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A New Pathway for the Synthesis of α-Ribazole-Phosphate in *Listeria innocua*

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Abstract

The genomes of *Listeria* spp. encode all but one of 25 enzymes required for the biosynthesis of adenosylcobalamin (AdoCbl; coenzyme B_{12}). Notably, all *Listeria* genomes lack CobT, the nicotinamide mononucleotide:5,6-dimethylbenzimidazole (DMB) phosphoribosyltransferase (EC 2.4.2.21) enzyme that synthesizes the unique α-linked nucleotide N^1-(5-phospho-α-D-ribosyl)-DMB (α-ribazole-5′-P, α-RP), a precursor of AdoCbl. We have uncovered a new pathway for the synthesis of α-RP in *Listeria innocua* that circumvents the lack of CobT. The *cblT* and *cblS* genes (locus tags *lin1153* and *lin1110*) of *L. innocua* encode an α-ribazole (α-R) transporter and an α-R kinase, respectively. Results from *in vivo* experiments indicate that *L. innocua* depends on CblT and CblS activities to salvage exogenous α-R, allowing conversion of the incomplete corrinoid cobinamide (Cbi) into AdoCbl. Expression of the *L. innocua cblT* and *cblS* genes restored AdoCbl synthesis from Cbi and α-R in a *Salmonella enterica cobT* strain. *Lin*CblT transported α-R across the cell membrane, but not α-RP or DMB. UV-visible spectroscopy and mass spectrometry data identified α-RP as the product of the ATP-dependent α-R kinase activity of *Lin*CblS. Bioinformatics analyses suggest that α-R salvaging occurs in important Gram-positive human pathogens.

INTRODUCTION

Cobamides, such as adenosylcobalamin (AdoCbl, Fig. 1) are complex cobalt-containing cyclic tetrapyrroles whose biosynthesis is restricted to bacteria and archaea (Escalante-Semerena & Warren, 2008). The lower ligand of AdoCbl is 5,6-dimethylbenzimidazole (DMB), a purine analog tethered to the corrin ring via a structure known as the nucleotide loop. *Salmonella enterica* assembles the nucleotide loop in four steps. One enzyme activates DMB, another activates the corrin ring, a third condenses the activated precursors, and a fourth yields AdoCbl, the final product of the pathway (Escalante-Semerena & Warren, 2008).

Most relevant to the work reported here is the activation of DMB. As shown in Fig. 1, the nicotinate mononucleotide (NaMN):DMB phosphoribosyltransferase (CobT, EC: 2.4.2.21) enzyme activates DMB into N^1-(5-phospho-α-D-ribosyl)-DMB (α-ribazole-5′-P, α-RP). (Trzebiatowski *et al.*, 1994;Cameron *et al.*, 1991;Friedmann & Harris, 1965). Notably, the genomes of bacteria of the genus *Listeria* lack a *cobT* homolog (Hain *et al.*, 2006;Buchrieser *et al.*, 2003), raising the question of how *Listeria* compensates for the absence of CobT. Bioinformatics analysis performed by others (Rodionov *et al.*, 2003) noted the lack of CobT in *Listeria*, and proposed that two genes of unknown function (dubbed *cblT* and *cblS*) might encode non-orthologous replacements for CobT. The authors of these studies hypothesized that the putative CblS protein might have CobT-like activity, and that the putative CblT protein might be a DMB transporter (Rodionov *et al.*, 2009;Rodionov *et al.*, 2003).

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We have identified the biochemical activities of CblT and CblS from *Listeria innocua*, and provide a physiological framework for their activities. We show that *Lin*CblT is an α-ribazole (αR) transporter and *Lin*CblS is α-R kinase. Together, *Lin*CblT and *Lin*CblS comprise a new pathway for salvaging α-R and for the CobT-independent synthesis of α-RP. The distribution and possible implications of this new pathway amongst AdoCbl producers is discussed.

RESULTS

L. innocua cblT and *cblS* functions allow a *S. enterica cobT* strain to synthesize α-RP

We initially took a genetic approach to investigate the function of *Lin*CblT and *Lin*CblS. In these experiments, we used *S. enterica cobT* strains to block the synthesis of α-RP (Trzebiatowski *et al.*, 1994). All *S. enterica* strains lacked the Cbl-independent methionine synthase (MetE) enzyme, so methionine synthesis depended on the Cbl-dependent methionine synthase (MetH) enzyme (Jeter *et al.*, 1984). *S. enterica* strains were grown under aerobic conditions to block *de novo* synthesis of the corrin ring (Escalante-Semerena & Roth, 1987), but the medium was supplemented with dicyanocobinamide ($[CN]_2$Cbi), a precursor of AdoCbl whose conversion to AdoCbl required the synthesis of α-RP.

The *Lin cblT* and *cblS* genes (locus tags *lin1153* and *lin1110*, respectively) were cloned individually or together into plasmid pBAD24, placing them under the control of the arabinose-inducible P_{BAD} promoter (Guzman *et al.*, 1995). The resulting plasmids were transformed into *S. enterica cobT* strain JE1244 (Table S1), a strain unable to make α-RP (Fig. 1). As expected, robust growth of strain JE1244 carrying plasmid pBAD24 was restored by the addition of CNCbl to the medium (Fig. 2, column A, row 6), but not in medium containing $(CN)_2$Cbi (Fig. 2, column A, row 2), or $(CN)_2$Cbi + DMB (Fig. 2, column A, row 3). Poor growth was obtained in medium containing $(CN)_2$Cbi + α-R (Fig. 2, column A, row 4). A culture of strain JE1244 reached the same density in medium supplemented with $(CN)_2$Cbi + α-RP or CNCbl. However, growth with $(CN)_2$Cbi + α-RP occurred at a slower rate (Fig. 2, column A, rows 5, 6), suggesting that α-RP was inefficiently taken up by *S. enterica*. Positive control experiments showed that a plasmid-encoded $cobT^+$ allele restored AdoCbl synthesis in strain JE1244, allowing growth in medium supplemented with $(CN)_2$Cbi, with or without addition of DMB, α-R, or α-RP (Fig. 2, column B).

A plasmid encoding *Lin* $cblT^+$ and $cblS^+$ (pCBLTS1) did not improve the growth of JE1244 in medium containing $(CN)_2$Cbi, $(CN)_2$Cbi + DMB, or $(CN)_2$Cbi + α-RP, even at high levels of induction (Fig. 2, column C, rows 2, 3, and 5). The latter results indicated that the *Lin*CblT and *Lin*CblS proteins did not have CobT-like activity. In contrast, even at a low concentration of inducer (250 μM arabinose), plasmid pCBLTS1 (*Lin* $cblTS^+$) restored AdoCbl synthesis in strain JE1244 growing in medium containing $(CN)_2$Cbi + α-R (Fig. 2, column C, row 4). Plasmid pCBLT1 (*Lin* $cblT^+$) did not support growth of strain JE1244 under the conditions tested (Fig. 2, column D), and even caused a slight inhibitory effect when expressed at high levels (5 mM arabinose) in medium containing CNCbl (Fig. 2, column D, row 6). Expression of plasmid-encoded *Lin* $cblS^+$ (pCBLS4) restored AdoCbl synthesis in strain JE1244 in medium supplemented with $(CN)_2$Cbi + α-R, but only when high level of inducer (5 mM arabinose) was present in the medium (Fig. 2, column E, row 4).

L. innocua cannot synthesize α-RP, but can salvage α-R

The above results suggested that *L. innocua*, which naturally lacks CobT, might rely on exogenous α-R for AdoCbl biosynthesis. To test this hypothesis, we developed a nitrogen-

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limited defined medium for growth of *L. innocua* (MLM, Table S2). The only nitrogen sources in MLM were low concentrations of adenine and the required amino acids cysteine, leucine, isoleucine, and valine. We tested whether wild-type *L. innocua* could use ethanolamine as a nitrogen source, which would require the activity of ethanolamine ammonia-lyase, an AdoCbl-dependent enzyme (Babior, 1982).

Under the conditions used, the cell density of a culture of *L. innocua* growing with ethanolamine as a nitrogen source was substantially higher when the medium was supplemented with CNCbl or NH_4Cl (Fig. 3, black squares, open circles *vs.* open squares). When $(CN)_2Cbi$ substituted for CNCbl in the medium, *L. innocua* grew poorly (Fig. 3, light grey triangles), suggesting that *L. innocua* could not synthesize α-RP *de novo*. Addition of DMB had only a very slight stimulatory effect (Fig. 3, grey circles). *L. innocua* grew well when provided with $(CN)_2Cbi$ and α-R (Fig. 3, dark grey diamonds), supporting the hypothesis that *L. innocua* contained a pathway for salvaging α-R from its environment.

A model for α-R salvaging

Ideas about the possible roles of the *Lin*CblT and *Lin*CblS proteins emerged from bioinformatics analyses. From the literature we knew that *Lin*CblT was a member of the ECF class of vitamin transporters (Rodionov *et al.*, 2003; Rodionov *et al.*, 2009), and our own PSI-BLAST searches (Altschul *et al.*, 1997) identified *Lin*CblS as a member of the phosphoribosylaminoimidazole synthetase (PurM) ATP-binding protein superfamily (McCulloch *et al.*, 2008, Li *et al.*, 1999). From this information, we surmised that *Lin*CblT and *Lin*CblS might comprise a system for the uptake of α-R and its conversion to α-RP. To our knowledge, an α-R salvaging pathway has not been described in any organism. The putative functions of *Lin*CblT and *Lin*CblS proteins were investigated *in vitro* and *in vivo*.

*Lin*CblT transports α-R across the cell membrane

A *S. enterica cobT* strain expressing *Lin cblT*$^+$ from a plasmid was grown to mid-log phase and tested for its ability to take up extracellular [^{14}C, C-2]DMB, [^{14}C, C-2]α-R, or [^{14}C, C-2]α-RP. Expression of *Lin cblT*$^+$ allowed *S. enterica* to accumulate α-R, but not DMB or α-RP (Fig. 4), indicating that the *Lin*CblT protein was a specific α-R transporter. No DMB, α-R, or α-RP accumulated in a strain lacking *Lin cblT* (Fig. 4).

The *Lin*CblS protein catalyzes the ATP-dependent phosphorylation of α-R

The *Lin*CblS protein was overproduced in *Escherichia coli* as a fusion protein with an *N*-terminal H_6 tag. H_6-*Lin*CblS protein was purified by Ni affinity chromatography, and the H_6 tag was removed using rTEV protease (Rocco *et al.*, 2008). A second Ni affinity chromatographic step yielded homogenous *Lin*CblS with three non-native *N*-terminal residues (Gly-Ala-Ser) (Fig. S1). *Lin*CblS (10 μg) was incubated with α-R (30 μM) and ATP (1 mM) for 3 h at 37°C in a 200-μl reaction mixture containing Tris-HCl (100 mM, pH 7.5 @ 25°C), $MgCl_2$ (1 mM), KCl (50 mM), and TCEP (1 mM). Components of the reaction mixture were separated by reverse-phase HPLC. The product that accumulated had an elution time, UV-visible absorbance spectrum, and mass spectrum identical to those of authentic α-RP (Fig. 5). No detectable product was formed (<0.04 nmol min^{-1} mg^{-1} protein) in reactions mixtures lacking *Lin*CblS, ATP, or TCEP (Fig. 5).

We optimized reaction conditions for the α-R kinase activity of *Lin*CblS (Fig. S2). Optimal pH was pH 7.0 with highest activity at 35°C. KCl was required, with optimal activity measured at 750 mM KCl; the activity of the enzyme varied at > 750 mM KCl. *Lin*CblS activity was optimal with 50 mM $MgCl_2$; reduced activity was observed when $CoCl_2$ or $MnCl_2$ (50 mM) were used instead of $MgCl_2$; activity was not observed with $CaCl_2$, $CuCl_2$,

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$NiCl_2$, or $ZnCl_2$. Under optimal conditions, product formation was linear up to 20 min, with 3, 5, or 7 μg of *Lin*CblS in the reaction mixture.

DISCUSSION

CobT-independent synthesis of α-RP

Prior to this work, there was no precedent in the literature for CobT-independent synthesis of α-RP. The genome of *L. innocua* does not encode a CobT homolog, the enzyme that synthesizes α-RP from DMB and NaMN. To circumvent this problem, *L. innocua* relies on an α-R transporter (*Lin*CblT, encoded by *lin1153*) and an α-R kinase (*Lin*CblS, encoded by *lin1110*) to synthesize α-RP. Thus *Lin*CblT and *Lin*CblS define a previously unknown pathway for α-R salvaging and α-RP synthesis (Fig. 6).

Not all bacteria encoding CblT or CblS homologs lack CobT (Table S3), suggesting that α-riboside salvaging and endogenous α-ribotide synthesis are not mutually exclusive. In fact, some genomes (*e.g. Propionibacterium acnes*, *Moorella thermoacetica*) encode two homologs of CobT; the significance of this apparent redundancy is unknown.

The results shown in Fig. 4 indicate that *S. enterica*, which is known to use exogenous DMB in AdoCbl biosynthesis (Escalante-Semerena & Warren, 2008), does not have a dedicated DMB importer. Based on results shown in figure 4, we also conclude that CblT does not function as a DMB importer, as previously proposed (Rodionov *et al.*, 2003). Thus, the existence of an active DMB transporter in any organism remains an open question.

Genes encoding functions of the α-R salvaging pathway are found in Gram-positive human pathogens

Bioinformatics searches identified CblT and CblS homologs only in a subset of Gram-positive bacteria, among which are a number of significant human pathogens, including *L. monocytogenes*, *Clostridium botulinum, C. tetani*, and *C. perfringens* (Tables S3) (Markowitz *et al.*, 2006). The *cblT* and *cblS* homologs of different species exist in different genetic contexts, and in many instances *cblT* and *cblS* are found in loci containing putative AdoCbl synthesis genes (Fig. S3).

We found three genomes (*Bacillus halodurans*, *Desulfitobacterium hafniense*, *P. acnes*), which encode CblS homologs, but not CblT homologs, and one (*B. coahuilensis*) that encodes a CblT homolog, but no CblS homolog. It is unclear why in some cases only one of these proteins is synthesized. It is possible that genomes that encode one but not the other protein contain non-orthologous replacements of the missing protein.

Multiple routes to the lower ligand

Some genomes that encode CblT or CblS homologs also encode BluB (O_2-dependent, $FMNH_2$-degrading DMB synthase) homologs (Table S3) (Taga *et al.*, 2007;Gray & Escalante-Semerena, 2007). The presence of BluB suggests that, in the presence of oxygen, these bacteria synthesize DMB, and that α-RP is endogenously synthesized by CobT. The presence of CblT and CblS suggests an alternative means of synthesizing α-RP, possibly in response to changing oxygen levels. Eight genomes encoding CblT or CblS homologs also encoded corrinoid amidohydrolase (cobyric acid-forming) CbiZ homologs (Table S3), suggesting that these bacteria have cobamide remodeling capabilities to ensure that the correct lower ligand base is incorporated into the final product of the pathway (Gray *et al.*, 2008;Gray & Escalante-Semerena, 2009).

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What does the existence of CblTS tell us about the environment?

The existence of CblT and CblS, especially in bacteria that lack CobT, implies that there is a reliable supply of α-R and other α-ribosides to support cobamide biosynthesis in the habitats occupied by these bacteria. This idea needs to be investigated.

How do CblT and CblS work?

Alignment of 29 CblT homologs (Fig. S4) reveals a conserved Gly-Phe-Pro-Leu motif in a predicted cytoplasmic loop of CblT. We hypothesize that this motif may be involved in substrate recognition. CblS is homologous to the PurM protein superfamily, but alignment of *Lin*CblS with the *E. coli* proteins of this family (PurM, ThiL, HypE, and SelD) shows few conserved residues (Fig. S5). Alignment of 32 CblS homologs revealed conserved motifs (Fig. S6), but their function is not clear. Detailed structure-function analyses of CblS are needed to understand the mechanism and substrate recognition properties of CblS. A better understanding of CblT and CblS function would be needed for the development of antimicrobial drugs targeting these proteins.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table S1. *E. coli* strains were grown at 37°C in lysogenic broth (LB, Difco) (Bertani, 1951;Bertani, 2004). *S. enterica* strains were derived from strain TR6583 (*metE205 ara-9*). *S. enterica* strains were grown at 37°C in nutrient broth (NB, Difco) or no-carbon essential (NCE) minimal medium (Berkowitz *et al.*, 1968) containing $MgSO_4$ (1 mM), glycerol (22 mM), and trace minerals (Balch & Wolfe, 1976). *Listeria innocua* DD680 was grown at 37°C in brain heart infusion (BHI, Difco) or in MLM defined medium (minimal *Listeria* medium, Table S2). For growth curves, starter cultures were grown aerobically overnight in NB containing ampicillin (for *S. enterica*) or BHI (for *L. innocua*) and used to inoculate fresh medium (5% v/v). Growth curves were obtained using an ELx808 Ultra Microplate Reader (Bio-Tek Instruments) in a volume of 200 μl per well. When present, ampicillin was at 100 μg ml^{-1}. When added, corrinoids were at 15 nM and DMB, α-R, and α-RP were at 250 or 500 nM, as indicated. Synthesis of α-R and α-RP is described below. All other chemicals were purchased from Sigma.

Preparation of α-R and α-RP

α-RP was prepared enzymatically from DMB and NaMN, using purified *S. enterica* NaMN:DMB phosphoribosyltransferase (CobT) enzyme, as described (Maggio-Hall & Escalante-Semerena, 1999; Trzebiatowski & Escalante-Semerena, 1997). α-R was prepared by alkaline hydrolysis of CNCbl by a modification of the method of Pakin *et al*. (Pakin *et al.*, 2005); 0.1 g of CNCbl was incubated for 50 min at 100°C in 20 ml of 2.5 N NaOH, then neutralized by addition of 10 ml 5 N HCl. Alkaline phosphatase (1000 U) was added in 30 ml of 1 M Tris-HCl (pH 8.0), and incubated 48 h at 37°C. α-R was purified by reverse-phase liquid chromatography by binding to a 70-ml column of LiChroprep® RP-8 resin (EM Separations), previously equilibrated at 5 ml min^{-1} with H_2O. The column was rinsed with 250 ml H_2O, and then α-R was eluted off the column with a 450 ml linear gradient from 0 to 100% methanol (MeOH). Fractions containing α-R were identified by their characteristic fluorescence (excitation wavelength 250 nm, emission wavelength 312 nm) using a SpectraMAX Gemini EM spectrofluorimeter (Molecular Devices), dried under vacuum, and resuspended in 10 ml of dimethlysulfoxide (DMSO). Yield of α-R was approximately 8 mg. Samples of α-R were further purified by RP-HPLC, using a Beckman Coulter System Gold® 126 HPLC system equipped with a 250 × 10 mm Luna 5μ C18(2) column (Phenomenex); elution off the column was monitored using a photodiode array detector (λ 200 – 600 nm).

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The column was equilibrated at 3 ml min^{-1} with 70% H_2O and 30% MeOH. Column development started 20 min after injection, with a 50-min linear gradient to 60% MeOH, followed by a 10-min linear gradient to 100% MeOH. Fractions containing α-R were collected, dried under vacuum, and re-suspended in DMSO. The identity of α-R was confirmed by mass spectrometry (University of Wisconsin-Madison Biotechnology Center) (Fig. S7). ESI mass spectra were obtained using an Applied Biosystems 3200 Q TRAP mass spectrometer. The molar extinction coefficients for DMB in DMSO (ε_{280} = 6027 M^{-1} cm^{-1}) or in MeOH (ε_{280} = 5260 M^{-1} cm^{-1}) (Maggio-Hall, 2001) were used to estimate the concentrations of purified α-R and α-RP in DMSO and MeOH.

Preparation of radiolabeled DMB, α-RP, and α-R

[C^{14}, C-2]DMB (43.24 μCi/μmol) was prepared as described (Claas *et al.*, 2010). [C^{14}]α-RP (9.51 μCi/μmol) was synthesized from [C^{14}-2]DMB and NaMN using *S. enterica* CobT as described (Maggio-Hall & Escalante-Semerena, 1999; Trzebiatowski & Escalante-Semerena, 1997). [C^{14}, C-2]α-R was prepared from 10 nmoles of [C^{14}, C-2]α-RP by addition of alkaline phosphatase (10 U) in 1 ml of Tris-HCl buffer (1 M, pH 8.0), incubation 24 h at 37°C, then bound to a 1 ml C18 Sep-Pak cartridge (Waters), rinsed with 40 ml H_2O, eluted with MeOH, and dried under vacuum, and resuspended in H_2O.

Genetic and molecular techniques

DNA manipulations were performed using described methods (Bloch, 1995; Moore & Dowhan, 2002; Struhl, 1987). Restriction and modification enzymes were purchased from Fermentas (Ontario, Canada) and used according to the manufacturer's instructions. All DNA manipulations were performed in *E. coli* DH5α (Raleigh *et al.*, 1989; Woodcock *et al.*, 1989). Plasmid DNA was isolated using the Wizard Plus SV Plasmid Miniprep kit (Promega). PCR products were purified with the Wizard SV Gel and PCR Clean-Up System kit (Promega). Genomic DNA was isolated from bacterial cultures using the Wizard SV Genomic DNA Purification kit (Promega). DNA sequencing reactions used non-radioactive BigDyeR protocols (ABI PRISM; Applied Biosystems) and were resolved at the Biotechnology Center of the University of Wisconsin-Madison. Primers used in this study are listed in Table S4. The identity of all inserts cloned into plasmid pBAD24 (Guzman *et al.*, 1995) was confirmed by sequencing with primers [1] and [2].

Construction of *Lin cblT*$^+$ plasmid

The *Lin cblT*$^+$ coding sequence was amplified using primers [3] and [4], and the resulting product was cloned into the *Eco*RI and *Xba*I sites of plasmid pBAD24 to yield plasmid pCBLT1.

Construction of *Lin cblS*$^+$ plasmids

The *Lin cblS*$^+$ coding sequence was amplified using primers [5] and [6], and the resulting product (with an ATG rather than the native TTG start codon) was cloned into the *Nhe*I and *Hin*dIII sites of plasmid pKLD116 (Rocco *et al.*, 2008) to yield plasmid pCBLS3. The identity of the insert was confirmed by sequencing with primers [7] and [8]. The *Lin cblS*$^+$ coding sequence plus 6 bp of 3′ sequence was excised from plasmid pCBLS3 and sub-cloned into the *Nhe*I and *Not*I sites of plasmid pTEV5 (Rocco *et al.*, 2008) to yield plasmid pCBLS5. The identity of the insert was confirmed by sequencing with primers [8] and [9]. The *Lin cblS*$^+$ coding sequence (with ATG start codon) was amplified using primers [10] and [11], and the resulting product was cloned into the *Eco*RI and *Hin*dIII sites of plasmid pBAD24 to yield plasmid pCBLS4.

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Construction of *Lin cblT*+ *cblS*+ plasmid

The *Lin cblT*+ coding sequence was amplified using primers [12] and [13], and the *Lin cblS*+ coding sequence (with ATG start codon) was amplified using primers [14] and [15]. The resulting PCR products were cloned into the *Eco*RI and *Pst*I sites of pBAD24 using the In-Fusion™ PCR Cloning System (Clontech) according to the manufacturer's instructions, yielding plasmid pCBLTS1.

Transport assays

Overnight cultures of *S. enterica* strains grown in NB containing ampicillin were sub-cultured (10% v/v) into 5 ml NCE glycerol medium containing ampicillin, arabinose (250 μM), and CNCbl (15 nM), then grown at 37°C with shaking to an optical density (OD_{600}) of 0.5 – 0.6. [^{14}C]DMB, [^{14}C]α-R, or [^{14}C]α-RP was added to 250 nM, and cultures were incubated at 37°C. 200-μl samples were removed at intervals, filtered through 0.45-μm filter discs (Pall Life Sciences) under vacuum, and washed with 5 ml of ice-cold NCE medium. Filter discs were placed in 8 ml of Scinti-Safe scintillation fluid (Fisher Scientific), and cell-associated radioactivity was quantified with a Tri-Carb 2100TR liquid scintillation counter (Packard).

Purification of *Lin*CblS protein

*Lin*CblS protein fused to a rTEV protease cleavable N-terminal H_6 tag was overproduced using plasmid pCBLS5 in *E. coli* BL21(DE3) (Novagen). 40 ml of an overnight culture of the overexpressing strain was inoculated into 2 liters of LB broth containing ampicillin. Cultures were grown 2 hours at 37°C with shaking, isopropyl-β-D-thiogalactopyranoside was added to 1 mM, and cultures were incubated for 20 h at 15°C with shaking. Cells were harvested by centrifugation (15 min at 5,000 × *g* at 4°C), resuspended in 10 ml of Tris-HCl buffer (20 mM, pH 7.9 at 4°C) containing NaCl (0.5 M) and imidazole (5 mM), and broken by sonication with a Fisher Scientific Sonic Dismembrator 550 (5 min at half duty). Cell lysate was cleared by centrifugation (1 h at 14,000 *g* at 4°C) and filtered through a 0.45-μm syringe filter (Nalgene). Tagged *Lin*CblS protein was purified using His-Bind® resin (Novagen) according to the manufacturer's instructions. The H_6 tag was removed by incubation for 3 h at 30°C with rTEV protease (Kapust & Waugh, 2000) present in the buffer at a 1:10 ratio of *Lin*CblS:rTEV protease. H_6-rTEV protease was resolved from de-tagged *Lin*CblS protein by passing the protein mixture over His-Bind® resin. The purity of proteins was monitored by SDS-PAGE (Laemmli, 1970) and Coomassie Blue staining (Sasse, 1991). Fractions containing de-tagged *Lin*CblS protein were pooled, dialyzed (MWCO = 10,000 membrane, Pierce) at 4°C against 2 liters of Tris-HCl buffer (20 mM, pH 7.9 at 4°C) containing NaCl (50 mM) and glycerol (10% v/v) with three buffer changes, and stored at −80°C after flash freezing in liquid N_2. Protein purity was assessed using the TotalLab software package (Nonlinear Dynamics Ltd).

α-R kinase activity assays

*Lin*CblS activity was assayed in 200-μl reaction mixtures containing 3 – 10 μg of *Lin*CblS. Reactions contained Tris-HCl buffer (100 mM, pH 7.0 @ 25°C), *tris*(2-carboxyethyl)phosphine (5 mM), KCl (0.75 M), $MgCl_2$ (50 mM), α-R (60 μM), and ATP (1 mM), unless otherwise indicated. Where indicated, $MgCl_2$ was replaced with 50 mM $CaCl_2$, $CoCl_2$, $CuCl_2$, $MnCl_2$, $NiCl_2$, or $ZnCl_2$. Buffers (100 mM) used to assess pH optimum were: 2-(N-morpholino)ethanesulfonic acid for pH 5.5, 6, and 6.5; Tris-HCl for pH 7, 7.5, 8, 8.5, and 9. Reactions were incubated at the indicated temperatures and stopped by incubation at 100°C for 10 min. Precipitated protein was removed by centrifugation (2 min at 7,500 × *g*) and samples were filtered with Spin-X filtration columns (2-μm pore size) (Costar).

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HPLC analysis of *Lin*CblS reaction products

The product of the *Lin*CblS reaction was resolved by a modification of Phenomenex HPLC application #15754 using a Beckman Coulter System Gold® 126 HPLC system equipped with a Beckman Coulter System Gold® 508 autosampler and a Phenomenex 150 × 4.6 mm Synergi 4μ Hydro-RP column. Products were detected by their absorbance at 287 nm using a photodiode array detector. The column was equilibrated at 1 ml min^{-1} with 97% 20 mM ammonium acetate, pH 4.5 and 3% acetonitrile (CH_3CN). After injection, the column was developed for 10 min with a linear gradient to 40% CH_3CN, then developed for 5 min with a linear gradient to 100% CH_3CN. α-RP was quantified by comparison with a standard curve of α-R (limit of detection = 10 pmol). The identity of the *Lin*CblS reaction product was confirmed by liquid chromatography mass spectrometry (University of Wisconsin-Madison Biotechnology Center), using a using an Agilent LC/MSD ESI-TOF with a mass accuracy of greater than 3 ppm. Separation was performed with an Agilent 1100 LC with a 2.1 × 50 mm Zorbax SB-C18 column (1.8 μm particle size), using a 20 min gradient of 100% solvent A (0.1% formic acid in water) to 15% solvent B (0.1% formic acid in acetonitrile). Reference masses of 121.05087 and 922.0098 amu from the Agilent API TOF reference mass solution kit were used as a lock mass standard.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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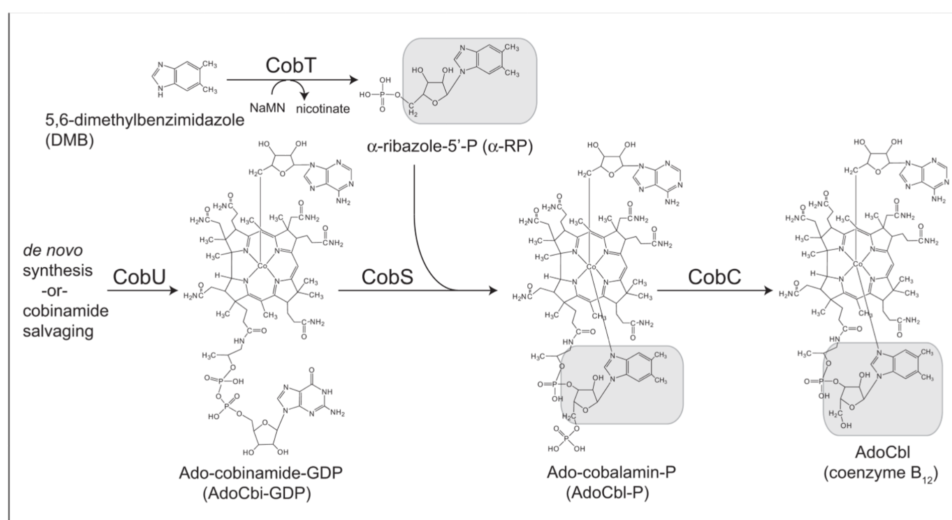

Figure 1. *De novo* assembly of the lower ligand of coenzyme B_{12} in *S. enterica*

The α-R moiety is indicated with grey boxes. Abbreviations: CobT, nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase; CobS, adenosylcobalamin-phosphate synthase; CobC, adenosylcobalamin-phosphate phosphatase; NaMN, nicotinate mononucleotide; Ado, 5′-deoxyadenosine; P, phosphate; GDP, guanosine diphosphate.

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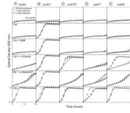

Figure 2. α-R salvaging in *S. enterica* expressing *cblT* and *cblS* alleles from *L. innocua*
Corrinoid-dependent aerobic growth of *S. enterica* JE1244 (*metE205 ara-9 cobT10*::Tn*10*d[tet^{+}]) derivatives in NCE containing glycerol (22 mM), $MgSO_4$ (1 mM), trace minerals, ampicillin (100 μg ml^{-1}), and arabinose (as indicated). Optical density at 650 nm was measured for 24 h at 37°C. Corrinoids were added at 15 nM. DMB, α-R, and α-RP were added at 250 nM. Plasmids used were: vector, pBAD24; p*cobT*$^{+}$, pCOBT48 (*S. enterica cobT*$^{+}$); p*cblTS*$^{+}$, pCBLTS1 (*Lin cblT*$^{+}$ *cblS*$^{+}$); p*cblT*$^{+}$, pCBLT1 (*Lin cblT*$^{+}$); p*cblS*$^{+}$, pCBLS4 (*Lin cblS*$^{+}$). Growth curves were obtained using an ELx808 Ultra Microplate reader (Bio-Tek Instruments). Each growth curve was performed in triplicate. Error bars of one standard deviation are indicated.

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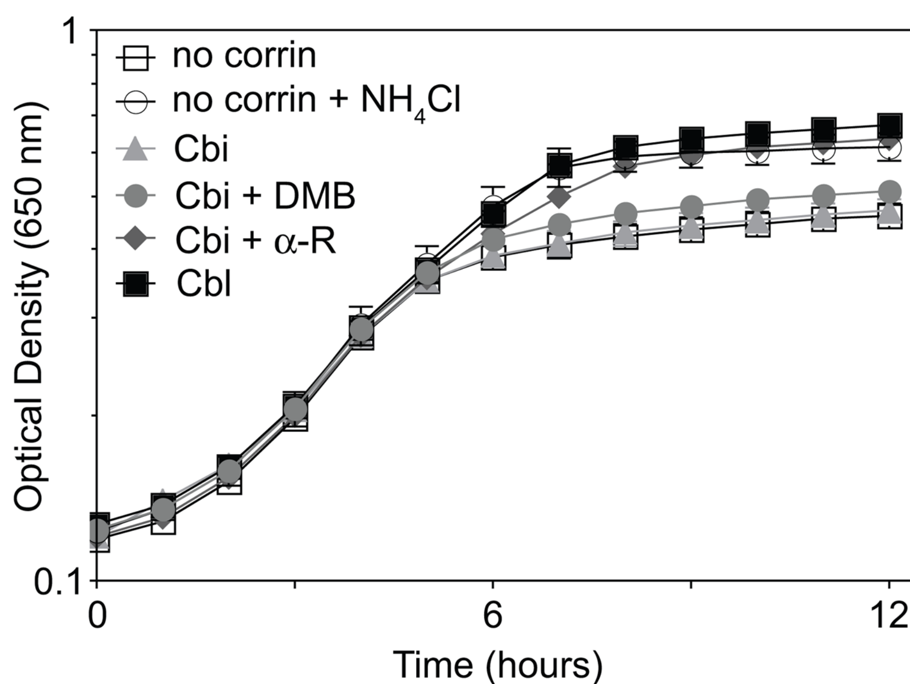

Figure 3. Corrinoid-dependent ethanolamine utilization in *L. innocua*

Aerobic growth of *L. innocua* DD680 in MLM containing ethanolamine (100 mM). Optical density at 650 nm was measured for 12 h at 37°C. Corrinoids were added at 15 nM, α-R was added at 500 nM, and NH_4Cl was added at 1 g l^{-1}. Growth curves were obtained using an ELx808 Ultra Microplate reader (Bio-Tek Instruments). Each growth curve was performed in triplicate. Error bars of one standard deviation are indicated.

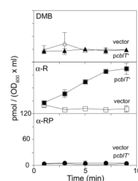

Figure 4. *Lin*CblT is an α-R transporter

Accumulation of radiolabeled compounds by *S. enterica* strains JE8511 (vector; open symbols; *metE205 ara-9 cobT10*::Tn*10*d[*tet*$^+$]/pBAD24 [*bla*$^+$]) and JE12550 (p*cblT*$^+$; filled symbols; *metE205 ara-9 cobT10*::Tn*10*d[*tet*$^+$]/pCBLT1 [*cblT*$^+$ *bla*$^+$]) in NCE containing glycerol (22 mM), $MgSO_4$ (1 mM), trace minerals, ampicillin (100 μg ml^{-1}), and arabinose (250 μM). At time zero, [^{14}C]DMB (top panel), [^{14}C]α-R (middle panel), or [^{14}C]α-RP (bottom panel) was added to exponentially growing cells to a concentration of 250 nM. Each accumulation curve was performed in triplicate. Error bars of one standard deviation are indicated.

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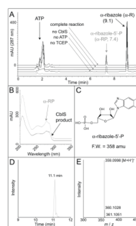

Figure 5. *Lin*CblS is an ATP-dependent α-R kinase

Reactions containing *Lin*CblS (10 μg), Tris-HCl (100mM, pH 7.5), $MgCl_2$ (1 mM), KCl (50 mM), α-R (30 μM), ATP (1 mM), and TCEP (1 mM) were incubated 3 h at 37°C. **A**. Products were separated by RP-HPLC with a 10-min linear gradient from 97% ammonium acetate (20 mM, pH 4.5) + 3% acetonitrile (CH_3CN) to 40% CH_3CN. Control reactions lacking *Lin*CblS, ATP, or TCEP are indicated. The authentic α-RP control is shown in grey. **B.** UV-visible spectra of the *Lin*CblS product (black trace) and authentic α-RP (grey trace). **C.** Structure and formula weight of α-RP. **D**. ESI-TOF LC/MS elution time of the *Lin*CblS product, separated with a 20-min linear gradient from 100% formic acid in H_2O (0.1% v/v), to 15% formic acid (0.1% v/v) in CH_3CN. **E.** Mass spectrum of the *Lin*CblS product, which was identical to that of authentic α-RP (not shown).

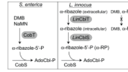

Figure 6. Comparison of lower ligand assembly in *S. enterica* and *L. innocua*

Abbreviations: *Lin*CblT, α-R transporter; *Lin*CblS, α-R kinase; AdoCbl-P, adenosylcobalamin-phosphate. The right panel reflects the findings reported in figure 4, which indicate that, most likely, in *L. innocua* CblT does not transport DMB nor α-ribazole-P (α-RP) into the cell.

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