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NOTES

The *eutD* Gene of *Salmonella enterica* Encodes a Protein with Phosphotransacetylase Enzyme Activity

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The EutD protein of *Salmonella enterica* is homologous to the catalytic domain of the phosphotransacetylase (Pta) enzyme. The Pta-like activity level of the EutD enzyme compared favorably to that of other Pta enzymes. High-pressure liquid chromatography and mass spectrometry verified that acetyl-coenzyme A was the product of the reaction. The EutD protein restored growth of an *S. enterica pta* strain on acetate as the source of carbon and energy.

Ethanolamine is used by *Salmonella enterica* serovar Typhimurium LT2 (hereafter referred to as *S. enterica*) as a source of carbon, nitrogen, and energy (8, 21, 22). In principle, the biochemistry of the conversion of ethanolamine to the central metabolite acetyl-coenzyme A (Ac-CoA) is relatively simple (Fig. 1A). The first step in the catabolism of ethanolamine is catalyzed by the well-characterized ethanolamine ammonia-lyase, a coenzyme B_{12}-dependent enzyme (2, 3, 5, 9, 10, 24). The products of the ethanolamine ammonia-lyase-catalyzed reaction are ammonia and acetaldehyde, whose oxidation to acetate may be coupled to the synthesis of Ac-CoA (1). There are two possible fates for Ac-CoA in this bacterium. When the cell is not starved for energy, Ac-CoA enters the glyoxylate bypass of the tricarboxylic acid cycle to fuel the energy generation system and serves as a building block for many intermediary and secondary metabolism pathways (16). Under conditions of energy limitation, Ac-CoA is converted to acetyl-phosphate (Ac-P) and ultimately to acetate by the action of the phosphotransacetylase (Pta [EC 2.3.1.8]) and acetate kinase (Ack [EC 2.7.2.1]) enzymes. Under these conditions, acetate is excreted and later recaptured (17). In this pathway, the conversion of Ac-P to acetate is coupled to substrate-level phosphorylation of ADP to yield ATP. The EutE protein has been proposed to be the enzyme responsible for converting acetaldehyde to Ac-CoA; however, the EutE protein has not been isolated or studied in detail. Ethanolamine catabolic functions in *S. enterica* are encoded by the 17-gene *eut* operon (15, 25). Many of the annotated functions of the *eut* genes are based exclusively on their homology to proteins of known function. For example, the predicted primary amino acid sequence of the EutD protein is 37% identical and 56% similar to that of the catalytic domain of the Pta enzyme of *S. enterica* and many other prokaryotes (11). However, no experimental support for this annotation has been reported. In this paper, we provide in vivo and in vitro evidence that shows that the EutD protein is a bona fide Pta enzyme.

The EutD protein compensates for the lack of Pta activity during growth on acetate. To investigate whether the EutD protein had Pta activity, the *eutD* gene was cloned under the control of an arabinose-inducible promoter and was introduced into a *pta acs* strain unable to grow on acetate. The *acs* gene encodes a high-affinity Ac-CoA synthetase enzyme (EC 6.2.1.1) that activates acetate to Ac-CoA when acetate is present at a low concentration in the environment (17, 18). Because the Ack/Pta and Acs systems are the only ones *S. enterica* has for the conversion of acetate to Ac-CoA, inactivation of both of these systems renders a strain unable to use acetate as a carbon and energy source. Arabinose-induced expression of *eutD* in the *pta acs*/pEUTD2 P_{araBAD}-*eutD*$^+$ strain (JE7245) restored growth on acetate to a rate comparable to that measured for the wild-type strain (Fig. 2) and to the activity associated with the Pta enzyme of the methanogenic archaeon *Methanosarcina thermophila* (Fig. 2).

EutD converts Ac-P to Ac-CoA in the presence of free CoA. Incubation of the H_6-EutD enzyme with Ac-P and CoA yielded Ac-CoA. Reverse-phase high-pressure liquid chromatography was used to separate components of the reaction mixture on the basis of their hydrophobicity characteristics. The chromatogram of the reaction mixture showed a compound eluting 104 min after injection (Fig. 3A). This compound was identified as Ac-CoA on the basis of its retention time and its mass spectrum (Fig. 3B). The mass spectrum of the unknown compound was identical to that obtained with authentic, commercially available Ac-CoA. For the sake of simplicity, only the signals diagnostic of the molecular ion are labeled in Fig. 3B. The latter were observed with mass/charge ratios (*m*/*z*) of 808.6 (M − 1H), 810.6 (M + 1H), and 846.4 (M − 2H + K), where M is the mass of the molecular ion, H is a proton, and K is a potassium ion. These data confirmed that EutD had Pta activity.

Isolation and initial characterization of the EutD Pta activity. Basic kinetic parameters of the Pta activity were obtained with homogeneous, N-terminally tagged H_6-EutD protein pu-

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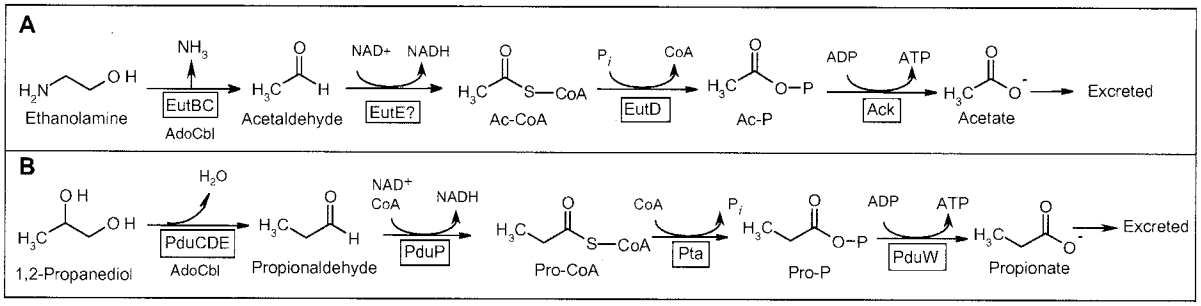

FIG. 1. Biochemical steps for the conversion of ethanolamine and 1,2-propanediol to the corresponding acyl-CoA derivative. (A) Ethanolamine is converted to Ac-CoA in two steps. EutD and Ack then convert Ac-CoA to acetate, which is excreted. (B) Similarly, 1,2-propanediol is converted from Pro-CoA to propionate by Pta and Ack. Propionate is eventually excreted.

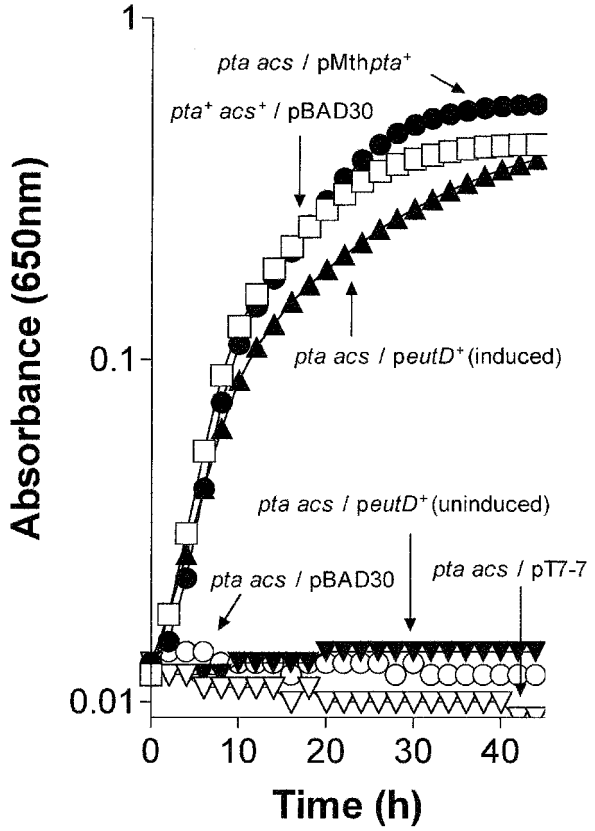

FIG. 2. EutD can restore growth of a *pta acs* strain on 50 mM acetate. Growth kinetics was analyzed using a 96-well microtiter plate (Becton Dickinson) and a computer-controlled Ultra microplate reader (Bio-Tek Instruments) equipped with KC4 software. The temperature of the incubation chamber was set at 37°C. Each well of the plate contained 198 μl of fresh medium, which was inoculated with 2 μl of an overnight culture of *S. enterica* grown on nutrient broth medium. Growth was monitored according to increases in the absorbance at 650 nm. Data were collected every 15 min; cultures were shaken for 800 s between readings. Plasmids carrying wild-type alleles of the *M. thermophila pta* (pML702 *pta*$^+$) or *S. enterica eutD* (plasmid pEUTD2 P_{araBAD}-*eutD*$^+$) genes were introduced into the strain. Expression of the *eutD* gene was induced by the presence of arabinose in the medium (250 μM). Strains were grown on a minimal medium (4) supplemented with $MgSO_4$ (1 mM), L-methionine (0.5 mM), and acetate (50 mM). pBAD30, cloning vector; p*eutD*$^+$, pEUTD2.

rified using Ni-affinity chromatography. H_6-EutD protein was overproduced in *Escherichia coli* strain BL21(λDE3) harboring plasmid pEUTD8 (pET15b *eutD*$^+$) after induction with isopropyl-β-D-thiogalactopyranoside (250 μM). Cells were broken open using a French press (Spectronic Unicam) at 1.034×10^4 kPa. Cell debris was pelleted by centrifugation at $39{,}191 \times g$ in an Avanti J-25I centrifuge (Beckman-Coulter) for 40 min at 4°C with a J25.50 rotor, and the clarified cell extract was loaded onto a 1-ml His-Bind nitrilotriacetic acid affinity chromatography column (Novagen) which was developed per the manufacturer's instructions. Fractions containing H_6-EutD protein were dialyzed overnight at 4°C with 1 liter of 50 mM Tris-Cl buffer (pH 7.2) containing 2 mM dithiothreitol, 10 mM EDTA, and 20 mM KCl. EutD protein was dialyzed using the buffer described above without EDTA and with 10% (vol/vol) glycerol as cryoprotectant. H_6-EutD protein (>95% homogeneous) was stored at −80°C until used. Assay conditions and detection of product formation were as described previously (20) except that the final volume of the reaction mixture was 1 ml, the reaction was started by the addition of substrate (i.e., potassium lithium Ac-P [1 μmol]), and the reaction was performed at 30°C. The purity and the concentration of the H_6-EutD protein were established as previously described (7, 19, 23) (data not shown). Pseudo-first-order kinetics yielded apparent K_m values of 46 μM for free CoA and 129 μM for Ac-P. The k_{cat} value calculated per micromole of EutD monomer was 1,927 s^{-1}, and the catalytic efficiency (k_{cat}/K_m) values for free CoA and Ac-P were 4.2×10^4 and 1.5×10^4, respectively. A pH activity profile for the H_6-EutD enzyme showed maximal activity at pH 7.2, with an approximately 90% loss of activity measured at pH 6.5 and 25% of the activity lost at pH 7.8 (data not shown). A thermal stability analysis showed that >50% of the enzyme's activity was lost after a 5-min incubation at 25°C, with >90% of the activity lost at >45°C (data not shown).

EutD Pta activity is a key difference between ethanolamine and 1,2-propanediol catabolism in *S. enterica*. Despite the fact that ethanolamine and 1,2-propanediol catabolisms in *S. enterica* occur via very similar biochemical reactions (Fig. 1) (6, 15), no evidence has been reported for the existence of a structural or functional homolog of Pta encoded by the propanediol utilization (*pdu*) operon. The catabolism of both compounds requires the assembly of a carboxysome-like structure, with increasing evidence supporting the hypothesis that both compounds are catabolized inside the carboxysome-like

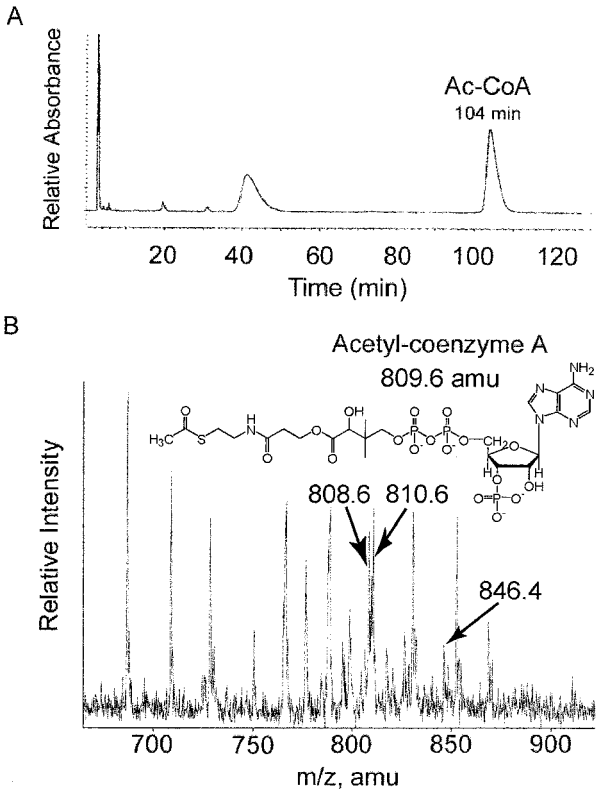

FIG. 3. Ac-CoA is the product of the EutD-catalyzed reaction. (A) Elution profile of components of a EutD-catalyzed reaction. Components of the reaction mixture were separated (as described previously) (14) using a Prodigy 5μ ODS-2 column (Phenomenex) (250 by 4.60 mm; 5 μm), a computer-controlled high-pressure liquid chromatography system equipped with a model 600 quaternary solvent delivery system (Waters, Milford, Mass.), and a model 996 Plus photodiode array detector. Elution of materials from the column was monitored at 260 nm. The column was first developed isocratically with acetonitrile and water (1.75/98.25) containing 0.2 M ammonium acetate for 20 min at a rate of 1 ml/min and then switched to a 100-min convex gradient (Waters curve 3) with acetonitrile and water (10/90) containing 0.2 M ammonium acetate. The column was maintained at 35°C, and the flow rate was kept constant at 1 ml/min. A reaction mixture containing authentic Ac-CoA (Sigma) but lacking EutD was processed in parallel and used to determine the retention time for Ac-CoA under the conditions used. (B) Mass spectrometry. Fractions containing the EutD reaction product were concentrated using a vacuum and a SpeedVac concentrator (Thermo Savant), resuspended in 50% acetonitrile, and analyzed by ion electrospray mass spectrometry (negative mode) at the University of Wisconsin—Madison Biotechnology Center. The labeled peaks identified prominent ions of the molecular ion. The fragmentation pattern of the sample was identical to that of authentic Ac-CoA purchased from Sigma (data not shown). amu, atomic mass units.

structure (12, 13, 15, 25). Why does ethanolamine catabolism require EutD in addition to the housekeeping Pta enzyme? Why is an additional Pta activity not required for 1,2-propanediol catabolism? Answers to these questions will shed light on the physiological restrictions confronted by *S. enterica* during the catabolism of these compounds.

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