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The Thiamine Kinase (YcfN) Enzyme Plays a Minor but Significant Role in Cobinamide Salvaging in *Salmonella enterica*[▽]

Michele M. Otte, Jesse D. Woodson,† and Jorge C. Escalante-Semerena*

Department of Bacteriology, University of Wisconsin—Madison, 144A Enzyme Institute, 1710 University Avenue, Madison, Wisconsin 53726-4087

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Cobinamide (Cbi) salvaging is impaired, but not abolished, in a *Salmonella enterica* strain lacking a functional *cobU* gene. CobU is a bifunctional enzyme (NTP:adenosylcobinamide [NTP:AdoCbi] kinase, GTP: adenosylcobinamide-phosphate [GTP:AdoCbi-P] guanylyltransferase) whose AdoCbi kinase activity is necessary for Cbi salvaging in this bacterium. Inactivation of the *ycfN* gene in a Δ*cobU* strain abrogated Cbi salvaging. Introduction of a plasmid carrying the *ycfN*$^+$ allele into a Δ*cobU* Δ*ycfN* strain substantially restored Cbi salvaging. Mass spectrometry data indicate that when YcfN-enriched cell extracts were incubated with AdoCbi and ATP, the product of the reaction was AdoCbi-P. Results from bioassays confirmed that YcfN converted AdoCbi to AdoCbi-P in an ATP-dependent manner. YcfN is a good example of enzymes that are used by the cell in multiple pathways to ensure the salvaging of valuable precursors.

Many bacteria, archaea, and eukaryotes (including humans) have enzymes that require adenosylcobalamin (AdoCbl) (also known as coenzyme B_{12}) as a cofactor, yet synthesis of AdoCbl is limited to archaea and bacteria (29). The gram-negative enterobacterium *Salmonella enterica* serovar Typhimurium LT2 (hereafter referred to as serovar Typhimurium) synthesizes AdoCbl de novo only under anoxic conditions but can salvage exogenous, preformed corrinoids (e.g., cobinamide [Cbi], cobyric acid [Cby]) from the environment under oxic and anoxic conditions (15). When Cbi or Cby enters the cell, it is adenosylated by the housekeeping corrinoid adenosyltransferase CobA enzyme, yielding adenosylcobinamide (AdoCbi) or adenosylcobyric acid (AdoCby), respectively (13, 23). The adenosylated precursor is converted to AdoCbl by the enzymes of the nucleotide loop assembly pathway (12, 17) (Fig. 1).

The CobU enzyme of the nucleotide loop assembly pathway is bifunctional (ATP/GTP:AdoCbi kinase, GTP:AdoCbi-phosphate [GTP:AdoCbi-P] guanylyltransferase) (19) and is important for de novo synthesis and for Cbi salvaging. The AdoCbi kinase activity is necessary only for Cbi salvaging, while the GTP:AdoCbi-P guanylyltransferase activity is required both for de novo synthesis of AdoCbl and for Cbi/Cby salvaging (26).

Archaea lack CobU (26). In lieu of CobU, archaea use CobY (ATP/GTP:AdoCbi-P nucleotidyltransferase) for de novo synthesis of AdoCbl and CbiZ (AdoCbi amidohydrolase) to salvage Cbi (32, 33). CbiZ converts Cbi to Cby (32), which, when condensed with aminopropanol-phosphate, yields AdoCbi-P (8), the substrate for CobY (26, 33) (Fig. 1).

De novo synthesis of AdoCbl can be restored in a Δ*cobU* mutant strain of serovar Typhimurium when a plasmid carrying the archaeal *cobY* gene is introduced into the strain (26).

Here, we report that a Δ*cobU*/p*cobY*$^+$ strain of serovar Typhimurium can salvage Cbi, albeit at a reduced rate, and that the ability of the Δ*cobU* strain to salvage Cbi is due to the existence of an alternate AdoCbi kinase enzyme in this bacterium. Results from in vivo experiments identified the *ycfN* gene as the one encoding the alternate AdoCbi kinase enzyme, and results from in vitro experiments support the idea that YcfN is responsible for the observed in vivo phosphorylation of AdoCbi.

MATERIALS AND METHODS

Microbiological techniques. (i) Bacteria, culture media, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. All serovar Typhimurium strains carry a null allele of the *metE* gene that encodes the cobalamin (Cbl)-independent methionine synthase (MetE) enzyme. *metE* strains require exogenous methionine to grow. Alternatively, a *metE* strain can use the Cbl-dependent methionine synthase (MetH) enzyme to methylate homocysteine when Cbl is available (25). Serovar Typhimurium strains were cultured in nutrient broth (NB; Difco), while *Escherichia coli* strains were cultured in lysogeny broth (3, 4). Plasmids were maintained by the addition of ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), or tetracycline (20 μg/ml) to NB. No-carbon essential (NCE) medium (28) supplemented with glycerol (30 mM) and $MgSO_4$ (1 mM) was used to grow cells under nutrient-defined conditions. Solid media contained 15 g of Bacto agar (Difco) per liter. Corrinoids were added to media at 15 nM. Cyanocobyric acid was a gift from Paul Renz (Institut für Biologische Chemie und Ernahrungswissenschaft, Universität-Hohenheim, Stuttgart, Germany); dicyanocobinamide [$(CN)_2$Cbi] and cyanocobalamin (CNCbl) were purchased from Sigma.

Genetics and recombinant DNA techniques. (i) Transposon mutagenesis. Phage P22 HT105/1 *int-201* (21, 22) was grown on a pool of ~100,000 serovar Typhimurium strains, each carrying one transposition-defective Tn*10d*(*tet*$^+$) element (30) in the chromosome. The resulting phage lysate was used as a donor to transduce strain JE7178 (Δ*cobUST*/p*cobYST*$^+$) to tetracycline resistance (Tc^r) on nutrient agar-tetracycline plates. Tc^r transductants were screened by replica printing for the loss of the ability to salvage $(CN)_2$Cbi.

(ii) Strain construction: construction of a Δ*cobU* Δ*ycfN* strain of serovar Typhimurium. A chromosomal in-frame deletion of the *cobU* gene was constructed in strain TR6583 as described previously (11). For this purpose, we used primers OL25 (5′-ATG ATG ATT CTG GTG ACG GGC GGG GCA CGT AGT GGT AAA AGC CGT CAT GCT GTG TAG GCT GGA GCT GCT TC-3′) and OL26 (5′-TCA TTT AAT TTT GAC TCC AAT ACC TGA GAC

* Corresponding author. Mailing address: Department of Bacteriology, University of Wisconsin—Madison, 144A Enzyme Institute, 1710 University Avenue, Madison, WI 53726-4087. Phone: (608) 262-7379. Fax: (608) 265-7909. E-mail: escalante@bact.wisc.edu.

† Present address: The Salk Institute Plant Biology Laboratory, La Jolla, CA 92037.

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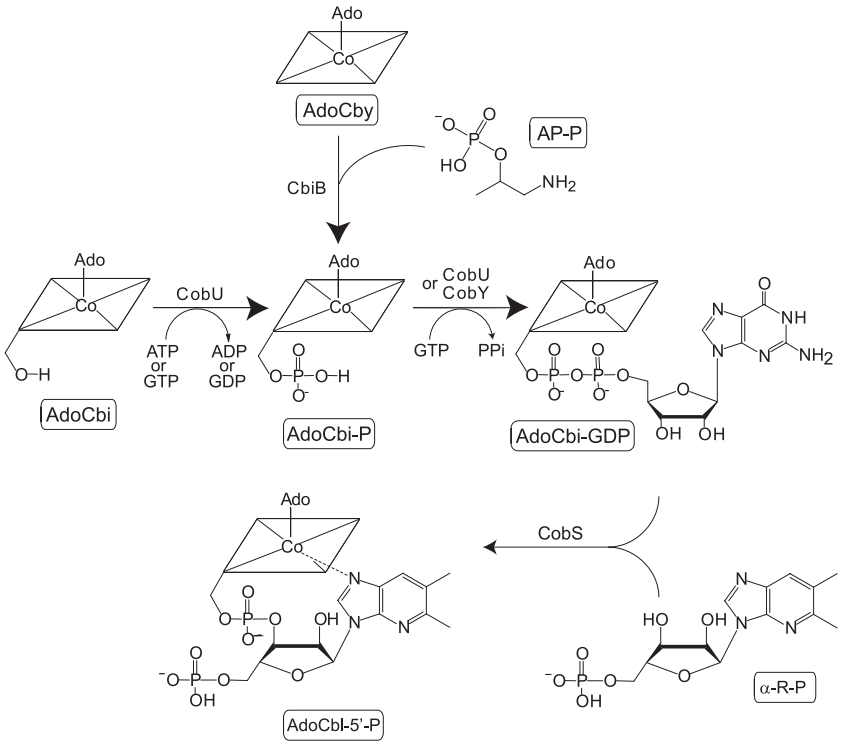

FIG. 1. Nucleotide loop assembly pathway in *Salmonella enterica* serovar Typhimurium LT2. Intermediates are boxed and shown below structures; enzyme names are shown above or left of arrows. Abbreviations: AP-P, *R*-1-amino-2-propanol *O*-2-phosphate; PPi, pyrophosphate; α-R-P, α-ribazole-5′-phosphate; CbiB, putative AdoCbi-P synthase; CobU, ATP/GTP:AdoCbi kinase, GTP:AdoCbi-P guanylyltransferase; CobS, AdoCbl (5′-phosphate) synthase.

TAC CAG CCA GAC CTC ATC CGC CAT ATG AAT ATC CTC CTT AG-3′); the resulting strain was JE8249 (Δ*cobU*). An in-frame deletion of the *ycfN* gene was constructed in strain JE8249 by using primers OL27 (5′-GTG CGG TCC AAC AAC AAT AAT CCC TTA ACG CGC GAC GAG ATC CTG TCG CGC GTG TAG GCT GGA GCT GCT TC-3′) and OL28 (5′-TTA TCC TTT CAT ACG TAG CTG GCG CCA GGT TTC ATC GGC CAG CCT GAT AAA CAT ATG AAT ATC CTC CTT AG-3′); the resulting strain was JE8268 (Δ*cobU* Δ*ycfN*). Both in-frame deletions were verified by DNA sequencing using BigDye protocols (ABI PRISM). Reaction mixtures were resolved by the University of Wisconsin—Madison Biotechnology Center.

(iii) Complementation of AdoCbi kinase activity. Plasmids pCLK740 (*ycfN*$^+$) and pCOBY38 (*cobY*$^+$) were electroporated into strain JE8268 (Δ*cobU* Δ*ycfN*) to assess $(CN)_2$Cbi salvaging; the resulting strain was JE8315. Plasmid pET-16b was used as a negative control. Plasmids pET-16b and pCOBY38 were electroporated into strain JE8268, yielding strain JE8314. Isolated colonies of strains tested were inoculated into NB and incubated at 37°C overnight (~16 h). A 5-μl aliquot of the overnight culture was diluted into 195 μl of NCE medium as described above and incubated at 37°C with shaking in a 96-well microtiter plate for 32 h using an ELx808 Ultra microplate reader (Bio-Tek Instruments).

Biochemical techniques. (i) Protein overproduction and purification. ***(a)*** **His_6-CobA protein.** A 100-ml volume of lysogeny broth supplemented with ampicillin was inoculated with a fresh transformant of *E. coli* strain BL21(DE3) (Novagen) carrying plasmid pCOBA17 (*cobA*$^+$) (9) and incubated at 37°C with shaking at 200 rpm for approximately 18 h. Cells were harvested by centrifugation at 4°C at 15,000 × *g* for 15 min using a JA-25.50 rotor in an Avanti J-25I Beckman/Coulter refrigerated centrifuge and resuspended in 5 ml of lysis buffer (His-Bind binding buffer [Novagen] containing protease inhibitor phenylmethylsulfonyl fluoride [1 mM] and BugBuster [Novagen]). After a 20-min incubation at room temperature with occasional mixing, cell extract was obtained by centrifugation at 4°C at 43,000 × *g* for 30 min as described above.

Particulates were removed from the cell extract by filtration through a 0.2-μm filter, and His_6-tagged CobA protein (His_6-CobA) was purified over a His-Bind Quick 900 cartridge (Novagen) equilibrated with binding buffer according to the manufacturer's protocol. EDTA was added to the eluted protein to a final concentration of 5 mM to remove excess nickel.

Protein concentration was determined using the Bradford method (7), and the protein was concentrated to 1 to 2 mg/ml using a Centriprep centrifugal filter unit with an Ultracel YM-10 membrane (Millipore). Protein was transferred to SnakeSkin dialysis tubing with a 10,000-Da molecular-mass cutoff (Pierce) and dialyzed against a series of five 750-ml buffer changes: (i) Tris hydrochloride buffer (Tris-HCl [50 mM, pH 8.0, at 4°C]) containing EDTA (10 mM) for approximately 12 h, (ii) Tris-HCl buffer (50 mM, pH 8.0, at 4°C) containing EDTA (5 mM) for 2 h, and (iii) Tris-HCl buffer (50 mM, pH 8.0, at 4°C) containing NaCl (100 mM) for 2 h. The third dialysis step was performed thrice. Dithiothreitol (DTT; 1 mM) and glycerol (10% [vol/vol] final concentration) were added to the protein, and the final protein concentration was determined using the Bradford method (7). Two-hundred-microliter samples of dialyzed His_6-CobA protein (>95% homogeneous [data not shown]) were stored at −80°C until use.

(b) **CobU protein.** CobU protein was overproduced and purified as described previously (27), with the following modifications. Cells from a 4-liter culture of the overproducing strain were lysed using a French press operating at 138 × 10^3 kPa. After the addition of ammonium sulfate (9.6% saturation at 4°C; UltraPure; ICN Biomedicals), we used fast protein liquid chromatography (ÁKTA explorer; GE Healthcare) to isolate CobU protein from the clarified cell extract in two additional steps. Step 1, hydrophobic interaction chromatography, was performed as follows. Cell extract was applied to two 5-ml HiTrap Phenyl (high-sub) FastFlow (high substitution) columns (GE Healthcare) connected in tandem and equilibrated with buffer A [Tris-HCl buffer (0.1 M, pH 8.0, at 4°C) containing DTT (10 mM), glycerol (10%, vol/vol), and $(NH_4)_2SO_4$ (9.6%, wt/vol)]. Protein was eluted off the columns at a flow rate of 2 ml per min using a linear gradient to 100% buffer B (Tris-HCl buffer [0.1 M Tris, pH 8.0, at 4°C] containing DTT [10 mM]). CobU was found in the flowthrough fractions. Step 2, dye ligand chromatography, was performed as follows. CobU-containing fractions from the phenyl column purification step were pooled, dialyzed against buffer C (Tris-HCl buffer [0.1 M, pH 8.0, at 4°C] containing DTT [10 mM] and $MgCl_2$ [5 mM]), and applied to a 5-ml HiTrap Blue HP column (GE Healthcare) equilibrated with buffer C. Protein was eluted at a flow rate of 1 ml per min with a linear gradient to 100% buffer D (buffer C containing NaCl [2 M]). CobU was found in the flowthrough fractions.

TABLE 1. Strains and plasmids used in these studies

Strain or plasmid	Relevant genotype and/or description	Reference or source
Strains		
Salmonella enterica[a]		
TR6583 (formerly SA2929)	*metE205 ara-9*	K. Sanderson via J. Roth
Derivatives of TR6583		
JE7127	Δ*cobUST ycfN121*::Tn*10*d(*tet*$^+$)/pCOBY35	
JE7178	Δ*cobUST272*/pCOBY35	Laboratory collection
JE8249	Δ*cobU1315*	
JE8268	Δ*cobU1315* Δ*ycfN112*	
JE8308	Δ*cobU1315*/pSU39	
JE8309	Δ*cobU1315*/pCOBY38	
JE8310	Δ*cobU1315* Δ*ycfN112*/pJO52	
JE8312	Δ*cobU1315* Δ*ycfN112*/pCOBY38	
JE8313	Δ*cobU1315* Δ*ycfN112*/pCLK740	
JE8314	Δ*cobU1315* Δ*ycfN112*/pCOBY38 pET-16b	
JE8315	Δ*cobU1315* Δ*ycfN112*/pCOBY38 pCLK740	
JE8407	Δ*cobU1315* Δ*ycfN112*/pCLK740 pTara	
JE8408	Δ*cobU1315* Δ*ycfN112*/pET-16b pTara	
Escherichia coli		
BL21(DE3)	B F$^-$ *ompT hsdS* (r_B^- m_B^-) *dcm gal* λ(DE3)	Invitrogen
Plasmids		
pBAD33	Cloning vector; *cat*$^+$	14
pCLK740	*E. coli ycfN*$^+$ cloned into plasmid pET-16b	18
pCOBA17	*cobA*$^+$ in pET-15b; encodes His_6-CobA	9
pCOBY35	*S. enterica cobST*$^+$ *Methanosarcina mazei cobY*$^+$ (*cobYST*$^+$) cloned into plasmid pBAD33	Laboratory collection
pCOBY38	*M. mazei cobY*$^+$ cloned into plasmid pSU39	31
pET-16b	N-terminal His_{10} tag vector; *bla*$^+$	Novagen
pGP1-2	T7 RNA polymerase$^+$ *kan*$^+$	24
pJO52	*cobU*$^+$ cloned into plasmid pT7-7	19
pSU39	Cloning vector; *kan*$^+$	2
pT7-7	Cloning vector; *bla*$^+$	24
Ptara	T7 RNA polymerase$^+$ *cat*$^+$	34

[a] All strains are derivatives of *S. enterica* serovar Typhimurium strain LT2. Unless otherwise stated, strains were constructed for this study.

CobU-containing fractions were pooled and concentrated in a Centricon Plus-80 device with an Ultracel PL membrane (Millipore). Concentrated CobU protein was dialyzed against HEPES (10 mM, pH 7.5) containing NaCl (100 mM) and DTT (10 mM). Protein concentration was determined by the Bradford method (7). CobU protein (>95% homogeneous [data not shown]) was flash frozen in liquid N_2 and stored at −80°C until use.

***(c)* His_{10}-YcfN.** The *E. coli* YcfN protein was overproduced fused to an amino-terminal decahistidine (His_{10}) tag. The synthesis of His_{10}-YcfN protein was directed by plasmid pCLK740 (kindly provided by T. Begley) in serovar Typhimurium strain JE8407 [Δ*cobU* Δ*ycfN*/pCLK740 (*ycfN*$^+$)/pTara (T7 *rpo*$^+$)]. One-hundred-milliliter cultures were grown at 37°C to an optical density at 600 nm (OD_{600}) of ~0.5, and IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 0.5 mM to induce *ycfN*$^+$ expression; incubation at 37°C continued for ~16 h after IPTG induction. Cells were pelleted and resuspended in 5 ml of Tris-HCl buffer (0.1 M, pH 8.0, at 37°C) containing phenylmethylsulfonyl fluoride (1 mM). Cells were placed on ice and lysed by sonication for 2 min (5-s pulse followed by 10 s of cooling) in a model 550 sonic dismembrator (Fisher). The His_{10}-YcfN protein was observed only in the insoluble fraction as a band that migrated with an approximate mass of 34 kDa and was estimated by densitometry to constitute 5% of the total protein.

(ii) Assessment of protein purity. Protein concentration was determined by the Bradford method (7). Purity was assessed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16), followed by staining with Coomassie brilliant blue (20). Protein purity was established by band densitometry using a computer-controlled Fotodyne imaging system with Foto/Analyst version 5.00 software (Fotodyne Incorporated) for image acquisition and TotalLab version 2005 software (Nonlinear Dynamics) for analysis.

(iii) Synthesis of corrinoid substrates. ***(a)* AdoCbi.** AdoCbi was prepared enzymatically as reported previously (19), except that 1 mg of purified His_6-CobA enzyme was added to the reaction mixture in lieu of crude cell extract. AdoCbi was purified from the reaction mixture under red light, using a Sep-Pak C_{18} cartridge (Waters) preequilibrated with double-distilled water (ddH_2O). The sample was eluted with 6 ml of 100% methanol and dried under a vacuum in a SpeedVac concentrator (Thermo Savant).

***(b)* AdoCbi-P.** All synthesis and purification steps were performed under red light. AdoCbi was resuspended in 10 ml of Tris-HCl buffer (100 mM, pH 8.5, at 25°C) containing $MgCl_2$ (25 mM). ATP (4 mM) and CobU protein (3 mg) were added to the reaction mixture, which was incubated at 37°C for 2 h. AdoCbi-P was resolved from AdoCbi by high-performance liquid chromatography (HPLC) using a System Gold HPLC system (Beckman/Coulter) equipped with an Alltima HP C_{18} AQ 5-μm column (150 by 4.6 mm) (Alltech). We employed System II for corrinoid separation (described elsewhere) without modifications (5).

(iv) In vitro assay for ATP:AdoCbi kinase activity. The reaction was performed under red light to avoid photolysis of the C—Co bond of the AdoCbi substrate. Reaction mixtures contained Tris-HCl buffer (0.1 M, pH 8.0, at 37°C) containing $MgCl_2$ (5 mM), ATP (2 mM), His_{10}-YcfN-enriched cell extract or cell extract obtained from cells carrying the empty cloning vector (25 μg), and AdoCbi (0.2 mM). The final volume of the reaction mixture was 20 μl. Reaction mixtures were incubated at 37°C for 16 h. Reactions were stopped by the addition of 2.5 μl of KCN (100 mM) to derivatize the adenosylated compounds to their dicyanated forms, followed by heating at 80°C for 10 min. Reaction mixtures were centrifuged for 1 min at 14,000 × *g* in a Beckman Coulter Microfuge 18 centrifuge to remove denatured protein. Formation of $(CN)_2$Cbi-phosphate [$(CN)_2$Cbi-P] was detected using the bioassay described below.

(v) Bioassay for detection of $(CN)_2$Cbi-P. A bioassay was used to detect the presence of $(CN)_2$Cbi-P in reaction mixtures. Strain JE8309 [Δ*cobU* Δ*ycfN*/pCOBY38 (*cobY*$^+$)] was used as an indicator strain. Cells in 1 ml of an overnight NB culture were washed three times with sterile saline and resuspended in 1 ml of sterile saline. A 400-μl aliquot of saline-washed cells (1 ml) was added to 5 ml of molten 0.7% (wt/vol) agar and overlaid on NCE agar plates supplemented

TABLE 2. Doubling times of Δ*cobU* mutants[a]

Strain	Relevant genotype	Doubling time (min)
TR6583	*cobU*$^+$ *ycfN*$^+$	52 ± 2
JE8308	Δ*cobU*/pSU39	NG
JE8310	Δ*cobU* Δ*ycfN*/p*cobU*$^+$	51 ± 1
JE8309	Δ*cobU*/p*cobY*$^+$	54 ± 2

[a] Doubling times of cultures grown in NCE glycerol medium supplemented with 15 nM Cby. Doubling times were calculated graphically from semilog plots of OD_{595} as a function of time and are shown as arithmetic means ± standard errors of the means ($n = 3$). NG, no growth. pSU39 is the empty vector control. pCOBY38 is p*cobY*$^+$ (*cobY*$^+$ cloned into pSU39). pJO52 is p*cobU*$^+$ (*cobU*$^+$ cloned into pT7-7).

with glucose (11 mM). A 5-μl aliquot of the in vitro reaction mixture was spotted onto the overlay; equal volumes of 7.5 μM CNCbl and $(CN)_2$Cbi-P (authentic standards) were spotted as positive controls. Growth was assessed after incubation at 37°C for 16 h.

(vi) MS. The remaining product of the in vitro Cbi kinase reaction mixture (15 μl) was loaded onto a Sep-Pak C_{18} cartridge (Waters) preequilibrated with ddH_2O. Purified corrinoids were eluted off the resin with 2.5 ml of 100% (vol/vol) methanol, and the sample was dried under a vacuum in a SpeedVac concentrator (Thermo Savant). The dried sample was resuspended in 15 μl of ddH_2O; a 5-μl sample was removed and analyzed by mass spectrometry (MS) at the Mass Spectrometry Facility of the University of Wisconsin—Madison Biotechnology Center. The remainder of the mixture was treated with shrimp alkaline phosphatase (SAP; Fermentas) according to the manufacturer's instructions, loaded onto a preequilibrated Sep-Pak C_{18} cartridge (Waters), and eluted and dried as described above. The dried sample was analyzed by MS. Mass spectra were obtained using a Bruker Daltronics (Billerica, MA) BILFLEX III matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS.

RESULTS AND DISCUSSION

Cbi salvaging is abolished in a strain lacking *cobU* and *ycfN* functions. As mentioned above, the archaeal CobY protein has the guanylyltransferase activity associated with CobU but lacks the kinase activity required to salvage Cbi (26). In the absence of CobU, serovar Typhimurium cannot convert the precursor AdoCby or AdoCbi to AdoCbl (Fig. 1). Consistent with previous results, a serovar Typhimurium Δ*cobU* strain carrying plasmid pCOBY38 (*cobY*$^+$) (JE8309) efficiently salvaged Cby (Table 2). Unexpectedly, strain JE8309 also salvaged $(CN)_2$Cbi, albeit less efficiently than strain TR6583 (*cobU*$^+$) (Fig. 2). Because CobY does not have AdoCbi kinase activity and its substrate is AdoCbi-P (26), the ability of the strain to salvage $(CN)_2$Cbi suggested the existence of an alternate AdoCbi kinase in serovar Typhimurium. To identify the alternate AdoCbi kinase, we performed transposon mutagenesis on strain JE7178 (Δ*cobUST*/p*cobYST*$^+$), using the method described above. We isolated one tetracycline-resistant (Tc^r) strain that failed to salvage $(CN)_2$Cbi. The location of the mini-Tn*10* element was determined by sequencing the DNA flanking the insertion as described previously (10) and matching the DNA sequence to a region of the serovar Typhimurium genome (1). This search showed that the transposon in strain JE7127 [Δ*cobUST ycfN121:*Tn*10*d(*tet*$^+$)/p*cobYST*$^+$] was located within the *ycfN* gene.

The chromosomal organization of *S. enterica* suggests that *ycfN* is in an operon. To confirm that the inability of strain JE7127 to salvage $(CN)_2$Cbi was due to the inactivation of *ycfN* and not due to polar effects of the mini-Tn*10* insertion, an in-frame deletion of the *ycfN* gene was generated in strain JE8249 (Δ*cobU*), yielding strain JE8268 (Δ*cobU* Δ*ycfN*). The latter strain failed to salvage $(CN)_2$Cbi (Fig. 2). Introduction of both *cobY* and the *E. coli ycfN*$^+$ gene into strain JE8268 (yielding strain JE8315 [Δ*cobU* Δ*ycfN*/p*cobY*$^+$ p*ycfN*$^+$]) resulted in a substantial restoration of $(CN)_2$Cbi salvaging, whereas introduction of *cobY* and the empty cloning vector (yielding strain JE8314 [Δ*cobU* Δ*ycfN*/p*cobY*$^+$ pET-16b]) did not (Fig. 2). Taken together, these results showed that, in a Δ*cobU* strain, the inactivation or deletion of *ycfN* was sufficient to abrogate the ability of serovar Typhimurium to salvage $(CN)_2$Cbi and suggested that YcfN was the alternate AdoCbi kinase.

In vitro evidence that the YcfN protein has AdoCbi kinase activity. We performed in vitro AdoCbi kinase assays to show that YcfN phosphorylated AdoCbi directly. YcfN protein was overproduced in strain JE8407 (Δ*cobU* Δ*ycfN*) as an N-terminal His_{10} fusion protein (His_{10}-YcfN), which, as reported earlier, was mainly insoluble (data not shown) (18). We used affinity chromatography in an attempt to isolate soluble His_{10}-YcfN protein. Cells from a 1-liter culture of strain JE8407 [Δ*cobU* Δ*ycfN*/pCLK740 (*ycfN*$^+$)/pTara (T7 *rpo*$^+$)] were lysed as described above, and the soluble fraction was passed over a His-Bind Quick 900 cartridge (Novagen) according to the manufacturer's protocol. The eluted fraction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by silver staining. We were unable to detect soluble His_{10}-YcfN protein (data not shown.) The eluted fraction also did not have AdoCbi kinase activity in vitro.

These results suggest that the amino terminus of the fusion protein may not be surface exposed, rendering the protein unable to bind to the affinity column, or that the purification process abrogated the protein's activity. Therefore, for our assays, we used His_{10}-YcfN-enriched, unresolved cell extracts.

Reaction mixtures included ATP, AdoCbi, $MgCl_2$, and ei-

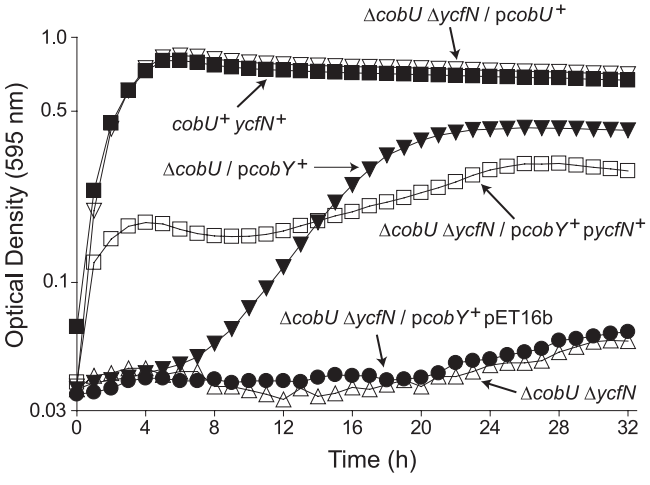

FIG. 2. YcfN allows a Δ*cobU* mutant to salvage Cbi. Results are shown for Cbl-dependent growth of serovar Typhimurium strains in NCE glycerol medium containing $(CN)_2$Cbi (15 nM) at 37°C. Strains used are methionine auxotrophs that must synthesize Cbl to satisfy their requirement for methionine. Growth is reported as OD_{595} as a function of time. Strains are indicated by their relative genotypes. Strains used were TR6583 (*cobU*$^+$ *ycfN*$^+$), JE8268 (Δ*cobU* Δ*ycfN*), JE8309 (Δ*cobU*/p*cobY*$^+$), JE8310 (Δ*cobU* Δ*ycfN*/p*cobU*$^+$), JE8314 [(Δ*cobU* Δ*ycfN*/p*cobY*$^+$ pET16b; empty-vector control)], and JE8315 (Δ*cobU* Δ*ycfN*/p*cobY*$^+$ p*ycfN*$^+$).

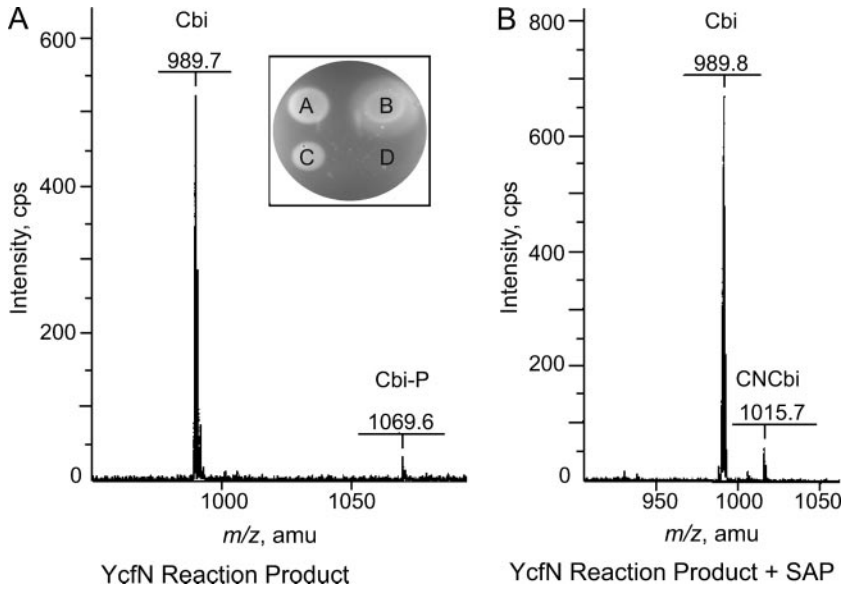

FIG. 3. The product of the YcfN reaction is Cbi-phosphate. Shown are the MALDI-TOF mass spectra and the results of bioassays used to identify the product of the in vitro YcfN reaction. (A) YcfN reaction product. Signals with *m/z* values of 989.7 and 1069.6 were consistent with the molecular masses of Cbi (990.1) and Cbi-P (1069.1), respectively. (Inset) Five microliters of the YcfN reaction product or 5 μl of 75 μM HPLC-purified corrinoids was used in the bioassay. A, HPLC-purified $(CN)_2$Cbi-P; B, authentic (CN)Cbl (vitamin B_{12}); C, YcfN reaction product; D, empty-vector control reaction product. (B) YcfN reaction product treated with SAP. Signals with *m/z* values of 989.8 and 1015.7 were consistent with the molecular masses of Cbi without (990.1) and with (1016.1) an upper cyano ligand, respectively. amu, atomic mass units.

ther His_{10}-YcfN-enriched extract or cell extract obtained from a strain carrying the empty cloning vector (pET-16b) used to overexpress *ycfN*. The reaction was allowed to proceed for 16 h at 37°C. The presence of AdoCbi-P in the reaction products was detected with a bioassay that used strain JE8309 (Δ*cobU* Δ*ycfN*/p*cobY*$^+$) as an indicator. Strain JE8309 is a methionine auxotroph that synthesizes methionine via the Cbl-dependent methionine synthase (MetH) enzyme. Because serovar Typhimurium requires very few (<100) molecules of Cbl per cell to satisfy its requirement for methionine (6), the bioassay is an extremely sensitive measure of Cbl synthesis. Cell growth around the site of application indicated synthesis of Cbl from AdoCbi-P through the pathway described in Fig. 1. Authentic CNCbl (vitamin B_{12}) and HPLC-purified $(CN)_2$Cbi-P were used as positive controls. The product of the His_{10}-YcfN-containing reaction supported growth, while the empty-vector control did not (Fig. 3A, inset).

The YcfN reaction product was cyanated, purified, and submitted to the Mass Spectrometry (MS) Facility at the University of Wisconsin—Madison Biotechnology Center for analysis. The MALDI-TOF mass spectrum of the YcfN reaction product contained a molecular ion signal with an *m/z* of 1069.6 atomic mass units, consistent with Cbi-P lacking an upper ligand (Fig. 3A). Loss of the upper ligand is frequently, but not always, observed during MS analysis. After treatment with SAP and subsequent repurification, the molecular ion signal shifted to an *m/z* of 1015.7 atomic mass units, consistent with Cbi with an upper cyano ligand (Fig. 3B). Together, the bioassay and MS data confirmed that YcfN phosphorylated AdoCbi in vitro.

We used a bioassay to quantify the amount of AdoCbi-P synthesized by YcfN in vitro. For this purpose, we grew strain JE8315 in minimal medium containing various concentrations of $(CN)_2$Cbi-P, and the kinetics of growth was monitored at 595 nm as a function of time (data not shown). The slopes of the exponential phase of the growth curves were plotted against the concentrations of $(CN)_2$Cbi-P in the medium, and a best-fit line was drawn (Fig. 4). Using the equation of this line, we determined the concentration of $(CN)_2$Cbi-P in the reaction mixture that contained His_{10}-YcfN and all the other

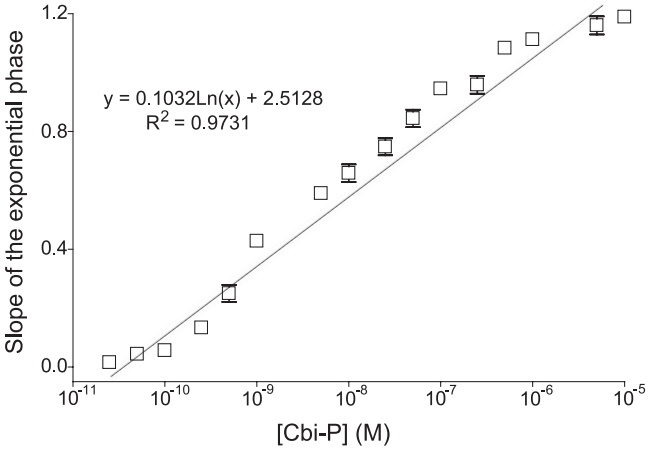

FIG. 4. Growth rate of the Δ*cobU* Δ*ycfN*/p*cobY*$^+$ mutant correlates with Cbi-phosphate concentration in the medium. Growth in NCE glycerol medium supplemented with various concentrations of Cbi-P was monitored at 595 nm. Slopes of the exponential phase growth rates were calculated graphically from semilog plots of OD_{595} as a function of time. Slopes are shown as a function of concentration of Cbi-P in the medium (n = 3). Equation of the best-fit line and the coefficient of determination (R^2 value) were found using Microsoft Excel.

substrates needed to phosphorylate AdoCbi. Using the above-described bioassay, we estimated the concentration of $(CN)_2$Cbi-P in the reaction mixture to be 370 pM ± 28 pM (average for three determinations).

In summary, we have identified the YcfN protein as a nonspecific AdoCbi kinase enzyme. This activity of YcfN is not associated with AdoCbl biosynthesis, but as shown, it allows a CobU-deficient strain of serovar Typhimurium to synthesize enough AdoCbl from Cbi so that the cell can grow. YcfN is required for the phosphorylation of thiamine in the thiamine-salvaging pathway of *E. coli*. Given the obvious structural differences between thiamine and Cbi, we speculate that the corrin ring of Cbi does not interact with YcfN and that most likely the active site is close to the surface of the enzyme.

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