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A new class of EutT ATP:Co(I)rrinoid adenosyltransferases found in *Listeria monocytogenes* and other *Firmicutes* does not require a metal ion for activity

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Abstract

ATP:Co(I)rrinoid adenosyltransferases (ACATs) are involved in *de novo* adenosylcobamide (AdoCba) biosynthesis, and in salvaging complete and incomplete corrinoids from the environment. The ACAT enzyme family is comprised of three classes of structurally and evolutionarily distinct proteins (*i.e.*, CobA, PduO, EutT). The structure of EutT is unknown, and an understanding of its mechanism is incomplete. The *Salmonella enterica* EutT (*SeEutT*) enzyme is the best-characterized member of its class and is known to be a ferroprotein. Here, we report the identification and initial biochemical characterization of an enzyme representative of a new class of EutTs that does not require a metal ion for activity. *In vivo* and *in vitro* evidence shows that the metal-less EutT homologue from *Listeria monocytogenes* (*LmEutT*) has ACAT activity, and that unlike other ACATs, the biologically active form of *LmEutT* is a tetramer. *In vitro* studies revealed that *LmEutT* was more efficient than *SeEutT* and displayed positive cooperativity. *LmEutT* adenosylated cobalamin but not cobinamide, showed specificity for ATP and 2'-deoxyATP, and released a triphosphate by-product. Bioinformatics analyses suggest that metal-less EutT ACATs are also present in other *Firmicutes*.

Keywords

corrinoid adenosyltransferases; corrinoid adenosylation; coenzyme B₁₂ biosynthesis; ATP:Co(I)rrinoid adenosyltransferase; ACAT; ethanolamine catabolism

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SUPPORTING INFORMATION.

Table S1, list of detection limits for elements analyzed by ICP-OES; Table S2, list of strains and plasmids used in this study; Table S3, primers used in this study; Figure S1 (chemical structure of dicyanocobinamide); Figure S2 (*LmEutT* homogeneity assessment); Figure S3 (Multiple sequence alignment of EutT homologues from enterobacteria and *Firmicutes*); Figure S4 (Phylogenetic tree of EutT homologues shown in Fig. S2); Figure S5 (Specificity of *LmEutT* for its nucleotide substrate). Figure S6 (Spectra of corrinoid adenosyltransferase reactions).

INTRODUCTION

Like most vitamins, vitamin B₁₂ (a.k.a. cyanocobalamin, CNB₁₂) needs to be converted to its coenzymic, biologically active form. In coenzyme B₁₂ (a.k.a. adenosylcobalamin, AdoCbl), the upper ligand is a 5'-deoxyadenosine (Ado) group that is attached to the corrin ring via a unique covalent organometallic C-Co bond (Fig. 1A). AdoCbl is used by cells to perform carbon-based radical chemistry, which is initiated by the homolysis of the C-Co bond. Ado• radicals are used to extract non-acidic hydrogen atoms to accelerate the cleavage of C-C, C-N, and C-O bonds to allow group migrations within the molecule [reviewed in ¹]. *Salmonella enterica* is an enterobacterium that synthesizes AdoCbl *de novo* or from precursors found in its environment [reviewed in ²]. Corrinoid adenosylation has been extensively studied in this bacterium.

Enzymes responsible for the attachment of the Ado upper ligand of the ring are known as ATP:Co(I)rrinoid adenosyltransferases (hereafter ACATs; PF01923). With the exception of plants and fungi, ACATs are found in cells from all domains of life [reviewed in ³]. ACATs facilitate the reduction of Co(II)balamin to Co(I)balamin by the generation of a Co(II)balamin four-coordinate intermediate in the enzyme active site ⁴⁻⁸ (Fig. 1B). Three classes of ACATs have been identified, namely CobA, PduO, and EutT. All three classes of ACATs are present in *S. enterica* and have been studied in detail. In *S. enterica*, CobA is the “housekeeping” ACAT, and its activity is required for *de novo* corrin ring biosynthesis and adenosylation of scavenged complete and incomplete corrinoids ⁹. PduO is encoded by the *pduO* gene that is part of a 21-gene operon whose functions are used to catabolize 1,2-propanediol ¹⁰⁻¹². AdoCbl is the coenzyme of the 1,2-propanediol dehydratase that converts 1,2-propanediol to propionaldehyde ¹³. In-depth studies of CobA and PduO have been reported, and structures with substrates bound to them have been elucidated ^{6, 14-16}. EutT class of ACATs are not as well understood. In *S. enterica*, EutT is encoded by the *eutT* gene of the 17-gene ethanolamine utilization (*eut*) operon ¹⁷⁻²⁰ that encodes functions necessary for the AdoCbl-dependent catabolism of ethanolamine [reviewed in ²¹].

To date, a structure of a EutT class ACAT has not been reported, but insights into the mechanism of the *S. enterica* enzyme have been published ^{8, 22, 23}. Unlike CobA and PduO, *S. enterica* EutT (*SeEutT*) requires ferrous ions for optimal activity, but substantial activity is observed when Zn(II) ions substitute for Fe(II) ions. It is known that Fe(II) binds to a cysteine-rich binding motif of *SeEutT* HX₁₁CCXXC ^{22, 24}, and recently published work suggests that the metal promotes the formation of the DMB-off, four-coordinate Co(II)Cbl substrate bound to the active site of *SeEutT* ²³.

Here, we report results of bioinformatics analyses of *SeEutT* homologues that revealed a group of EutT homologues in the *Firmicutes* that lack the metal-binding motif present in *SeEutT*. We also report the initial biochemical characterization of a metal-less EutT homologue from *Listeria monocytogenes* 10403S. Our data indicate that metal-less EutT homologues comprise a new class of EutT enzymes. The accompanying paper by Stracey *et al* reports spectroscopic evidence that the mechanism of catalysis of *LmEutT* proceeds via a four-coordinate Co(II)balamin intermediate. Consistent with our findings, spectroscopic

evidence reported by Stracey *et al* show that the enzyme does not adenosylate Co(II)binamide ²⁵.

MATERIALS AND METHODS

Bacterial strains and growth media

Strains used in this study were all derivatives of *Salmonella enterica* sv Typhimurium LT2 (Table S1). MudJ is an abbreviation of the MudI1734 transposon ²⁶. Mutant strains were constructed using the γ Red recombinase system as described ²⁷. Transductional crosses needed for strain construction were performed using a high-frequency, general transducing mutant of bacteriophage P22 ²⁸.

Difco nutrient broth (NB) (8 g/L) with NaCl (5 g/L) was used as rich medium. All chemicals were purchased from Sigma-Aldrich Chemical Company unless otherwise stated. All chemicals were reagent grade and were used without further purification. Cyanocobalamin (CNCbl) was added as such to culture media. Hydroxycobalamin (HOCbl) was used in *in vitro* activity assays, dicyanocobinamide [(CN)₂Cbi] (Fig. S1) was decyanated as described elsewhere ²⁹, and commercially available adenosylcobalamin (AdoCbl) was used as a standard. Two minimal media were used in this work, one medium was no-carbon E (NCE) ³⁰, which contained a source of nitrogen but not a source of carbon, and a second medium lacked nitrogen and carbon (NCN) ³¹ supplemented with MgSO₄ (1 mM) ³², and Wolfe's trace minerals ³³. When NCN medium was used, glycerol (22 mM) was added as the carbon source and ethanolamine (30 mM) was added as the nitrogen source. When used as carbon and energy source ethanolamine was present at 90 mM; both media were supplemented with CNCbl (200 nM), and IPTG (100 μ M). When lactose was used as carbon and energy, it was present at 29 mM. Ethanolamine (1 mM) and CNCbl (50 nM) were added to allow EutR-dependent activation of the *eut* operon, and IPTG (10 μ M) was added to induce expression of plasmid-encoded *LmEutT* or *SeEutT*. For plasmid maintenance, 100 μ g/mL or 20 μ g/mL ampicillin was added to rich and minimal medium, respectively.

Plasmid construction

All plasmids were constructed using described protocols ^{34,35}. *Listeria monocytogenes* 10403S *eutT*⁺ and *Salmonella enterica eutT*⁺ genes were amplified from genomic DNA and codon-optimized synthesized DNA (GeneScript) and cloned into the NcoI and SalI sites of vector pTAC-85 ³⁵. *L.m. eutT*⁺ was also cloned into the expression vector pTEV18 using primers listed in Table S3 and protocols described elsewhere ³⁴.

Phenotypic analysis

Strains were grown in biological triplicate in nutrient broth (NB, Difco) medium with ampicillin (100 μ g/mL) at 37°C overnight (~16 h) shaking at 155 rpm. Overnight cultures were sub-cultured into a microtiter plate containing 200 μ L of medium per well. When using ethanolamine as a carbon or nitrogen source, cultures were sub-cultured at a 5% inoculum (v/v). Cultures were subcultured at a 2.5% inoculum (v/v) in medium containing lactose. Cultures were grown in a plate reader (Biotek, model ELx808) whose chamber was set at 37°C for 48 h with continuous shaking on medium intensity.

Protein purification

BL21-CodonPlus cells (Agilent) containing plasmid pEUT140 (Table S1) were grown to an $OD_{600} = 0.5\text{--}0.6$ at $37\text{ }^{\circ}\text{C}$ in 4 L flasks containing 2 L of Terrific Broth (TB) medium [tryptone (24 g/L), yeast extract (24 g/L), glycerol (4 mL/L), KH_2PO_4 (17 mM), K_2HPO_4 (72 mM), MgSO_4 (2 mM)]³⁶. Metal springs were placed in the flasks to increase aeration. To maintain the plasmids, ampicillin (100 $\mu\text{g/mL}$) and chloramphenicol (25 $\mu\text{g/mL}$) were added to the medium. Gene expression was induced by the addition of IPTG (1 mM), and protein over production proceeded for 36 h at $15\text{ }^{\circ}\text{C}$ and shaking (150 rpm). Cells were harvested by centrifugation at $6,000 \times g$ for 15 min at $4\text{ }^{\circ}\text{C}$ in a refrigerated Avanti J-20 XPI equipped with a JLA 8.1 rotor. Cell pellets (~ 1 g of wet cell mass) were resuspended in 5 mL of buffer A [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 50 mM, pH 7.5 at $4\text{ }^{\circ}\text{C}$), NaCl (0.5 M), imidazole (20 mM), and *tris*(2-chloroethyl)phosphate (TCEP, 1 mM)]. Cell suspensions were incubated at $4\text{ }^{\circ}\text{C}$ for 10 min with lysozyme (1 mg/mL) and DNase (0.1 mg/mL), after which the cell slurry was passed once through a cell disruptor (Constant Systems) at 1.91 kPa; serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF, 1 mM) was added to the cell lysate immediately after lysis. Lysates were centrifuged at $40,000 \times g$ for 30 min at $4\text{ }^{\circ}\text{C}$ in an Avanti J-25I centrifuged equipped with a JA 25.50 rotor. Supernatants were filtered with a $0.45\text{ }\mu\text{m}$ polyethersulfone (PES) filter (Millipore). Clarified cell lysate was loaded onto a 5 mL HisTrap Fast Flow (FF) column (GE) equilibrated with buffer A. Protein was eluted off the column with an imidazole gradient (20–500 mM). The His₆ tag was cleaved with in-house-purified recombinant tobacco etch virus (rTEV) protease³⁷ for three hours at room temperature, and protein was dialyzed thrice in buffer A at $4\text{ }^{\circ}\text{C}$. Protein was re-loaded onto a 5 mL HisTrap FF column and flow-through was collected. Flow-through material was dialyzed against HEPES buffer (50 mM, pH 7.5 at $4\text{ }^{\circ}\text{C}$) + NaCl (500 mM) + TCEP (1 mM), concentrated using an Amicon Ultra 15 centrifugal filter unit (10 kDa molecular mass cut off), and stored in liquid nitrogen at $-80\text{ }^{\circ}\text{C}$ until used. For preparation of protein for metal analysis, protein was dialyzed in a final HEPES buffer (20 mM, pH 7.5 at $25\text{ }^{\circ}\text{C}$), containing NaCl (100 mM) and TCEP (1 mM).

Purification of *LmEutT* under anoxic conditions—Protocols for protein overexpression and cell harvesting remained unchanged. Cell pellets were moved into an anoxic chamber (Coy) and resuspended in N_2 -sparged buffer A. Lysozyme (1 mg/mL), DNase (0.1 mg/mL) and PMSF (1 mM) were added to the lysate, and the cell slurry was sonicated on ice thrice (2 s on, 2 s off, 1 min total) with five-minute intervals between sonications. Samples were transferred into 50-ml stainless steel centrifuge tubes with an airtight seal and removed from the chamber. Samples were centrifuged at $40,000 \times g$ for 30 min at $4\text{ }^{\circ}\text{C}$, and the centrifuge tube was returned to the anoxic chamber. Cell lysates were applied to a Ni-NTA affinity chromatography resin (1 mL, Qiagen) equilibrated with buffer A. Five-mL fractions of flow-through were collected, followed by 5 mL washes with buffer A, buffer A + 5% buffer B [HEPES (50 mM, pH 7.5 at $25\text{ }^{\circ}\text{C}$) + NaCl (500 mM) + imidazole (500 mM) + TCEP (1 mM)], buffer A + 6% buffer B, and buffer B. The His₆ tag was cleaved with rTEV protease for two hours at room temperature and the protein was dialyzed thrice against buffer A at $25\text{ }^{\circ}\text{C}$. Protein was re-loaded onto the Ni-NTA column and fractions of the flow-through, the buffer A wash, and the buffer B wash were collected.

Fractions from the buffer A wash were collected and dialyzed against HEPES buffer (50 mM, pH 7.5 at 25 °C) containing NaCl (500 mM) and TCEP (1 mM), and finally against HEPES buffer (20 mM, pH 7.5 at 25 °C) containing NaCl (100 mM) and TCEP (0.25 mM). Protein was dispensed into 10-mL glass Balch vials, sealed with a butyl rubber stopper and aluminum seal; samples were stored at –80 °C until used.

Assessment of *LmEutT* activity in the absence and presence of metals

Purified *LmEutT* was thawed on ice and dialyzed in 1 L of buffer (50 mM HEPES, 500 mM NaCl, 1 mM TCEP pH 7.5 at 4 °C) with 1 mM EDTA for 1 h at 4 °C. *LmEutT* was then dialyzed thrice in 1 L of buffer treated with Chelex (4 g L⁻¹) to remove the EDTA but maintain the protein demetallated. *LmEutT* was then assayed for activity in the absence of metal and the presence of Fe(II), Ni(II), Co(II), Cu(II), Ca(II), and Zn(II) (400 nM each) with the microtiter Co(I)balamin assay described below. All buffers and solutions used in the assay were Chelex treated, and all glassware was acid washed overnight with 1 N HCl and rinsed with MilliQ H₂O prior to use.

Assessment of protein homogeneity

To quantify the homogeneity of *LmEutT*, purified *LmEutT* samples were loaded onto a 12% (w/v) SDS-PAGE³⁸ gel containing a molecular mass marker in one lane (Precision Plus, Bio-Rad) and 2 µg of protein in the other lane. The gel was developed by applying 200 V for 50 min. Protein was visualized using a Coomassie Blue R staining solution (25% isopropanol, 10% acetic acid, 0.05% Coomassie Brilliant Blue R)³⁹. The gel was submerged in the staining solution and microwaved for 45 s. After heating, the gel was rinsed and excess dye was removed using acetic acid (10%, v/v). The destaining procedure was repeated twice and the gel was placed in fresh destaining solution and rocked overnight at room temperature. The gel image shown in Figure 8B was captured using a FotoDyne digital imaging system and analyzed by TotalLab 1D gel analysis software. The protein purity was calculated by determining the total pixel intensity of the protein of interest to the total protein pixel intensity. The purity of *LmEutT* protein used in this work was ~90% as shown in Figure S2.

Inductively coupled plasma optical emission spectroscopy (ICP-OES)

For both normoxically and anoxically purified protein, *LmEutT* was dialyzed in buffer with the lowest concentration of buffer and salt possible for the purpose of this experiment. Anoxically purified protein was handled in the anoxic chamber for the following procedure. HEPES buffer (20 mM, pH 7.5 @ 25 °C) containing NaCl (100 mM) and TCEP (0.25 mM). The protein was then loaded onto a microfuge concentrator and diluted with Chelex 100 (BioRad Laboratories)-treated buffer (0.4% w/v Chelex). Protein was centrifuged in the concentrator, and the wash was collected and stored; these steps were repeated twice. Two samples of the wash, two samples of the Chelex-treated buffer, and four samples of the *LmEutT* protein solution were submitted to the Center for Applied Isotope Studies (CAIS) at the University of Georgia for ICP-OES analysis of twenty elements including iron and zinc.

ACAT activity assays

LmEutT activity was assayed using the Co(I) and Co(II) assays described elsewhere^{15, 22, 29, 40} in either a microtiter plate or cuvette under anoxic conditions. For both methods the assay buffer [Tris•HCl (100 mM, pH 7.5 at 37 °C), KCl (500 mM)], MgCl₂ (1 M), and Ti(III)citrate stock (200 mM) were each prepared and N₂-sparged in Balch vials³³. Ti(III)citrate was prepared as described elsewhere²⁴. Other substrates were weighed out and transferred into the anoxic chamber before being suspended in anoxic buffer or anoxic water. Aquacobinamide, (H₂O)₂Cbi, was synthesized using a protocol described elsewhere, and was resuspended in anoxic water²⁹.

Microtiter plate setup for *LmEutT* activity assays—When assays were performed in microtiter dishes, the total volume of the reaction mixture was 300 µL. To set up the reaction, the buffer volume was split into two wells containing a final volume of 150 µL each. The corrinoid (HOCbl or (H₂O)₂Cbi) and reducing system were incubated in one well. In a second well MgCl₂, protein, and ATP were incubated. This allowed for both pre-incubation of the enzyme with the substrate that binds first, and reduction of Co(III) to Co(II) or Co(I). Both wells were equilibrated at 37°C for 20 min before the reaction components were mixed together and absorbance was monitored for 45 min at 525 nm for HOCbl ($\epsilon = 7877 \text{ M}^{-1} \text{ cm}^{-1}$) or 458 nm for (H₂O)₂Cbi ($\epsilon = 9300 \text{ M}^{-1} \text{ cm}^{-1}$). The pathlength of the reactions was also monitored to standardize the values to the equivalent of a cuvette with a pathlength of 1 cm.

Cuvette setup for *LmEutT* activity assays—For these assays we used a 1.0-mL quartz cuvette capped with a rubber butyl stopper. The headspace of the cuvette was flushed with O₂-free nitrogen gas for 5 min prior to the addition of reaction mixture components. After flushing, assay buffer (1 mL), Ti(III)citrate (2.5 mM), HOCbl (50 µM), and protein (varied concentrations) were added to the cuvette using pre-flushed Hamilton syringes. Reaction mixtures were then incubated at 37°C for 30 min. The reaction was started by the addition of ATP (1 mM) and formation of AdoCbl was spectrophotometrically monitored at 525 nm.

Determination of kinetic parameters—Pseudo-first order kinetic data were obtained at a range of Cbl concentrations (1–30 µM) with a saturating concentration of ATP (1 mM). Pseudo-first order kinetic data were obtained at a range of concentrations for ATP (1–30 µM) and dATP (1–30 µM) at a saturating concentration of HOCbl (50 µM). The data displayed positive cooperativity and was fit to a sigmoidal equation to obtain the maximum velocity (V_{max}), the K_{half} ($K_{0.5}$), and the Hill constant (h):

$$Y = \frac{V_{max} \times X^h}{(K_{0.5}^h + X^h)} \quad (1)$$

To obtain the turnover constant (k_{cat}), the following equation was used, where the enzyme concentration is represented by E:

$$k_{cat} = \frac{V_{max}}{E} \quad (2)$$

Catalytic efficiencies were obtained by dividing $k_{cat}/K_{0.5}$.

Bioassays—*SeEutT* and *LmEutT* proteins were incubated in assay buffer (1 mL) containing $MgCl_2$ (1 mM), $Ti(III)citrate$ (2.5 mM), $HOCbl$ (50 μM), ATP (1 mM) and EutT oligomer (50 nM). A reaction mixture lacking enzyme, and a reaction mixture lacking ATP were used as controls. Reaction mixtures were kept in the dark for two hours at 37°C, then filtered with a 0.22- μm filter (Amicon). Some reactions were exposed to light for 15 min after the two-hour incubation. AdoCbl and HOCbl standards (Sigma) were prepared at the same concentration as the HOCbl concentration present in the enzyme reaction mixture (50 μM). The reactions were filtered, and 8 μL of the filtered reactions were added to each well of a 96-well plate containing 200 μL of NCE medium containing Wolfe's trace minerals ³³, $MgSO_4$ (1 mM), ethanolamine hydrochloride (90 mM, pH 7), L-methionine (0.5 mM), and a 2.5% (v/v) inoculum of strain JE23613 (*pduO cobA eutT*) grown overnight in NB + ampicillin (100 $\mu g/mL$) medium. Microtiter plates were incubated shaking at 37°C and growth was monitored spectrophotometrically at 630 nm every 90 min. UV-vis scans (250–700 nm) of the product of the *LmEutT* reaction mixtures were obtained before and after irradiation. Samples were irradiated using a bank of 8×60W incandescent light bulbs placed 30 cm away from the sample. Spectra of the authentic AdoCbl and irradiated AdoCbl were also obtained.

Determination of the phosphate by-product of the *LmEutT* reaction

A 1-mL cuvette reaction mixture containing assay buffer, $MgCl_2$ (2 mM), $Ti(III)citrate$ (1 mM), $HOCbl$ (100 μM), ATP (3 mM), and *LmEutT* (20 $\mu g/mL$) was incubated in the presence of light to continuously regenerate the $Co(II)Cbl$ substrate and maximize ATP usage. After 5 h, ethylenediaminetetraacetic acid (EDTA) was added to each reaction to a final concentration of 20 mM. Deuterated water (D_2O) was added to the reaction to a final concentration of 17% (v/v). ¹H-Decoupled ³¹P-NMR spectra of the samples were obtained using a Bruker AVANCE III 400 MHz spectrometer (Chemical Sciences Magnetic Resonance Facility at the University of Georgia) set at the default parameters of the spectrometer with the following modifications: a spectral width of 40 ppm, centered on -10 ppm. The data was collected in 512 scans per sample. A phosphoric acid standard was also analyzed to normalize the chemical shift between samples.

Gel permeation liquid chromatography

Purified *LmEutT* was dialyzed against 1 L of HEPES buffer (50 mM, pH 7.5 at 4 °C), NaCl (500 mM), and TCEP (1 mM), three times. The first two dialyses lasted 1 h, the third dialysis was performed overnight (~14 h). All *LmEutT* samples were quantified using a Nanodrop 1000 (Thermo Scientific). The column (Superose 12 10/300 GL, GE) was equilibrated with four column volumes of dialysis buffer at a rate of 0.75 mL/min. A sample of *LmEutT* (100 μL) at a concentration of 4.1 mg/mL was loaded onto the column and the

column was developed until the sample was eluted off. After *LmEutT* was eluted, the column was washed with two column volumes before a new sample was loaded. This procedure was repeated with three samples. The mobile phase was applied at a rate of 0.75 mL min⁻¹ and peak fractionation was set to 10 mAU (280 nm) min⁻¹. One major peak eluted, and the eluents from each experiment were collected separately. These eluents were concentrated with a 0.5 mL centrifugal filter (Amicon), quantified using the protein's extinction coefficient (17,400 M⁻¹ cm⁻¹) and absorbance at 280 nm, and diluted to 0.582 mg/mL. These samples were assayed for activity using the Co(I)balamin assay mentioned above with HOCbl (50 µM) and ATP (1 mM).

RESULTS

Bioinformatics and phylogenetic analyses reveal EutT homologues without a metal-binding site

Iterative pBLAST⁴¹ searches identified putative EutT homologues and were aligned using Geneious software⁴² and annotated with ESPript⁴³ (Fig. 2; an expanded alignment can be found in Fig. S3). Assembly of a phylogenetic tree revealed two distinct groups of EutT homologues (Fig. S4). We found that these EutT homologues could be split into two classes, class I and class II. Class I homologues were found in *Enterobacteriaceae*, including *S. enterica*. Notably, all members of class I EutT proteins contained the critical residues of the metal-binding motif HX₁₁CCXXC found in *SeEutT*^{20, 22} (Fig. 2). Class II EutT homologues were found in several *Firmicutes* (e.g., *Clostridia*, *Listeria*, *Brevibacillus* and *Paenibacillus* species) and did not contain any of the metal-binding residues found in class-I enzymes (Fig. 2, S3). Results of *in vivo* and *in vitro* assays showed that class-II EutT homologues from *Clostridium tetani* E88 and *Listeria monocytogenes* subs. 10403S had ATP-Co(I)rrinoid adenosyltransferase activity (data not shown). *L. monocytogenes* EutT (*LmEutT*) was analyzed further.

Purified *LmEutT* does not contain a metal

The metal content of normoxically and anoxically purified *LmEutT* samples were analyzed by inductively coupled plasma-optical emission spectrometry (ICP-OES), probing for the presence of twenty elements including Zn and Fe (Table 1). Unlike *SeEutT*²², neither iron nor zinc were detected in significant quantities in the proteinaceous solution, a result that was consistent with the absence of the metal-binding motif found in class-I EutT homologues. The detection limits for all elements tested are shown in Table S1.

LmEutT does not require a metal for activity

Purified *LmEutT* was dialyzed in buffer containing EDTA to remove any bound divalent metal ions. EDTA was subsequently dialyzed away in buffer treated with Chelex to maintain a metal-free enzyme and buffer. Various metals were incubated in a reaction mixture with *LmEutT* protein anaerobically, and the activity of *LmEutT* was assayed anaerobically using the Co(I)balamin assay²⁰. *LmEutT* activity without treatment (41 nmol AdoCbl/min/mg of protein) was comparable to chelated *LmEutT* without addition of metal (39 nmol AdoCbl/min/mg of protein) and did not show an improvement in activity with the addition of ferrous iron, zinc, cobalt, or nickel, with specific acidity values ranging from 39 – 44

nmol of AdoCbl/min/mg of protein. A one-way unpaired ANOVA analysis determined no significance among these data, with $p = 0.14$. These data are in contrast to *SeEutT*, which is inactive without the addition of Zn(II) or Fe(II) ions ²².

***In vivo* evidence that *LmEutT* has ATP:Co(I)rrinoid adenosyltransferase (ACAT) activity**

The *L. monocytogenes* 10403S *eutT*⁺ allele was cloned into vector pTAC-85, placing the gene under the control of the isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible promoter. Primers used to amplify *eutT* are described in Table S3. The resulting plasmid (pEUT159) was transformed into a *S. enterica* strain lacking all ACATs known to exist in this bacterium, *i.e.*, CobA, PduO, EutT (hereafter referred to as the ACAT strain JE23613, Table S2). Plasmids encoding *SeEutT* or *LmEutT* restored growth with ethanolamine as a carbon and energy source to different degrees, with *LmEutT* being noticeably less efficient than *SeEutT* (Fig 3A, black vs gray triangles). To rule out problems with inefficient mRNA translation due to differences in codon usage between *L. monocytogenes* and *S. enterica*, the *L. monocytogenes eutT*⁺ gene was synthesized (GenScript) for optimal expression in *S. enterica*. Although expression of codon-optimized *L. monocytogenes eutT*⁺ resulted in improved growth, we observed an ~10-h lag before the onset of exponential growth and limited cell yield (Figure 3A, gray diamonds).

To test a condition with a lower cellular requirement for AdoCbl, the same strains were grown in minimal medium containing glycerol as the carbon and energy source and ethanolamine as the nitrogen source. Under these growth conditions all EutT homologues restored growth at similar rates, but exponential growth of the strain that synthesized *LmEutT* was delayed for ~8h, a delay that was not observed with the strain synthesizing *SeEutT* (Fig. 3B, diamonds and inverted triangles vs gray triangles). These data suggested that while *LmEutT* was active *in vivo* and did to a certain extent support ethanolamine ammonia-lyase function, an unidentified problem prevented *LmEutT*-supported wild-type growth with ethanolamine in this heterologous system. Experiments described below attempted to determine whether *LmEutT* synthesized sufficient AdoCbl to grow on lactose as a result of the expression of a *lacZ*⁺ transcriptional fusion under the control of the *eut* promoter, a condition that requires AdoCbl synthesis but not a functional ethanolamine metabolosome.

***In vivo* assessment of *LmEutT* function independent of ethanolamine catabolism**

We used an alternative approach to determine the level of *LmEutT* activity *in vivo* independent of ethanolamine catabolism. For this purpose, the vector encoding *LmEutT* was transformed into a ACAT strain carrying a chromosomal MudJ (*lacZ*⁺) ²⁶ transcriptional fusion in the *eut* operon (strain JE22174) ²⁰. The rationale for this approach was centered on the AdoCbl requirement for the EutR-dependent expression of the *eut* operon ^{19, 20}. We reasoned that if *LmEutT* synthesized AdoCbl *in vivo*, it would bind to the EutR/ethanolamine binary complex triggering the transcription of the *eut* operon and the concomitant synthesis of LacZ (*i.e.*, β -galactosidase). Growth with lactose as the source of carbon and energy would mean that *LmEutT* synthesized sufficiently high levels of AdoCbl to ensure high levels of *eut* operon expression. We observed that the native and codon-optimized *L. monocytogenes eutT*⁺ genes, and the *S. enterica eutT*⁺ gene supported robust

growth with lactose (Fig. 4, gray diamonds and gray triangles). These results suggested that *LmEutT* synthesized sufficient AdoCbl. These results also suggested that the observed ethanolamine phenotypes (Fig. 3) were probably caused by a reason other than insufficient AdoCbl.

***LmEutT* is a *bona fide* ATP:Co(I)rrinoid adenosyltransferase (ACAT)**

LmEutT was purified to ~90% purity in HEPES buffer (50 mM, pH 7.5 at 4 °C) containing NaCl (0.5 M) and TCEP (1 mM) (Fig. S2). The optimal buffer for the ACAT activity assay was determined to be Tris•HCl (0.1 M, pH 7.5) containing KCl (0.5 M). To confirm that the product of the reaction was AdoCbl, *LmEutT* and *SeEutT* were assayed for activity. The reaction products were filter-sterilized and spotted into wells containing a *Δ*ACAT strain whose growth required AdoCbl. Growth was assessed using NCE minimal medium supplemented with 90 mM ethanolamine without corrinoid supplementation. This composition was needed to assess the presence of AdoCbl in reaction mixtures.

Supplementation of the reaction product from both *SeEutT* and *LmEutT* supported growth on ethanolamine (Fig. 5A). A portion of the *LmEutT* reaction was exposed to light for 15 min before it was added to the bioassay. The *Δ*ACAT indicator strain inoculated into medium containing irradiated AdoCbl did not grow, further confirmation that the product formed was AdoCbl (Fig. 5A). This was also confirmed by comparing the spectral signature of the *LmEutT* reaction before and after irradiation to an authentic AdoCbl standard (Fig. 5B). When *LmEutT* was incubated with Cbl without any added ATP, the reaction conferred some growth to the *Δ*ACAT indicator strain compared to the Cbl standard, suggesting that purified *LmEutT* might have had ATP bound to it.

Specificity of *LmEutT* for its NTP and corrinoid substrates

To gain insights into the specificity of *LmEutT* for its NTP substrate, enzyme activity was assayed as a function of the NTP added to the reaction mixture. Unexpectedly, the specific activity of *LmEutT* when ATP and HOCbl were used as substrates (360 nmol AdoCbl min⁻¹ mg⁻¹ of protein) was somewhat lower than the specific activity of the enzyme when 2'-deoxy-ATP (hereafter dATP) substituted for ATP (450 nmole AdoCbl min⁻¹ mg⁻¹ of protein) (Fig. S5). No enzyme activity significant from the baseline was detected when ADP, AMP, TTP, GTP, CTP, UTP, or ITP substituted for ATP (Fig. S5).

The substrate specificity for HOCbl and (H₂O₂) Cbi were also tested for *LmEutT*. The Co(I) assay uses Ti(III)citrate, which reduces the corrinoid directly to the supernucleophilic state required for the adenosylation step. Thus, the Co(I) assay solely determines whether *LmEutT* can bind and position the substrates correctly, but it does not provide information on its ability to facilitate the reduction of the corrinoid from Co(II) → Co(I). In contrast, the Co(II) assay is used to reduce Co(III) to Co(II), and requires that an ACAT facilitate the second reduction step³. Previous work established that CobA interacts with reduced flavodoxin A (FldA) to drive the reduction of Co(II)Cbl to Co(I)Cbl. In contrast to CobA, *SeEutT* can use free dihydroflavins to drive the reduction of Co(II)Cbl to Co(I)Cbl^{22, 44}. *LmEutT* adenosylated both HOCbl and (H₂O₂) Cbi when the substrate was reduced to the Co(I) state using Ti(III)citrate, indicating that *LmEutT* can bind both corrinoids in the

substrate binding pocket (Fig. 6, third set of columns). However, *LmEutT* only adenosylated HOcbl when the substrate was reduced to the Co(II), whether FldA-mediated or by free dihydroflavins (Fig. 6, first two set of columns). The results of these assays were further confirmed by observing the spectral changes of these corrinoids in the absence and presence of each ACAT (Fig. S6).

Triphosphate is the by-product of the *LmEutT* reaction

We used ^{31}P -NMR to analyze the products of the *LmEutT* reaction. Signals observed in the spectrum of the complete reaction mixture indicated that triphosphate was the by-product of the reaction (Fig. 7A). *LmEutT* did not cleave ATP in the absence of cobalamin (Fig. 7B). The signals observed in the above spectra were compared to authentic standards resuspended in the same buffer (Fig. 7C).

Pseudo-first order kinetics analysis of the reaction catalyzed by *LmEutT*

Kinetic parameters for the reaction catalyzed by *LmEutT* were obtained with Cbl when MgATP (1 mM) was saturating. Under these conditions, the enzyme displayed positive cooperativity (Fig. 8A). The analysis was repeated under conditions when cobalamin (50 μM) was saturating and ATP or dATP concentrations were varied. We observed positive cooperativity under all conditions tested, as observed with *SeEutT* previously (Fig. 8B, 8C)²². A summary of the kinetic parameters is reported in Table 2.

LmEutT forms a tetramer

We determined the oligomeric state of active *LmEutT* using gel permeation liquid chromatography. The retention time of *LmEutT* was consistent with a molecular mass of 121 kDa, a mass that closely matched that of tetrameric *LmEutT* (118 kDa) (Fig. 9). We assayed the activity of the *LmEutT* tetramer collected from the column and compared its activity to that of the protein loaded onto the column using the Co(I) assay. The measured specific activities were $309 \pm 23 \text{ nmol min}^{-1} \text{ mg}^{-1}$ and $311 \pm 28 \text{ nmol min}^{-1} \text{ mg}^{-1}$, respectively. These results confirmed that the major peak was *LmEutT*.

DISCUSSION

Listeria monocytogenes and other *Firmicutes* synthesize a new sub-class of metal-less EutT ATP:Co(I)rrinoid adenosyltransferases

The EutT homologue encoded by the *L. monocytogenes* genome shares only 29% end-to-end amino acid identity with *SeEutT* (Fig. S2). In spite of such limited identity, the *LmEutT* protein has *bona fide* ACAT activity (Fig. 5). Importantly, *LmEutT* and EutT homologues from other *Firmicutes* lack the metal-binding motif that is critical for metal binding and function in *SeEutT* (Fig. 2, Fig. S3).

The absence of the metal in *LmEutT* was confirmed by results of ICP-OES analysis of ~90% homogeneous, enzymatically active *LmEutT* enzyme (Fig. S2, Table 1). Consistent with the idea that *LmEutT* does not require a metal ion for activity, EDTA-treated *LmEutT* was as active as untreated enzyme. The lack of metal in *LmEutT* is of interest, because in *SeEutT* Fe(II) somehow disrupts the coordination bond between DMB and the Co(II) ion of the ring.

The resulting DMB-off form of the corrinoid substrate results in a Co(II) four-coordinate species whose redox potential is within physiological range and can be readily reduced to Co(I) cobalamin by dihydroflavins^{22, 23, 45}. At present, it is unclear how *LmEutT* generates the Co(II) four-coordinate species of the cobalamin in the absence of a metal ion.

***LmEutT* is a *bona fide* ATP:Co(I)rrinoid adenosyltransferase**

Three lines of evidence support this conclusion. First, *LmEutT* compensated for the absence of *SeEutT* in a *S. enterica* strain devoid of ACAT activities during growth on NCE plus ethanolamine minimal medium (Fig. 3). Second, *LmEutT* supported robust growth of a *S. enterica* strain lacking all three known ATP:Co(I)rrinoid adenosyltransferases (*i.e.*, CobA, PduO, EutT) with lactose as the sole source of carbon and energy. The β -galactosidase enzyme needed to grow on lactose was encoded by a *lacZ* transcriptional reporter inserted within the *eut* operon of *S. enterica* (Fig. 4). Hence, growth on lactose could only happen if *LmEutT* synthesized sufficient AdoCbl to trigger the expression of the *eut* operon. Furthermore, *in vitro* activity data indicate that *LmEutT*, with a specificity of $5.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for ATP and $5.45 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for HOCbl has as strong, if not stronger, ACAT activity than *SeEutT*; whose values were previously reported as $1.7 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for HOCbl (Table 2)²². Third, results from bioassays further confirmed that the product of the *LmEutT* reaction was AdoCbl (Fig. 5).

In spite of having strong ACAT activity, *LmEutT* does not support robust growth of a *S. enterica eutT* strain with ethanolamine

It is of interest that when ethanolamine was provided as the sole source of carbon and energy, *LmEutT* did not support growth of a Δ ACAT strain as well as *SeEutT* did (Fig. 3A). These results are inconsistent with those from experiments that demanded growth on lactose (Fig. 4). Hence, transcriptional and translational issues do not explain the growth differences between strains expressing *SeEutT* or *LmEutT* with ethanolamine as carbon and energy source. We speculate that the limited growth of strains that synthesized *LmEutT* may be the result of sub-optimal interactions of *LmEutT* with other *S. enterica* Eut proteins, or alternatively, the inability for *LmEutT* to localize to the metabolosome. These possibilities merit further investigation.

***LmEutT* prefers dATP as its nucleotide substrate, and unlike *SeEutT*, it releases triphosphate as its by-product**

Our data indicate that *LmEutT* uses only ATP or dATP *in vitro* (Fig. S5). Comparison of the catalytic efficiencies of *LmEutT* when ATP or dATP were used as co-substrates supports this conclusion (Fig. S5). While this is an interesting result, the physiological importance of this result is unclear, since it is not known whether the lumen of the metabolosome contains any dATP. An additional difference between class I and class II EutT enzymes is the phosphate by-product. While class-I *SeEutT* releases PP_i and P_i ²⁴, class-II *LmEutT* releases PPP_i , which is also the phosphate byproduct of CobA and PduO ACATs^{46, 47}.

Concluding remarks

This study of *LmEutT* sheds light on the enzymatic properties of a previously unknown subclass of EutT homologues belonging to ethanolamine-utilizing *Firmicutes*, and expands the functional diversity of ATP:Co(I)rrinoid adenosyltransferases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AdoCba	adenosylcobamide
HOcbl	hydroxycobalamin
AdoCbl	adenosylcobalamin
(CN)₂Cbi	dicyanocobinamide
ACAT, ATP	Co(I)rrinoid adenosyltransferase
TCEP	<i>tris</i> (2-chloroethyl)phosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
PMSF	phenylmethanesulfonyl fluoride
NTA	nitrilotriacetic acid
PES	polyethersulfone
NCE	No-carbon essential
NCN	no-carbon, no-nitrogen
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
dATP	2'-deoxyATP
ICP-OES	inductively coupled plasma optical emission spectroscopy
EDTA	ethylenediaminetetraacetic acid

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Figure 1. Chemical structures of vitamin B₁₂, coenzyme B₁₂, and the pathway that converts the vitamin to the coenzyme

A. Structures of cyanocobalamin (a.k.a. CNCbl, vitamin B₁₂) and adenosylcobalamin a.k.a. AdoCbl, coenzyme B₁₂). Vitamin B₁₂ is converted to coenzyme B₁₂ in two one-electron reduction steps. The first step is thought to be enzyme independent, and can be driven by dihydroflavins; the second electron can also be donated by dihydroflavins once ATP and the corrinoid substrate are bound to the active site of the ATP:Co(I)rrinoid adenosyltransferase (ACAT). The ACAT catalyzes the nucleophilic attack of Co(I) to the 5' C of ATP. B. General corrinoid adenosylation pathway catalyzed by ACATs. ATP binds before the 5-coordinate (5c) Co(II)balamin substrate. The latter is converted to four-coordinate (4c) Co(II)balamin upon binding by displacement of the lower ligand base. The 4c Co(II)balamin species is reduced by dihydroflavins to generate the Co(I)balamin supernucleophile that attacks ATP, releasing a phosphate byproduct.

		70	80	90	100
Class I	<i>Salmonella enterica</i> serovar Typhimurium LT2	QQPQPVHGLTSS.DTHPQ.ACC	ELCRQPVVVK	KPD	TLTHTLTADKMMVAKS
	<i>Citrobacter koseri</i> ATCC 27028	QQLQPVHGLTSS.DTHPK.ACC	ELCRQPVVAK	KPD	TLTHTLTADKMMVAKS
	<i>Escherichia coli</i> UMN026	QQPQPVHGLTSS.DEHPQ.ACC	ELCRQPVVAK	KPD	TLTHTLSAEKMMVAKS
	<i>Escherichia coli</i> O157:H7	QQPQPVHGLTSS.DEHPQ.ACC	ELCRQPVVAK	KPD	TLTHTLSAEKMMVAKS
	<i>Klebsiella pneumoniae</i> subsp. pneumoniae	QTPQPVHGLTSS.DHPPQ.ACC	ELCHQPVVKK	KPD	TLTHTLTADTLVAKN
	<i>Yersinia intermedia</i> ATCC 29909	PKLQAVHGLTSQDSVSV..ASC	ELCHQTVVMH	KPD	TLTHTLNAQVMMVAKN
Class II	<i>Shewanella benthica</i> KT99	PELEKVHPLKSNVVRPTN..A	MLCHSDVSD	KPALM	THLDDDKMLVPEKT
	<i>Clostridium tetani</i> E88	...KDVKNIESK.EKRFIPRYEH	INGGFFENK	PEHMT	QLNGNKLVTIKD
	<i>Clostridium perfringens</i> ATCC 13124	.PKEIIRREVRE.KEY...KYET	VFGAKLDEK	PEHMT	HLTGNLLVFKD
	<i>Listeria innocua</i> ATCC 33091	...PVKKETTR.A...KFQT	IYGGSVDEK	PEHMT	HLRGNLLVFKD
	<i>Listeria monocytogenes</i> 10403S	...PVKTETTR.A...KFQT	IYGGALDEK	PEHMT	HLRGNLLVFKD
	<i>Brevibacillus laterosporus</i> LMG15441	...APVPTEIS.NKPSI.RYET	IYGGYLESK	PEHMT	HLRGNLLVFKD
	<i>Paenibacillus alvei</i> DSM29	VKVQVTKNLDEVPATEGKKRFR	TLYGGFLET	KPEHMT	HLRGNLLVFKD

Figure 2. A EutT sequence alignment reveals two classes of EutT proteins

A portion of interest from the multiple sequence alignment was selected for this figure, the complete sequence alignment is presented in figure S1. Homologues from strains in bold were tested for preliminary *in vitro* activity. Amino acids that participate in metal-binding are highlighted in yellow. The two classes of EutTs are indicated by the labels 'Class I' and 'Class II'. The numbers above the sequence alignment correspond to the EutT amino acid sequence of *S. enterica*.

Figure 3. *LmEutT* is active *in vivo*

S. enterica strains were grown in minimal medium with ethanolamine as carbon and energy source (panel A) or minimal medium with glycerol as carbon and energy source and ethanolamine as nitrogen source (panel B). Strains were grown in 200 μ L of medium in microtiter dishes and growth was monitored at 630 nm with a plate reader. The experiment was repeated three times, and the error bars represent the standard error between three biological replicates of one experiment.

Figure 4. *LmEutT* synthesized sufficient AdoCbl to activate the transcription of the *eut* operon
 All strains were grown in NCE plus lactose minimal medium. Strains were grown in 200 μ L of medium as described in Figure 3. The experiment was repeated three times, and the error bars represent the standard error between three biological replicates. The Δ ACAT *eutE::MudJ* (*lacZ⁺*) strain did not grow with lactose as the carbon and energy source (closed circles, open circles respectively). Strains carrying a chromosomal *eutE::MudJ* (*lacZ⁺*) fusion and a plasmid encoding an active EutT grew with lactose as carbon and energy source.

Figure 5. *LmEutT* is active *in vitro*

A. The product of reaction mixtures containing *SeEutT* and *LmEutT* proteins incubated with the indicated substrates was fed to a strain that required AdoCbl for growth on ethanolamine (*cobA pdu eut*). Authentic AdoCbl and HOCbl standards were added as controls; + h ν indicates that the reaction was exposed to light. Growth was monitored in a 96-well plate reader. The experiment was repeated twice, and error bars represent the standard error of a biological triplicate. B. UV-vis spectra of the AdoCbl product of the *LmEutT*-catalyzed reaction, before (dark gray line) and after (light gray line) irradiation. Authentic AdoCbl (Sigma) was used for comparison before (dark gray dotted line) and after (light gray dotted line) irradiation (inset graph).

Figure 6. *LmEutT* is specific for cobalamin

LmEutT activity with cobalamin or cobinamide were tested using the Co¹⁺ assay [Ti(III)citrate)], the Fre-dependent Co(II) assay⁴⁰, or the FldA-dependent Co(II) assay. *SeEutT* and *SeCobA* were also assayed as controls. *LmEutT* adenosylated HOCbl under all assay conditions, but only adenosylated Co(I)Cbi.

Figure 7. *LmEutT* releases triphosphate as the by-product

All samples were in Tris•HCl buffer (100 mM Tris, pH 7.5 at 37 °C) containing KCl (500 mM), MgCl₂ (2 mM) and Ti(III)citrate (1 mM). A. In the presence of *LmEutT* (20 μg mL⁻¹), HOCbl (100 μM) and ATP (3 mM) the formation of a triphosphate product was evident, (−6.03, −6.15; −20.57, −20.70, and −20.82 ppm). Integration of these peaks gave values of 1.05 (doublet), 1.08 (triplet), and 1.00 (doublet) for the α, β, and γ phosphate of ATP; and 0.35 (doublet) and 0.14 (triplet) for the α and β phosphate of the PPP_i byproduct, respectively. B. This triphosphate product was not present when HOCbl was omitted from the reaction. Regions of interest are magnified in the NMR spectra inset. Integration of these peaks gave values of 1.01 (doublet), 0.97 (triplet), and 1.00 (doublet) for the α, β, and β phosphate of ATP, respectively. C. ATP and HOCbl concentrations were as in the experiment shown in panel A, but phosphate product standards were each added to a final concentration of 1 mM. Shifts for the phosphate standards were the following: P_i (−2.46 ppm), PP_i (−6.87 ppm), PPP_i (−6.07, −6.19, −20.62, −20.75, −20.87 ppm). Integration of these peaks provided the following: 1.01 (doublet), 0.99 (triplet), and 1.00 (doublet) for the α, β, and γ phosphate of ATP, respectively; 0.21 (doublet) and 0.11 (triplet) for the PPP_i standard; 0.12 (singlet) for the PP_i standard; 0.27 (singlet) for the P_i standard.

Figure 8. *LmEufT* kinetics

The kinetics of AdoCbl formation was analyzed with A. Varying concentrations of Cbl (x-axis) and saturating ATP (1 mM); B. Varying concentrations of ATP (x-axis) and saturating Cbl (50 μ M); C. Varying concentrations of dATP (x-axis) and saturating Cbl (50 μ M).

Figure 9. *LmEutT* forms a tetramer

Purified *LmEutT* was applied onto a size exclusion chromatography column (Superose 12 10/300 GL, GE) and the elution time was compared to markers of known molecular masses (γ -globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa, vitamin B₁₂, 1.35 kDa). A. Data were plotted as the log_{10} of the molecular mass as a function of elution time. The white squares denote the standards, and the gray circle represents the mean elution time of *LmEutT* in three different experiments. B. A sample trace of *LmEutT* eluting from the size exclusion chromatography column is shown. The large peak corresponds to an *LmEutT* tetramer. The small peak appears to be a protein aggregate >1000 kDa. The identity of the aggregated protein was not established.

Table 1
ICP-OES analysis of *LmEutT* purified under normoxic and anoxic conditions

*BD: below detection, numbers in parenthesis in these columns reflect the detection limits reported by the Center for Applied Isotope Studies (CAIS) at the University of Georgia (UGA). Values reflect the average concentrations of metal found in the protein sample and the standard error between samples.

	[Protein]	[Iron]	[Zinc]
<i>LmEutT</i> normoxic purification	15 ± 0.4 µM	*BD (<0.4 µM)	*BD (<1.5 µM)
<i>LmEutT</i> anoxic purification	9 ± 0.6 µM	0.9 ± 0.4 µM	*BD (<1.5 µM)

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Table 2

Kinetic parameters of *Lm*EntT

The kinetic parameters were obtained from data in Figure 8. Units for each parameter are in parentheses where applicable. The k_{cat} was calculated assuming four active sites per *Lm*EntT tetramer.

	$K_{0.5}$ (μ M)	h	V_{max} (nM s ⁻¹)	k_{cat} (s ⁻¹)	$k_{cat}/K_{0.5}$ (M ⁻¹ s ⁻¹)
Cbl	4.2 $\pm$ 0.2	1.9 $\pm$ 0.2	41.8 $\pm$ 1.3	0.21 $\pm$ 0.01	5.00 $\times$ 10 ³
ATP	4.4 $\pm$ 0.3	2.3 $\pm$ 0.3	47.7 $\pm$ 1.7	0.24 $\pm$ 0.01	5.45 $\times$ 10 ³
dATP	3.8 $\pm$ 0.2	1.8 $\pm$ 0.2	55.7 $\pm$ 1.4	0.28 $\pm$ 0.01	7.37 $\times$ 10 ³