes encoding the enzymes that activate phenol and *p*-cresol in *S. ovata*. Notably, *S. ovata* can also synthesize benzimidazolyl-Cba when

benzimidazole is present in its environment (Stupperich and Eisinger, 1989b), indicating that this bacterium has enzymes that can synthesize α-*N*-glycosidic and α-*O*-glycosidic ribotides.

In this paper we report that the newly discovered heterodimeric enzyme ArsAB [for alpha-ribotide synthesis (Ars) proteins A and B] of *S. ovata* activates phenol, *p*-cresol, and DMB to α-phenolyl, α-*p*-cresolyl-ribotide, and α-DMB-ribotide, respectively. We used a heterologous complementation system to isolate the genes *arsA* and *arsB* [encoding ArsA (36.65 kDa) and ArsB (36.18 kDa)], from a *S. ovata* gene library. Bioinformatics analyses of the putative amino acid sequences showed that *So*ArsA and *So*ArsB were homologous to *Se*CobT. However, unlike *Se*CobT, *So*ArsA and *So*ArsB function as a heterodimer, and can activate phenol, *p*-cresol, in addition to DMB. We also report that the AdoCba biosynthetic machinery of *S. enterica* incorporates phenolic bases into the final cobamide when $arsA^+$ and $arsB^+$ were provided in trans. We note that neither phenolyl-nor *p*-cresolyl-Cba supported *S. enterica* growth on ethanolamine or 1,2-propanediol as a carbon and energy sources, consistent with the need of ethanolamine ammonia-lyase and diol dehydratase for a base-on AdoCba.

Results

Isolation of *S. ovata* genes encoding functions needed for the activation of phenol and *p*-cresol

Our search for *S. ovata* genes encoding phenol and *p*-cresol activating functions assumed that, in *S. ovata*, the activation of these compounds was catalyzed by a homolog of *Se*CobT. At the time of this work, the sequence of the *S. ovata* was not known, precluding the use of bioinformatics approaches for the identification of genes encoding *Se*CobT homologs. To circumvent this problem, we took a function-based approach. For this purpose, we constructed a *S. ovata* gene library (Fig. S1) comprised of approximately 12,500 fosmids, each of which carried one 40-kbp (average size) fragment of *S. ovata* genomic DNA. Since we assumed that the size of the *S. ovata* genome was 5 Mbp, the total amount of *S. ovata* DNA in the library represented 100× coverage. The search for fosmids encoding *S. ovata* CobT-like functions was performed using strain JE11215, a derivative of *E. coli* EPI300 (Epicentre, Table 1) carrying a marker-less deletion of *metE* (encodes the Cbl-independent methionine synthase) and a replacement of *cobT* with the kan^+ gene encoding kanamycin resistance. In the absence of exogenous methionine or Cbl, a CobT-like function encoded on the fosmid is required for growth of strain JE11215 under aerobic conditions on minimal medium supplemented with cobinamide (Cbi), a precursor of cobamides. We describe the method used to isolate the wild type alleles of *arsA* and *arsB* in the *Experimental procedures* section and supplemental material. The initial search yielded seven fosmids with distinct restriction patterns that restored AdoCba synthesis in JE11215. Among these fosmids, pSolibC17 was selected for further characterization. A strategy that combined sub-cloning and complementation of function yielded a fragment of *S. ovata* DNA that was sequenced in its entirety (2,267 bp; GenBank Accession # JF895493; Fig. S2). The *S. ovata* genes encoding *Se*CobT-like function were named *arsA* and *arsB* for alpha ribotide synthesis; *arsA* and *arsB* were found together, with a sequence overlap of 8 bp that included the stop codon of *arsA* (Fig. S2). Both proteins were 350 aa long, and shared end-to-end homology with *Se*CobT (356 aa) (Fig. 3). A notable difference between *So*ArsB and *So*ArsA and *Se*CobT was an 11-residue gap located approximately in the middle of the primary sequence of *So*ArsB. This gap was confirmed by sequencing *arsA* and *arsB* directly from *S. ovata* genomic DNA.

S. ovata arsA$^+$ and *arsB*$^+$ are necessary and sufficient to restore synthesis of AdoCba in a *S. enterica* strain lacking CobT function

We performed complementation studies in *S. enterica* to establish whether *Se*CobT-like activity was associated with *arsA*, *arsB* or their combination. For this purpose, *arsA*$^+$ and *arsB*$^+$ were placed individually or together under the control of the L-(+)-arabinose-inducible promoter in plasmid pBAD33 (Guzman *et al.*, 1995), resulting in plasmids pARSA4 (*arsA*$^+$), pARSB2 (*arsB*$^+$), and pARSAB4 (*arsA*$^+$ *arsB*$^+$). These plasmids were moved individually into strain JE2501 (*S. enterica metE cobT cobB*), resulting in strains JE13867 (*metE cobT cobB* / pBAD33), JE13871 (*metE cobT cobB* / pARSA4), JE13872 (*metE cobT cobB* / pARSB2), and JE13874 (*metE cobT cobB* / pARSAB4) (Table 1). The *cobB* gene, which encodes an NAD^+-dependent protein deacetylase (Starai *et al.*, 2002), was inactivated to eliminate all CobT-like activity in the cell (Trzebiatowski *et al.*, 1994; Tsang and Escalante-Semerena, 1998).

As shown in figure 4, co-expression of *arsA*$^+$ and *arsB*$^+$ compensated for the lack of CobT function in JE13874, allowing growth of the strain (Fig. 4B). In contrast, expression of *arsA*$^+$ or *arsB*$^+$ alone did not restore AdoCba biosynthesis in JE13871 and JE13872 (Fig. 4C, 4D). We note that the addition of phenol and *p*-cresol extended the lag time prior to the onset of exponential growth of strain JE13874 co-expressing *arsA*$^+$ and *arsB*$^+$ (Fig. 4B). Such an increase in the lag time was not observed when DMB substituted for phenol or *p*-cresol.

S. enterica synthesizes phenolyl- and *p*-cresolyl-Cba when *So*ArsA and *So*ArsB substitute for CobT and phenol or *p*-cresol are present in the culture medium

We confirmed that growth of strain JE13874 (*metE cobT cobB* / pARSAB4 *arsA*$^+$ *arsB*$^+$) under the conditions described above was due to the synthesis of phenolyl-Cba or *p*-cresolyl-Cba. Figure 5 (panels A, B, and C) shows the RP-HPLC separation (isolation), UV-visible spectrum and mass spectrum (identity) of *p*-cresolyl-Cba synthesized by strain JE13874. Equivalent data for phenolyl-Cba are presented in figure 5D, E, and F. The UV-visible spectra shown in figures 5B, E confirmed that the isolated compounds were corrinoids, but the data did not distinguish between complete and incomplete corrinoids (compare to $(CN)_2$Cbi and CNCbl, Fig. S3). However, the mass spectra data for *p*-cresolyl-Cba [*m/z* = 1291, $(M+H)^+$] and phenolyl-Cba [*m/z* = 1277, $(M+H)^+$] (Fig. 5C, F, respectively) were consistent with the predicted formula mass of phenolyl- and *p*-cresolyl-Cba without an upper ligand. We did not detect any phenolic-Cba in the cell extract when phenol or *p*-cresol was not added to the culture medium.

We used a bioassay to further establish that the isolated corrinoids were complete. To do this, we used a bioassay that employed *S. enterica* strain JE8248 (*metE cobS*), a cobamide auxotroph that cannot catalyze the penultimate step of the pathway (Fig. 2) (Zayas and Escalante-Semerena, 2007). As shown in figure 6, phenolyl-Cba and *p*-cresolyl-Cba supported growth of strain JE8248 on glucose under conditions that required the synthesis of methionine from homocysteine to occur via the Cba-dependent methionine synthase (MetH) enzyme. However, 10-fold higher concentrations of phenolyl-Cba or *p*-cresolyl-Cba were needed to match the response to cobalamin. In contrast to the above results, phenolyl-Cba and *p*-cresolyl-Cba did not support growth of JE8248 on ethanolamine or 1,2-propanediol (data not shown), consistent with the inability of phenol or *p*-cresol to form a coordination bond with the Co ion of the ring.

In *S. enterica*, the phenolyl moiety of phenolyl-Cba is derived from exogenous, not endogenous sources

To confirm the incorporation of phenol into the cobamide, we grew strain JE13874 (*metE cobT cobB* / pARSB4 *arsA*$^+$ *arsB*$^+$) in minimal medium supplemented with [^{13}C-1]-phenol (99% enriched). The cobamide synthesized under these conditions was isolated as described above, and was analyzed by ^{13}C heteronuclear multiple bond correlation (HMBC) NMR spectroscopy (Bax and Summers, 1986), and mass spectrometry. The spectrum of the [^{13}C-1]-phenolyl-Cba was compared to the spectrum of [^{13}C-1]-phenol (Fig. 7A,B). HMBC experiments detect long-range H-C couplings while suppressing H-H correlations, therefore the chemical shifts of protons in an HMBC spectrum are the result of only long range effects of the ^{13}C nucleus. As seen in figure 7B, [^{13}C-1]-phenolyl-Cba showed detectable long-range H-C couplings, which suggested that [^{13}C-1]-phenol was incorporated into the cobamide. If the ^{13}C label were not present in the cobamide, no chemical shifts would have been detected with the HMBC experiment. The difference in chemical shifts between free [^{13}C-1]-phenol and [^{13}C-1]-phenolyl-Cba was due to the differences in the magnetic environment of the protons relative to the free hydroxyl group in phenol and the ether-linked ribose in phenolyl-Cba. The complexity of the signals observed in the [^{13}C-1]-phenolyl-Cba spectrum, relative to the free [^{13}C-1]-phenol spectrum, was likely due to diverse magnetic environments affecting the phenolyl moiety of the cobamide. As expected, the mass [^{13}C-1]-phenolyl-Cba was one atomic mass unit greater than the mass of [^{12}C-1]-phenolyl-Cba (Fig. 5F vs Fig. 7C).

S. ovata ArsA and ArsB are soluble and active as a heterodimer

Our attempts to purify *So*ArsA and *So*ArsB individually resulted in insoluble proteins (data not shown). Given the in vivo observation that cobamide biosynthesis in strain JE2501 (*cobT cobB*) was restored only when *arsA*$^+$ and *arsB*$^+$ were co-expressed, we sought to co-purify *So*ArsA and *So*ArsB (*So*ArsAB) by over-expressing *arsA*$^+$ and *arsB*$^+$ together. To facilitate protein purification, a recombinant tobacco etch virus (rTEV) protease cleavable hexahistidine (H_6) tag was fused to the *N*-terminus of *So*ArsA using cloning vectors described elsewhere (Rocco *et al.*, 2008). *So*ArsB encoded by the resulting construct (pARSAB7, Table 1) was not tagged.

Two protein bands were resolved by SDS-PAGE after nickel affinity purification and rTEV protease treatment of soluble proteins (Fig. 8A). Since *So*ArsB was not tagged but co-purified with *So*ArsA, we hypothesized that the proteins interacted. Results of size exclusion chromatography experiments supported this idea (Fig. 8B). *So*ArsAB heterodimers had a retention time of 14.34 min, which corresponded to a mass of ~66 kDa. Results of peptide tandem mass spectrometry MALDI TOF (MS/MS) fingerprinting analysis of a tryptic digest of the fraction containing ArsAB confirmed that the two soluble proteins were *So*ArsA and *So*ArsB (Table S1). In addition, the fraction containing purified *So*ArsAB had NaMN:DMB phosphoribosyltransferase activity with a specific activity of 19 ± 3 nmol/min/mg of protein (Table S2).

*So*ArsAB synthesize α-phenolyl-, α-*p*-cresolyl-, and α-DMB-ribotide

The homology of *So*ArsA and *So*ArsB to *Se*CobT suggested that *So*ArsAB had NaMN:phenol/*p*-cresol phosphoribosyltransferase activity. Data reported in Table S2 show that *So*ArsAB had phosphoribosyltransferase activity, and that it activated phenol and DMB with similar efficiency. Out of the conditions tested, *So*ArsAB was most active in a glycine pH 9 buffer. The product of *So*ArsAB when phenol, *p*-cresol, or DMB was used as substrate was purified by RP-HPLC and identified by mass spectrometry (Fig. 9). To confirm the products of *So*ArsAB were ribotides, each of the products was dephosphorylated using alkaline phosphatase (see *Experimental procedures*), re-purified by RP-HPLC and their

mass was assessed by mass spectrometry (Fig. S4). In all instances, the observed masses were in agreement with the calculated formula masses. To test whether the *So*ArsAB products were biologically active, we supplemented the growth medium of *S. enterica* strain JE2501 (*cobT cobB*) with $(CN)_2$Cbi and RP-HPLC-purified α-phenolyl- and α-*p*-cresolyl-ribotide. Five-fold higher concentrations of α-phenolyl- and α-*p*-cresolyl-ribotides were required to support growth, relative to the concentration of α-DMB-ribotide required (Fig. 10).

The tandem chromosomal arrangement of *S. ovata arsA* and *arsB* and the 11-residue deletion of *So*ArsB are uncommon

We searched the finished and unfinished genome databases for other tandem genes encoding CobT homologs similar to the one we found in *Sporomusa ovata*. We searched for CobT (Pfam domain DBI_PRT pfam02277) sequences in Pfam (http://pfam.sanger.ac.uk/) for tandem gene sequences within the genomes having multiple CobT sequences. We identified consecutive genes encoding for CobT homolog (EC 2.4.2.21) sequences in the Integrated Microbial Genomes (IMG, http://www.jgi.doe.gov/) database using the EC number as a filter. We also searched the orthologs neighborhood regions in IMG once a tandem *cobT* homolog sequence was identified. At present, only some species of *Veillonella* and *Dialster invisus* share the tandem organization of the *S. ovata arsA* and *arsB* genes. Interestingly, the 11-residue deletion in *So*ArsB is found only in *Dialister invisus* ArsB, but not in *Veillonella parvula* ArsB (Fig. S5).

Discussion

The *arsA* and *arsB* genes of *S. ovata* encode a novel enzyme that activates phenolic bases during AdoCba biosynthesis

To date, the only organism known to synthesize phenolic cobamides is *Sporomusa ovata*. However, the identity of the enzymes responsible for the activation of phenol and *p*-cresol remained unknown. Using a combination of in vivo and in vitro approaches we isolated two genes of *S. ovata* whose products convert phenol and *p*-cresol to their corresponding α-ribotides, which are precursors in the biosynthesis of phenolic cobamides (Fig. 2). To reflect the function associated with their gene products, we propose the name *arsA* and *arsB*, which stands for alpha-ribotide synthesis.

The *So*ArsAB enzyme has sufficient NaMN:Base phosphoribosyltransferase activity to functionally replace the CobT enzyme of *S. enterica* in vivo to satisfy the methionine requirement of the cell. There are, however, notable differences between *Se*CobT and *So*ArsAB. First, *Se*CobT is active as a homodimer, while *So*ArsAB is active as a heterodimer. Second, *So*ArsAB can activate phenol, *p*-cresol, and DMB, that is, *So*ArsAB catalyze the formation of α-*O*- and α-*N*-glycosidic bonds. In contrast, *Se*CobT cannot activate phenol or *p*-cresol. On the other hand, *Se*CobT and *So*ArsAB have common features. First, they share a common ancestor, as shown by the end-to-end homology and the degrees of identity and similarity between *So*ArsA and *So*ArsB with *Se*CobT (Fig. 3). And second, based on what is known about *Se*CobT, it is likely that the mechanism of catalysis of *So*ArsAB is similar to that of *Se*CobT, as suggested by the conservation of the putative catalytic glutamyl residue at the *C*-terminus of both proteins (Fig. 3, S2). Mutational, structural and kinetic analyses of *So*ArsAB are needed to better understand the functional differences between this enzyme and *Se*CobT.

At present, the assignment of the starting methionine of *So*ArsA is tentative. As shown in figure 3, there is a methionine residue at position #5 in the sequence. Although the *So*ArsA protein we isolated has the sequence shown in figure 3, it is possible that in vivo the starting

methionine of *So*ArsA is Met5. We are currently eliciting antibodies against *So*ArsAB so we can isolate the complex from *S. ovata* and can resolve this issue.

We are interested in learning about the structural features that allow *So*ArsAB to have broader base specificity than *Se*CobT. For example, we need to determine whether or not the heterodimeric state of *So*ArsAB reflects on how substrate specificity is achieved. At present, we do not know whether *So*ArsAB can activate purine and benzimidazole analogs (Fig. 1).

The 11-residue deletion in *So*ArsB (also present in *D. invisus* ArsB) is unique among CobT homologs (Fig. S5). It is unclear how this deletion impacts enzyme activity, substrate specificity, or any other aspect of *So*ArsAB function. These questions await further biochemical and kinetic analyses of *So*ArsAB function.

Limitations of the use of *S. enterica* and *E. coli* for the in vivo analysis of phenolic cobamide function

The use of *S. enterica* and *E. coli* as heterologous systems for the isolation and analysis of *So*ArsAB function was successful. However, there are limitations to the use of these systems. For example, it is clear that the *So*ArsAB enzyme supported Cba-dependent growth of a *S. enterica cobT* strain in the absence of any exogenous base (i.e., phenolics or DMB; Fig. 4B, solid circles). This result shows that *S. enterica* makes substrates that *So*ArsAB can use; the data also show that DMB is the preferred substrate for *So*ArsAB in this system. Although these complications do not change the conclusion that *So*ArsAB activates phenolic bases and DMB, they make the in vivo analysis of *So*ArsAB function more challenging. Another limitation of these heterologous systems is revealed by the concentration dependent growth response observed with different cobamides (Fig. 6). There are at least two possible interpretations of these results. First, transport of phenolic cobamides by the BtuBCDEF system responsible for the assimilation of corrinoids in *S. enterica* and *E. coli* (Cherezov *et al.*, 2006; Hvorup *et al.*, 2007; Van Bibber *et al.*, 1999) is less efficient for phenolic cobamides than for cobalamin. And second, it is possible that the cobamide-dependent MetH enzyme has less affinity for phenolic cobamides than for cobalamin. Lastly, it appears like either *S. enterica* transports α-ribotides with different efficiencies, or the observed differences in growth as a function of the exogenous α-ribotide provided reflects different levels of functionality of the cobalamin 5'-P synthase (CobS) enzyme that consumes α-ribotides (Fig. 10).

Potential use of *S. ovata* enzymes for the study of cobamide specificity

Although the *S. ovata* predominantly synthesizes phenolic cobamides (Stupperich and Eisinger, 1989b; Stupperich *et al.*, 1989), we now know that *So*ArsAB can also activate DMB (Table S2), thus *S. ovata* has the capability of making cobalamin. It is of interest to identify growth conditions that demand synthesis of different cobamides in *S. ovata*. It is possible that *S. ovata* regulates the synthesis of phenolic cobamides versus other cobamides by controlling the activity or expression of *arsAB* in response to environmental cues. However, it is also possible that *S. ovata* synthesizes phenolic cobamides due to the lack of an endogenous source of DMB or other lower ligands. Previous studies revealed that a 40-kDa protein that contained a bound phenolic cobamide was induced by the presence of methanol in *S. ovata*. These findings suggested that the above-mentioned protein was involved in methanol and 3,4-dimethoxybenzoate metabolism in this bacterium (Stupperich *et al.*, 1992; Stupperich and Konle, 1993). Of note is the reported inhibition of *S. ovata* growth by exogenous DMB (Stupperich *et al.*, 1990). Whether the observed inhibition by DMB was due to the specific growth conditions that demanded the synthesis of phenolic cobamides remains an open question.

On the basis of the ability of *So*ArsAB to activate DMB, it is possible that there are conditions where exogenous DMB is not inhibitory to *S. ovata* growth, and under such conditions this bacterium synthesizes cobalamin. It is also possible, however, that the ability of *So*ArsAB to activate DMB is a remnant of the evolution of the enzyme that is of no use to *S. ovata*, either because this bacterium does not synthesize DMB, and because it does not encounter this base in its environment.

Results from in vivo analyses showed that phenolic cobamides supported growth of a *S. enterica* strain requiring the function of MetH (methionine synthase, Fig. 6). Since MetH is known to bind cobalamin in the base-off form (Drennan et al., 1994), it is not surprising that phenolic cobamides, whose lower ligand cannot coordinate to the cobalt atom, supported MetH function. We note that there were differences in the growth behavior when the medium was supplemented with Cbl or different phenolic cobamides (Fig. 6). These results can be explained by decreased binding affinities of MetH for phenolic cobamides relative to its affinity for Cbl.

Phenolic cobamides did not support the growth of *S. enterica* on 1,2-propanediol or ethanolamine (data not shown). These results were consistent with reports of diol dehydratase and ethanolamine ammonia-lyase requiring axial coordination of the lower ligand base to the cobalt atom for function (Abend *et al.*, 1999; Shibata *et al.*, 2010; Yamanishi *et al.*, 1998), a coordination bond that phenolic cobamides cannot form. A previous study, which showed that *p*-cresolyl-Cba was a competitive inhibitor for the 1,2-propanediol dehydratase is consistent with the involvement of a base-on coenzyme in the function of this enzyme (Poppe *et al.*, 1997).

We predict that the genome of *S. ovata* encodes a homolog of the cobinamide amidohydrolase (CbiZ) enzyme (Woodson and Escalante-Semerena, 2004), which is involved in lower ligand remodeling (Gray and Escalante-Semerena, 2009). The existence of a CbiZ-like activity in *S. ovata* would be a good complement to the broad specificity of *So*ArsAB for its base substrate, and would provide *S. ovata* with the ability to synthesize diverse cobamides when the need for a specific cobamide arises. Unfortunately, the pace at which this work can be performed is limited by the lack of a *S. ovata* genome sequence. There is, however, information in the literature about Cba-dependent methyltransferases from *S. ovata* (Stupperich *et al.*, 1992; Stupperich and Konle, 1993). Nevertheless, we are now in a good position to explore the specificity of these enzymes for different cobamides.

Experimental procedures

Strain constructions

All strains used in this study were derivatives of *Salmonella enterica* sv Typhimurium strain LT2 or *Escherichia coli* K12. Strain genotypes are described in Table 1. Chromosomal mutations were introduced into *E. coli* by phage P1-mediated transduction (Cronan *et al.*, 1969). Plasmids were introduced into *S. enterica* by electroporation (O'Toole *et al.*, 1993), and into *E. coli* by heat-shock transformation (Hanahan *et al.*, 1991). The pEAK2 temperature-sensitive plasmid carrying the *recA* gene was transformed into *E. coli* EPI300 strain prior to P1-mediated transductions (Kouzminova and Kuzminov, 2004). The Flp recombinase encoded in plasmid pCP20 was used to resolve the *kan* gene encoding kanamycin resistance in strains from the Keio collection (Datsenko and Wanner, 2000).

Construction of a *S. ovata* gene library and identification of *S. ovata arsA, arsB* genes

Sporomusa ovata type strain (ATCC® 35899, DSM 2662) was obtained from ATCC and grown as described (Möller *et al.*, 1984). *S. ovata* genomic DNA was isolated as described (Pospiech and Neumann, 1995), and was sheared with a 200-μL pipet tip. A gene library

was constructed using the Copy Control Fosmid Library Production Kit (Epicentre) (Fig. S1). Briefly, sheared DNA was resolved on 10×15cm 0.75% low melting point agarose gel. Forty-kb fragments were excised from the gel, melted and digested with agarase enzyme provided in the fosmid production kit before ethanol precipitation. The ends of the DNA fragments were blunted and phosphorylated with a mixture of T4 DNA polymerase, T4 polynucleotide kinase, ATP and dNTPs (provided in the kit) before ligation to *Eco*72I blunt-ended plasmid pCC1FOS, and packaging into empty lambda phage heads in vitro. Each fosmid contained approximately 40 kb of *S. ovata* genomic DNA. The lambda phage suspension was used to infect strain JE11215 (*E. coli* EPI300 Δ*metE cobT400::kan*$^+$) plating on LB + chloramphenicol. Assuming that the *S. ovata* genome is 5 Mb, we obtained about 100X coverage of the genome with approximately 12,500 clones. Twelve pools of ~1,000 clones were combined and inoculated into liquid culture, and plasmids were induced to multi-copy by the addition of L-(+)arabinose (1.3 mM). Approximately 10^4 cells were plated onto minimal medium. Strain JE11215 requires methionine in the absence of a complete cobamide. Derivatives of strain JE11215 harboring a fosmid were selected for growth on NCE minimal glycerol (22 mM) plates supplemented with $(CN)_2$Cbi (150 nM), thiamine (3 μM), leucine (150 μM), L-(+)-arabinose (1.3 mM); the same culture medium supplemented with phenol (75 μM) or *p*-cresol (75 μM) was also used; plates were incubated at 30°C for two days. Twenty-eight plasmids that supported growth of JE11215 on the selective media described above were screened by restriction analysis using NcoI (Fermentas). Plasmids were incubated with NcoI for 2 h, and restriction fragments were resolved in a 1% (w/v) agarose gel at 100 V for 1 h (Voytas, 2000). Seven plasmids with different restriction patterns were analyzed further; the pCC1FOS vector backbone has one NcoI restriction site. One of these plasmids (hereafter referred to as pSolibC17) was fragmented by sonication and the ends blunted as described above. Two 5-kb fragments were isolated by agarose gel electrophoresis (see above), followed by DNA extraction using Promega's Gel and PCR Cleanup Kit™. DNA fragments were ligated into *Eco*72I blunt-ended plasmid pCC1FOS and introduced into strain JE11215 by heat-shock transformation. Inheritance of the plasmid carrying *S. ovata* CobT-like functions supported growth on minimal medium containing glycerol, $(CN)_2$Cbi and DMB, phenol or *p*-cresol. The fragment of *S. ovata* DNA in pSolibC17 was sequenced using primers flanking the *Eco*72I insertion site (5'-GGATGTGCTGCAAGGCGATTAAGTTG-3' and 5'-CTCGTATGTTGTGTGGAATTGTGAGC-3'), and subsequent sequences were obtained by primer walking. Plasmid pSolibC17 encoded two CobT homologs, namely *So*ArsA and *So*ArsB. A scheme for the above procedure can be found in figure S1 (supplemental information).

Culture media, conditions and analyses

Strains were grown at 37°C in no-carbon essential (NCE) minimal medium (Vogel and Bonner, 1956) supplemented with glucose (11 mM), glycerol (22 mM), ethanolamine (90 mM), or 1,2-propanediol (90 mM) as carbon and energy source where indicated. Minimal medium also contained $MgSO_4$ (1 mM), trace minerals (Balch *et al.*, 1979). When added, ampicillin, kanamycin or chloramphenicol was present at 100 μg/mL, 50 μg/mL, or 12.5 μg/mL, respectively. For all growth experiments, strains were grown in triplicate in 96-well microtiter dishes; samples (2 μL) of overnight cultures grown in lysogeny broth (LB) (Bertani, 1951, 2004) was used to inoculate 198 μL of fresh medium plus supplements. Growth was analyzed using a computer-controlled BioTek ELx808-1 Ultra microplate reader (BioTek Instruments). Cell density measurements at 630 nm were acquired every 1800 s for 36 h; plates were shaken for 1745 s between readings. Data were analyzed using the GraphPad Prism v4 software package (GraphPad Software).

Plasmid constructions

Plasmid pARSA4—*arsA*$^+$ was amplified from plasmid pSolibC17 with primers 5'-CGAGCTCGTAATGGAGGTTATTATGAGTTTACTGC-3' and 5'-CTCTAGACGCTGACACTGTTCCATCGC-3'. The resulting fragment was cloned into the cloning vector pGEM (Promega) yielding plasmid pARSA2. The latter was cut with SacI and XbaI and ligated into the same sites in vector pBAD33 (Guzman et al., 1995) yielding plasmid pARSA4. Plasmid pARSA4 was used in functional complementation studies.

Plasmid pARSB2—*arsB*$^+$ was amplified from plasmid pSolibC17 with primers 5'-CGAGCTCGCTCTATGTGGCCATTAAGC-3' and 5'-CTCTAGAGCTTGCTAATCTCTAACATCCTTG-3'. The resulting fragment was cloned into pGEM (Promega) yielding plasmid pARSB1. The latter was cut with SacI and XbaI, and the fragment was ligated into the same sites in pBAD33, yielding plasmid pARSB2. Plasmid pARSB2 was used in functional complementation studies.

Plasmid pARSAB4—*arsA*$^+$ and *arsB*$^+$ were amplified together from plasmid pSolibC17 with primers 5'-CGAGCTCGTAATGGAGGTTATTATGAGTTTACTGC-3 and 5'-CTCTAGAGCTTGCTAATCTCTAACATCCTTG-3'. The resulting fragment was cloned into the pGEM (Promega), yielding plasmid pARSAB2. The latter was cut with SacI and XbaI and ligated into the same sites in pBAD33, yielding plasmid pARSAB4. Plasmid pARSAB4 was used in functional complementation studies.

Plasmid pARSAB7—*arsA*$^+$ and *arsB*$^+$ were amplified together from plasmid pSolibC17 with primers 5'-AGCTCGCCCGGGGATGGAGGTTATTATGAGTTTACTGCAAGC-3' and 5'-GCAGCTAGCGCTTGCTAATCTCTAACATCCTTGC-3'. The fragment was cut with SmaI and NheI enzymes and ligated into the StuI and NheI sites of plasmid pH6T, yielding plasmid pARSAB7. Plasmid pH6T is a derivative of cloning vector pET31b, which directs the synthesis of proteins of interest fused to an *N*-terminal hexahistidine (H_6) tag and an rTEV protease cleavage site. pH6T lacks two tyrosine residues in the sequence before the rTEV recognition site in pKLD37 (Rocco et al., 2008). Plasmid pARSAB7 directed the synthesis of H_6-ArsA and tag-less *So*ArsB. Plasmid pARSAB7 was used to overproduce *So*ArsAB proteins.

Biosynthesis and isolation of phenolyl- and *p*-cresolyl-Cba

Biosynthesis—An overnight culture of strain JE13874 (*cobT cobB* / pARSAB4 *arsA*$^+$ *arsB*$^+$) was grown in LB supplemented with chloramphenicol, and was used to inoculate (1 ml/L of fresh medium) into minimal NCE medium containing glycerol (55 mM), $(CN)_2$Cbi (300 nM), phenol or *p*-cresol (75 μM), L-(+)-arabinose (1.3 mM), and chloramphenicol (12.5 μg/mL). Two 1-liter cultures were grown for two days and harvested by centrifugation (Beckman/Coulter Avanti J20-XPI refrigerated centrifuge, equipped with a JLA-8.1000 rotor; 15 min at 4 °C; 6,000 × *g*), and kept at −80 °C until used.

Extraction—Frozen cells were resuspended in 30 mL of a 2:1 mixture of methanol and KH_2PO_4 (50 mM, pH 6.5) + KCN (5 mM). Once resuspended, cells were placed in a Max^{Q}Mini 4000 Barnstead/Lab-Line e Class shaking incubator heated to 65 °C; cells were continuously shaken (220 rpm) for 2 h. Cell debris was removed by centrifugation (Beckman/Coulter Avanti J25-I refrigerated centrifuge, equipped with a JA-25.50 rotor; 1 h at 4°C; 43,000 × *g*). The supernatant was filtered (Whatman™ Puradisc™ 25 mm PES filter, 0.45 mm pore size) and dried under vacuum using a Savant SPD111V concentrator running at room temperature. The pellet was resuspended in KH_2PO_4 buffer A [(50 mM, pH 6.5) + KCN (5 mM)] and applied onto a C_{18} Sep-Pak (Waters) previously conditioned with 10 mL of 100% methanol followed by 10 mL of dH_2O. Corrinoids bound to the column were

washed with 20 mL of dH_2O before elution with 100% methanol. Corrinoids dissolved in methanol were concentrated under vacuum. The pellet was resuspended in buffer A.

Isolation—Cobamides were resolved by reverse-phase high performance liquid chromatography (RP-HPLC) using a Beckman Coulter System Gold 126 system equipped with a Phenomenex Synergi Hydro-RP (150 by 4.6 mm) using a modified system I mobile phase described elsewhere (Blanche *et al.*, 1990). Briefly, the column was equilibrated at 23% buffer B [KH_2PO_4 (50 mM, pH 8.0) + KCN (5mM) + 50% (v/v) acetonitrile) and 77% buffer C [KH_2PO_4 (100 mM, pH 6.5) + KCN (10 mM)] at a flow rate of 1 mL/min and developed on a linear gradient to 53% buffer B for 43.2 min. The column was further developed to 100% buffer B for 5 min. Corrinoids were detected at 367 nm. Concentrations of phenolyl- and *p*-cresolyl-Cba were estimated using the molar extinction coefficient of $(CN)_2$Cbi at 367 nm (21,800 M^{-1} cm^{-1}).

Mass spectrometry of phenolyl- and *p*-cresolyl-cobamide

Corrinoids with a retention time of 25.1 and 21.5 min were collected, desalted and dried under vacuum. The molecular mass of each of these corrinoids was determined on a 4800 Matrix-Assisted Laser Desorption/Ionization-Time of Flight-Time of Flight (MALDI TOF-TOF) mass spectrometer (Applied Biosystems) scanning 700–4,000 Da mass range using 1000 shots acquired from 20 randomized regions of the sample spot at 3800 intensity of OptiBeam™ on-axis Nd:YAG laser with 200Hz firing rate and 3 to 7 ns pulse width in positive reflectron mode.

[^{13}C-1]-phenol labeling of cobamides and NMR spectroscopy

A cobamide was extracted and purified from strain JE13874 (*metE cobT cobB* / pARSAB4 *arsA*$^+$ *arsB*$^+$) grown in minimal glycerol medium containing $(CN)_2$Cbi and 99%-enriched [^{13}C-1]-phenol (75 μM) (Cambridge Isotope Laboratories). ^{13}C-labeled phenolyl-Cba was isolated, desalted, and dried under vacuum as described under *Experimental procedures*. The sample was resuspended in D_2O (Cambridge Isotope Laboratories) to an approximate concentration of 7.5 μM, which was calculated using the molar extinction coefficient of $(CN)_2$Cbi (see above). Authentic [^{13}C-1]-phenol dissolved in D_2O was used as standard. Both samples were subjected to a ^{13}C heteronuclear multiple bond correlation (HMBC) experiment on a 750.13 MHz, Avance III Bruker NMR spectrometer to detect long-range ^{1}H - ^{13}C connectivity with one bond couplings suppressed with a low-pass J-filter; no decoupling was performed during acquisition using gradient pulses for selection (Bax and Summers, 1986).

Purification of *So*ArsAB proteins

Wild type *So*ArsA and *So*ArsB proteins were overproduced in strain JE13607 (*E. coli* BL21(DE3) *cobT::kan*$^+$) from plasmid pARSAB7. Proteins were over-produced in *E. coli* BL21(DE3) *cobT::kan*$^+$ to ensure that native *Ec*CobT did not interfere. Proteins synthesis was induced in early-log-phase cultures (OD_{600} ~0.5) by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.5 mM; after the addition of IPTG, cultures were grown for 16 h at 30 °C. Cultures were harvested by centrifugation (15 min at 4 °C; 6,000 × *g*) in a Beckman/Coulter Avanti J20-XPI centrifuge equipped with a JLA-8.1000 rotor. Cells were stored at −80 °C until used. Frozen pellets were resuspended in buffer D [HEPES, (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer (50 mM, pH 8) containing NaCl (300 mM), imidazole (10 mM), and lysozyme (1 mg/mL)]. Cells were broken by sonication (60 s, 28% duty, 2 s pulses, setting 9) with a 500 Sonic Dismembrator (Fisher Scientific). Cell debris was removed by centrifugation (45 min at 4°C; 43,000 × *g*). Clarified extract was applied onto a 1-mL Ni-charged column (Ni-NTA,

Qiagen), after loading, the column was washed with 10 ml buffer D containing twice as much imidazole (20 mM). *So*ArsAB was eluted off the column with buffer D containing 300 mM imidazole. Fractions containing *So*ArsAB were pooled and H_7-rTEV protease (Blommel and Fox, 2007) was added at a *So*ArsAB: H_7-rTEV ratio of 1:100 to remove the H_7 tag. H_7-rTEV-treated protein was loaded onto a Ni-charged column to separate H_7-rTEV and other contaminants from tag-less *So*ArsAB in the flow through. *So*ArsAB was dialyzed in HEPES buffer (50mM, pH 8) containing NaCl (200 mM), glycerol (20%, v/v), flash frozen with liquid N_2, and stored at −80°C until used.

Determination of the oligomeric state of active *So*ArsAB

Purified *So*ArsAB sample (1 ml, 1 mg/mL) was applied onto an ÄKTA FPLC *Explorer* system (Amersham Biosciences) equipped with a Superdex 200 HR 10/30 (GE Healthcare) column. The column was developed with Na_2HPO_4 buffer (50 mM, pH 8) containing NaCl (200 mM). To generate a standard curve, the log of the molecular mass of selected proteins (Bio-Rad Laboratories gel filtration standards: thyroglobulin, 670 kDa; globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; vitamin B_{12}, 1,355 Da) was plotted against their retention time. The retention time of *So*ArsAB was interpolated to obtain an approximate mass of the *So*ArsAB complex.

Peptide fingerprinting of purified *So*ArsAB

Purified *So*ArsAB proteins were reduced with 25 mM dithiotreitol (DTT in 25 mM NH_4HCO_3) for 30min at 56°C, alkylated in the dark with 55 mM iodoacetamide (IAA in 25mM NH_4HCO_3) at room temperature for 30 min, washed twice in H_2O for 30 s, equilibrated in 25 mM NH_4HCO_3 for 1 min, dehydrated for 5 min in acetonitrile (ACN)/ H_2O/NH_4HCO_3 (50%:50%:25mM) then once more for 30 s in 100% ACN, dried again and rehydrated with 20 μL of trypsin solution [10 ng/μL trypsin Gold (PROMEGA) in 25 mM NH_4HCO_3 / 0.01% ProteaseMAX (w/v) (PROMEGA)]. Additional 30 μL of digestion solution [25 mM NH_4HCO_3 / 0.01% ProteaseMAX (w/v)] was added to facilitate complete rehydration and excess overlay needed for peptide extraction. *So*ArsAB digestion was conducted for 3 h at 42°C, after which peptides generated by the digestion protocol were transferred to a new tube and acidified with 2.5% trifluoroacetic acid (TFA) to 0.3% final. Degraded ProteaseMAX was removed via centrifugation [max speed, 10 min] and the peptides were solid-phase extracted (*ZipTip*® *C18* pipette tips Millipore, Billerica, MA). Peptides were eluted off the C18 column with 1 μL of ACN/H_2O/TFA (60%:40%:0.2%) into 0.5 ml Protein LoBind tube (Eppendorf), 0.5 μL was deposited onto the Opti-TOF™ 384 Well plate (Applied Biosystems) and re-crystalized with 0.50 μL of matrix [10 mg/ml α-cyano-4hydroxycinnamic acid in acetonitrile/H_2O/TFA (60%:40%:0.2%)]. Peptide mass fingerprint analysis was performed on a 4800 Matrix-Assisted Laser Desorption/Ionization-Time of Flight-Time of Flight (MALDI TOF-TOF) mass spectrometer (Applied Biosystems). The peptide fingerprint was generated scanning 700–4,000 Da mass range using 1000 shots acquired from 20 randomized regions of the sample spot at 3800 intensity of OptiBeam™ on-axis Nd:YAG laser with 200Hz firing rate and 3 to 7 ns pulse width in positive reflectron mode. Raw data was deconvoluted using GPS Explorer™ software and submitted for peptide mapping search analysis against NCBInr *E. coli* user modified database and translated *So arsA* and *So arsB* sequences with an in-house licensed Mascot search engine (Matrix, Science, London, UK http://www.matrixscience.com/) with cysteine carbamidomethylation and methionine oxidation as variable modifications.

*So*ArsAB in vitro activity assay

*So*ArsAB phosphoribosyltransferase activity was assayed in vitro using a modification of the described *Se*CobT assay (Claas *et al.*, 2010). The reaction was performed in buffer (100 mM) at the indicated pH, and the reaction mixture (35 μL) contained 120 ng *So*ArsAB/mL,

NaMN (3 mM), non-labeled phenol (1.9 mM), and [^{14}C-U]-phenol (0.1 mM, 60 mCi/mmol). In reactions where DMB substituted for phenol, the reaction mixture contained non-labeled DMB (1.98 mM) and [^{14}C-2]-DMB (20 μM, 43 mCi/mmol). The effect of pH on the activity was investigated using several buffer systems: i) at pH 7, 3-(*N*-morpholino)propanesulfonic acid (MOPS); ii) at pH 8, HEPES; at iii) pH 9 and 10 glycine-NaOH. The reaction was initiated by the addition of phenol or DMB to reaction mixtures pre-incubated at 37 °C for 10 min. Ten-μL samples were removed after 5, 10, and 15 min, and the reaction was stopped by mixing the sample with 2 μL of KCl (3 M); the entire mixture (12 μL) was spotted on a silica TLC plate (10 × 10 cm, PE SIL G/UV, Whatman™). The TLC plate was developed with a 3:2 (v/v) chloroform:methanol mobile phase, allowed to dry for 10 min, and exposed to a MultiPurpose Phosphor Screen (Packard). Radioactivity was detected using a Cyclone Storage Phosphor System (Packard) equipped with OptiQuant v 04.00 software (Packard). The detection range of the instrument was established using known amounts of ^{14}C-phenol or ^{14}C-DMB for reactions using either substrate.

Preparation and separation of α-ribotides

The above assay was scaled up to 200 μL and incubated for 6 h; none of the reagents was radiolabeled. α-Ribotides synthesized by *So*ArsAB were resolved on a Beckman Coulter System Gold® 126 HPLC system equipped a Phenomenex Synergi Hydro-RP (150 by 4.6 mm) column as reported elsewhere (Gray and Escalante-Semerena, 2010). Products were collected and dried under vacuum. The concentration of α-ribotides was estimated using the molar extinction coefficients (in water) of phenol (1373 M^{-1} cm^{-1}, at 270 nm), *p*-cresol (1700 M^{-1} cm^{-1}, at 277 nm), and DMB (5670 M^{-1} cm^{-1}, at 278 nm). α-Ribotides were dephosphorylated using alkaline phosphatase (Fast AP, Fermentas) according to the manufacturer's recommendations. Protein was removed by filtration using an Amicon® Ultra-0.5 centrifugal filter (MWCO = 10kDa), and α-ribosides were separated by RP-HPLC using a described protocol (Gray and Escalante-Semerena, 2010). Retention times (min) were as follows: i) α-phenolyl-ribotide, 6 min; ii) α-*p*-cresolyl-ribotide, 7.7 min; iii) α-DMB-ribotide, 7.7 min; iv) α-phenolyl-riboside, 8.5 min; v) α-*p*-cresolyl-riboside, 10.4 min α; vi) α-DMB-riboside, 9 min. After RP-HPLC purification, α-ribosides were desalted using a C_{18} Sep-Pak and dried under vacuum.

Mass spectrometry of α-ribosides and α-ribotides

The molecular masses of α-ribosides and α-ribotides were determined by electrospray ionization (ESI) mass spectrometry on an Agilent LC/MSD TOF spectrometer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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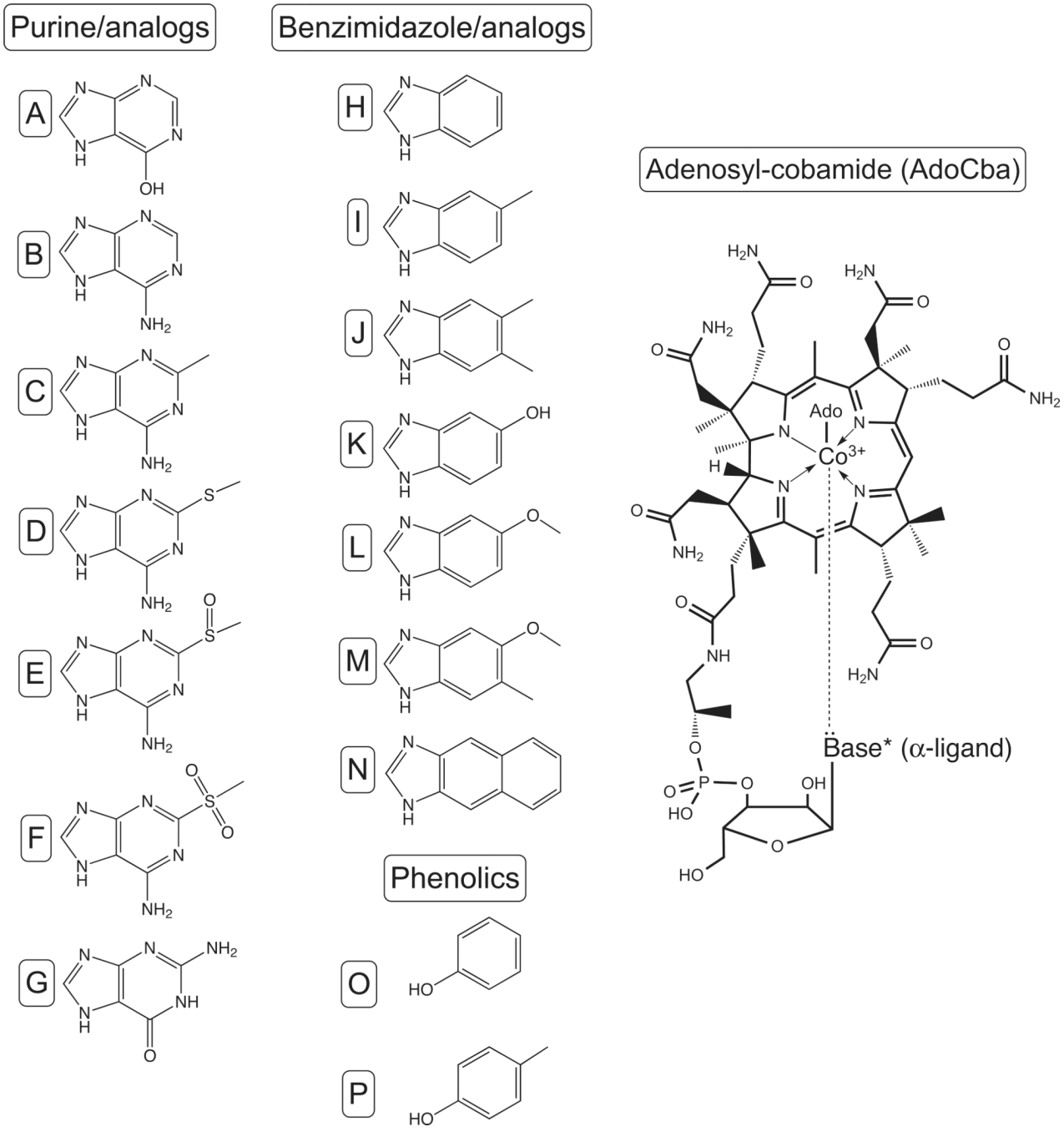

Figure 1. Structure of AdoCba and diversity of lower ligand bases found in Cbas

Purines/analogs: A, Hypoxanthine; B, adenine; C, 2-methyladenine; D, 2-methylmercaptoadenine; E, 2-methylsulfinyladenine; F, 2-methylsulfonyladenine; G, guanine. *Benzimidazole/analogs*: H, benzimidazole; I, 5-methylbenzimidazole; J, 5,6-dimethylbenzimidazole; K, 5-hydroxybenzimidazole; L, 5-methoxybenzimidazole; M, 5-methoxy-6-methylbenzimidazole; N, naphthimidazole. *Phenolics*: O, phenol; P, *p*-cresol. * Phenol and *p*-cresol do not form coordination bonds with the Co ion of the ring.

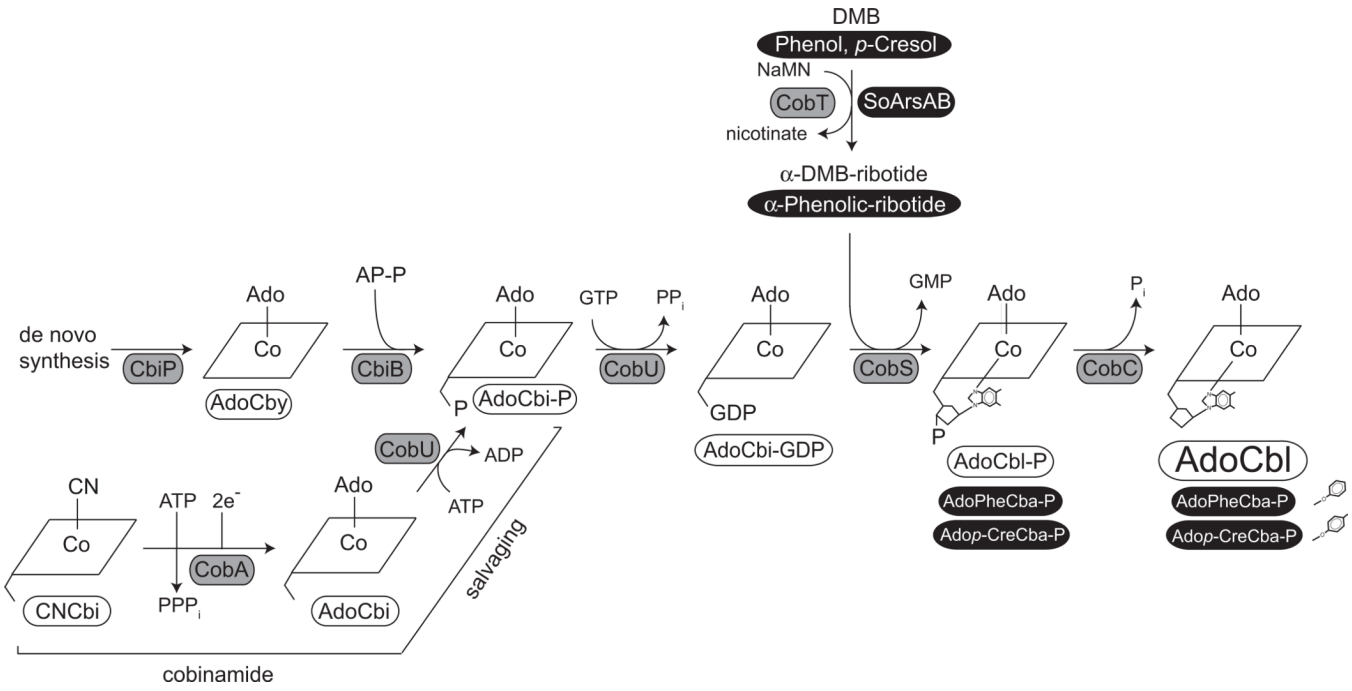

Figure 2. Late steps in AdoCbl biosynthesis in *S. enterica*

For simplicity purposes, the corrin ring is represented as a rhomboid, and lower ligands hydroxyl groups or water molecules are not shown. AdoCby, adenosylcobyric acid; AdoCbi-P, adenosylcobinamide-phosphate; AdoCbi-GDP, adenosylcobinamide-GDP; AdoCbl-P, adenosylcobalamin-phosphate; AdoCbl, adenosylcobalamin; AP-P, aminopropanol phosphate; DMB, 5, 6-dimethylbenzimidazole; NaMN, nicotinate mononucleotide. CbiP (AdoCby synthase) and CbiB (AdoCbiP synthetase) catalyze the last two steps of the de novo corrin ring biosynthetic pathway. CobU (AdoCbi kinase/AdoCbi-P guanylyltransferase), CobS (AdoCbl-P synthase), CobT, NaMN:DMB phosphoribosyltransferase, and CobC (AdoCbl-P phosphatase) comprise the nucleotide loop assembly pathway, also known as the late steps. CobA (ATP :Co(I)rrinoid adenosylytransferase) is the housekeeping corrinoid adenosylating enzyme required for de novo corrin ring biosynthesis, and for the salvaging of incomplete corrinoids like cobinamide. The phenolic substrates for ArsAB and the resulting Cbas synthesized using the α-phenolic-ribotide products are highlighted in black and white. Structures of *O*-glycosidic phenolic ligands are shown next to corresponding cobamide name. Phenolic ligands do not form a coordination bond with the Co ion of the ring.

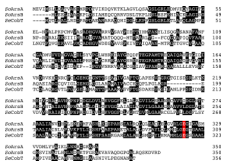

Figure 3. Alignment of *So*ArsA, *So*ArsB with *Se*CobT

Conserved regions are highlighted. *So*ArsA and *So*ArsB are 37% identical, 57 % similar to each other; *So*ArsA is 37% identical, 56% to *Se*CobT, and *So*ArsB is 35% identical, 55% similar to *Se*CobT. The conserved predicted active site glutamyl residue is highlighted in red.

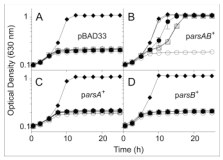

Figure 4. *So*ArsAB compensate for the lack of CobT activity during AdoCba biosynthesis in *S. enterica*

Strains of *S. enterica* were grown in minimal medium supplemented with glycerol (22 mM); $(CN)_2$Cbi (15 nM) was added to all cultures except to the control medium (open circles). Phenol (75 μM, open squares); *p*-cresol, (75 μM, open triangles), or DMB (150 μM, open diamonds); the growth response in medium lacking phenol, *p*-cresol or DMB is also shown (solid circles). CNCbl (15 nM, solid diamonds) was used as positive control. A. Strain JE13867 (*metE cobT cobB* / pBAD33); B. JE13874 (p*arsAB*$^+$ = pARSAB4); C. JE13871 (p*arsA*$^+$ = pARSA4); D. JE13872 (p*arsB*$^+$ = pARSB2).

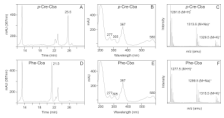

Figure 5. Isolation and characterization of phenolyl-Cba (Panels A–C) and *p*-cresolyl-Cba (Panels D–F) synthesized by *S. enterica*

Phenolyl- and *p*-cresolyl-Cba were synthesized by strain JE13874 (*metE cobT cobB* / pARSAB4 *arsA*$^{+}$ *arsB*$^{+}$) when phenol and *p*-cresol were supplemented in the growth medium. Panels A, D: Isolation of the cobamides by RP-HPLC. Panels B, E: UV-visible spectra of the isolated cobamides. Panels C, F: ESI mass spectra of the purified cobamides. Details of the analyses can be found in the *Experimental procedures* section.

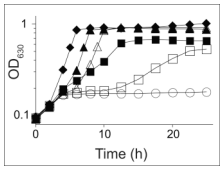

Figure 6. Phenolyl-Cba and *p*-cresolyl-Cba substitute for cobalamin in *S. enterica*

Strain JE8248 (*metE cobS*) was grown on medium supplemented with glucose (11 mM) + CNCbl (15 nM, diamonds), phenolyl-Cba (15 nM, open squares), phenolyl-Cba (150 nM, solid squares), *p*-cresolyl-Cba (15 nM, open triangles), or *p*-cresolyl-Cba (150 nM, solid triangles); no corrinoid control (circles). Growth on strain JE8248 depended on the function of MetH, the cobamide-dependent methionine synthase that converts homocysteine to methionine in this bacterium.

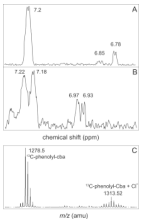

Figure 7. HMBC NMR spectroscopy and mass spectrometry of ^{13}C-labeled phenolyl-Cba

A. ^{13}C HMBC NMR spectra of [^{13}C-1]-phenol (A), and ^{13}C-labeled phenolyl-Cba (B). C. MALDI-TOF spectrum of the ^{13}C-labeled phenolyl-Cba. Number of acquisitions for [^{13}C-1]-phenol = 64,000. Number of acquisitions for ^{13}C-labeled phenolyl-cobamide = 16,384.

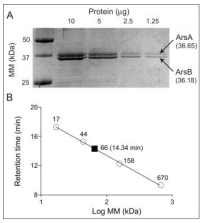

Figure 8. Purity and oligomeric state of *So*ArsAB

A. SDS-PAGE separation of purified tag-less *So*ArsAB. Predicted molecular masses for *So*ArsA and *So*ArsB were 36.65, 36.18 kDa, respectively. B. Gel filtration chromatography of tag-less *So*ArsAB. The retention time of protein standards of known molecular masses were plotted against their retention time. *So*ArsAB (solid square) eluted at 14.34 min, which corresponded to a mass of ~66 kDa.

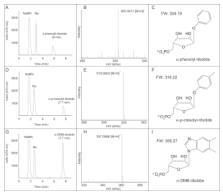

Figure 9. *So*ArsAB dependent synthesis of α-phenolyl-ribotide (panels A–C), α-*p*-cresolyl-ribotide, (panels D–F), and α-5,6-dimethylbenzimidazolyl (DMB)-ribotide (panels G–I)
Conditions for the in vivo synthesis of α-ribotides are described under *Experimental procedures*. Panels A, D, G, RP-HPLC resolution of reaction substrates from products; panels B, E, H, ESI mass spectra of α-ribotides synthesized by *So*ArsAB; panels C,F,I, structure and formula weight of α-ribotides synthesized by *So*ArsAB.

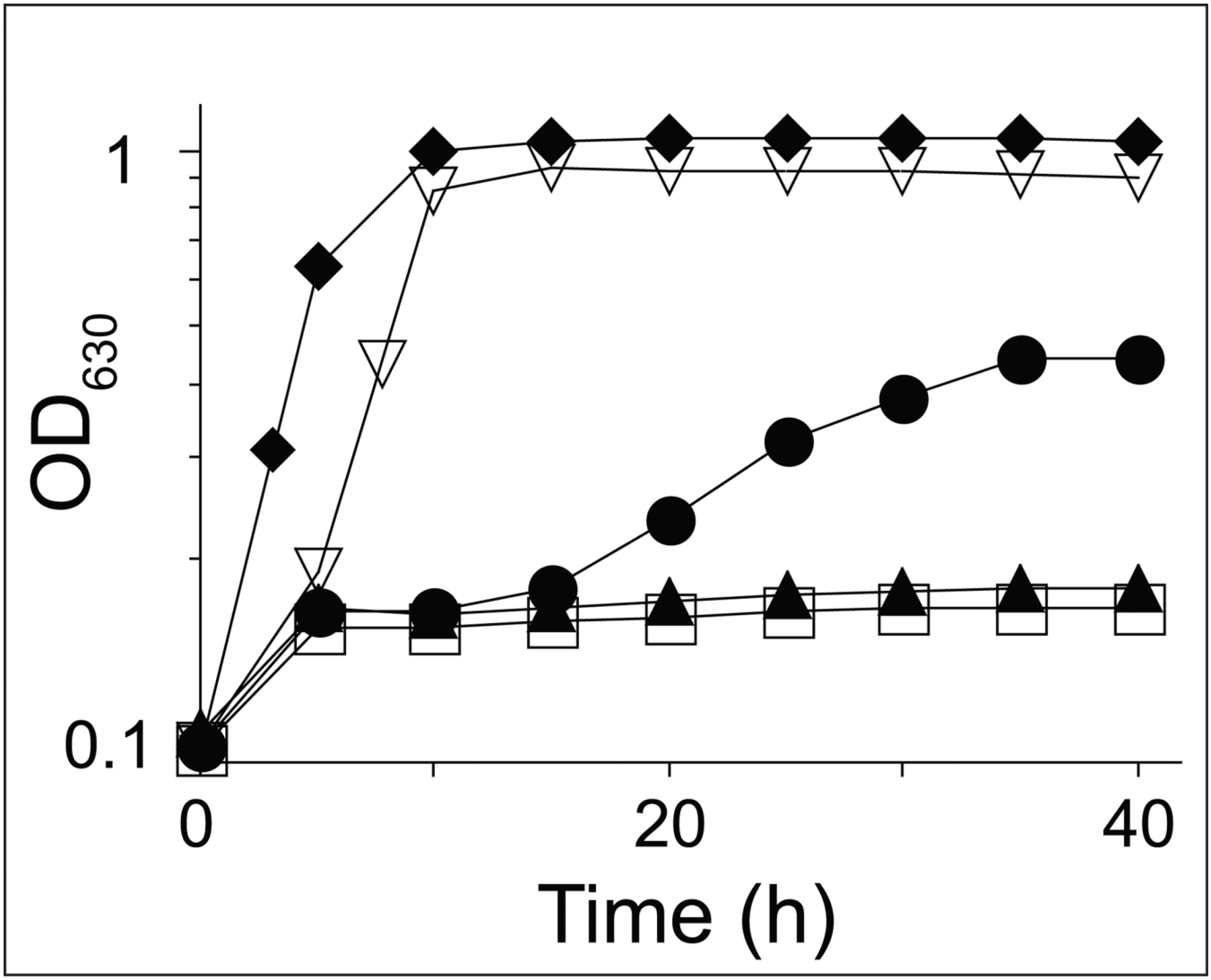

Figure 10.
Growth of strain JE2501 (*metE cobT cobB*) on NCE minimal medium supplemented with glucose (11 mM) with no corrinoids (squares); $(CN)_2$Cbi (15 nM, solid triangles); $(CN)_2$Cbi (15 nM) + α-DMB-ribotide (15 μM, diamonds); $(CN)_2$Cbi (15 nM) + α-*p*-cresolyl-ribotide (75 μM, inverted triangles); or $(CN)_2$Cbi (15 nM) + α-phenolyl-ribotide (75 μM, circles). Under the conditions used, growth of JE2501 demanded the conversion of Cbi to Cba by the nucleotide loop assembly enzymes (see Fig. 2), and the use of the resulting Cba by the methionine synthase (MetH) enzyme.

Table 1

Strains and plasmids used in this study

***S. enterica* strains**	**Genotype**	**Source**
TR6583	*metE205 ara-9*	K. Sanderson via J. Roth
TR6583 derivatives		
JE2501	*cobT109*::MudJ[a] *cobB1176*::Tn*10*d(*tet*$^+$)[b]	laboratory collection
JE8248	Δ*cobS1313*	(Otte and Escalante-Semerena, 2009)
JE13867	*cobT109*::MudJ *cobB1176*::Tn*10*d(*tet*$^+$) / pBAD33	
JE13871	*cobT109*::MudJ *cobB1176*::Tn*10*d(*tet*$^+$) / pARSA4	
JE13872	*cobT109*::MudJ *cobB1176*::Tn*10*d(*tet*$^+$) / pARSB2	
JE13874	*cobT109*::MudJ *cobB1176*::Tn*10*d(*tet*$^+$) / pARSAB4	
***E. coli* strains EPI300**	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) (StrR) Φ80d*lacZ*ΔM15 Δ*lac*X74 *recA1 endA1 araD139* Δ(*ara, leu*)*7697 galU galK* λ– *rpsL nupG trfA tonA dhfr*	Epicentre
EPI300 derivatives		
JE11098	*metE400*::*kan*$^+$	
JE11106	Δ*metE401*	
JE11215	EPI300 Δ*metE401 cobT150*::*kan*$^+$	
JE14470	EPI300 Δ*metE401 cobT150*::*kan*$^+$ / pSolibC17	
BL21(DE3)	F$^-$, *ompT*, *hsdS*$_B$ (r_B^-, m_B^-), *dcm*, *gal*, λ(DE3)	laboratory collection
JE13607	BL21 *cobT150*::*kan*$^+$	
Keio collection strains BW25113	*rrnB3* Δ*lacZ4787 hsdR513* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1*	(Baba *et al.*, 2006)
	metE400::*kan*$^+$	(Baba et al., 2006)
	cobT150::*kan*$^+$	(Baba et al., 2006)
Plasmids pBAD33 derivatives	complementation vector, P_{araBAD} *cat*$^+$	(Guzman et al., 1995)
pARSA4	*S. ovata arsA*$^+$	
pARSB2	*S. ovata arsB*$^+$	
pARSAB4	*S. ovata arsA*$^+$ *arsB*$^+$	
pTEV5 derivatives	Over-expression vector that fuses the *N*-terminus of the protein of interest to a H_6 tag, which can be removed by rTEV protease, *bla*$^+$	(Rocco et al., 2008)
pARSAB7	*S. ovata arsA*$^+$ *arsB*$^+$	
Other plasmids		
pEAK2	*bla*$^+$ *recA*$^+$	(Kouzminova and Kuzminov, 2004)
pCC1FOS	Fosmid construction plasmid *cat*$^+$	Epicentre

[a] MudJ is an abbreviation of MudI1734 (Castilho *et al.*, 1984)

[b] Tn*10*d(*tet*$^+$) is an abbreviation of Tn*10*Δ*16*Δ*17* (Way *et al.*, 1984)