The Journal of Microbiology (2010) Vol. 48, No. 5, pp. 629-636
DOI 10.1007/s12275-010-0091-0

Characterization of *Escherichia coli* EutD: a Phosphotransacetylase of the Ethanolamine Operon

Federico P. Bologna, Valeria A. Campos-Bermudez, Damián D. Saavedra, Carlos S. Andreo, and María F. Drincovich*

Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI); Universidad Nacional de Rosario; Suipacha 531, Rosario, Argentina

(Received March 8, 2010 / Accepted May 11, 2010)

The *Escherichia coli* genes *pta* and *eutD* encode proteins containing the phosphate-acetyltransferase domain. EutD is composed only by this domain and belongs to the ethanolamine operon. This enzyme has not been characterized yet, and its relationship to the multimodular *E. coli* phosphotransacetylase (Pta) remains unclear. In the present work, a detailed characterization of EutD from *E. coli* (EcEutD) was performed. The enzyme is a more efficient phosphotransacetylase than *E. coli* Pta (EcPta) in catalyzing its reaction in either direction and assembles as a dimer, being differentially modulated by EcPta effectors. When comparing EutD and Pta, both from *E. coli*, certain divergent regions of the primary structure responsible for their unique properties can be found. The growth on acetate of the *E. coli pta acs* double-mutant strain, was complemented by either introducing EcEutD or by inducing the *eut* operon with ethanolamine. In this case, the expression of a phosphotransacetylase different from Pta was confirmed by activity assays. Overall, the results indicate that EcEutD and Pta, although able to catalyse the same reaction, display differential efficiency and regulation, and also differ in the induction of their expression. However, under certain growth conditions, they can fulfil equal roles in *E. coli* metabolism.

***Keywords*:** *E. coli*, phosphotransacetylase, ethanolamine

Three *Escherichia coli* genes (*maeB*, *pta*, and *eutD*) encode proteins with the PTA_PTB domain found in phosphate acetyltransferases and butaryltransferases. MaeB is a NADP-dependent malic enzyme, which contains the PTA_PTB domain at the C-terminal end (Bologna *et al*., 2007). However, this domain, while not catalytically active in MaeB, is involved in metabolic allosteric regulation (Bologna *et al*., 2007). EcPta also contains the PTA_PTB domain at the carboxyl-terminus. In this case, this domain is catalytically active and involved in acetate generation and utilization (Campos-Bermudez *et al*., 2010). Finally, EcEutD belongs to the ethanolamine utilization operon (Kofoid *et al*., 1999; Fig. 1A) and it is composed only by the PTA_PTB domain (Fig. 2A). The *eutD* gene from *Salmonella enterica* encodes an enzyme with phosphotransacetylase activity (Brinsmade and Escalante-Semerena, 2004), although the product of the *E. coli eutD* gene has not been characterized yet.

Some *E. coli* strains can use ethanolamine as a source of carbon and nitrogen (Scarlet and Turner, 1976; Blackwell *et al*., 1977; Blackwell and Turner, 1978). The proteins for ethanolmine utilization are codified by a 17-gene ethanolamine utilization operon (*eut*) (Fig. 1A; Kofoid *et al*., 1999), which also encodes proteins involved in the structural assembly of the protein microcompartment (called metabolosome) where ethanolamine catabolism takes place (Sagermann *et al*., 2009). This structure optimizes the activity of ethanolamine catabolic enzymes, restricts acetaldehyde diffusion avoiding its toxic effects, and maintains the pool of free CoA (Kofoid *et al*., 1999; Brinsmade *et al*., 2005; Starai *et al*., 2005; Sagermann *et al*., 2009). Regarding the biochemistry of ethanolamine catabolism, this compound is converted into acetaldehyde by ethanolamine ammonia lyase (EutBC, Mori *et al*., 2004), which is then activated to acetyl-CoA by the acetaldehyde dehydrogenase (EutE, Fig. 1B). In *Salmonella enterica*, the acetyl-CoA derived from ethanolamine metabolism is converted into acetyl-P by the EutD phosphotransacetylase enzyme, with no apparent involvement of the housekeeping phosphotransacetylase (Pta) (Brinsmade and Escalante-Semerena, 2004; Brinsmade *et al*., 2005; Starai *et al*., 2005). Acetyl-P is then used to store energy via substrate-level phosphorylation, yielding acetate, which is released and later recaptured (Brinsmade *et al*., 2005; Starai *et al*., 2005; Fig. 1B). In *Salmonella* and *E. coli*, the ethanolamine degradative pathway is induced by ethanolamine plus vitamin B12, which is necessary for EutBC activity (Scarlet and Turner, 1976; Blackwell *et al*., 1977; Blackwell and Turner, 1978; Sheppard and Roth, 1994). It is noteworthy that some *E. coli* strains, as MG1655, have an insertion element that disrupts certain genes in the *eut* operon (Blattner *et al.*, 1997; Fig. 1A). However, this element, presumably present in ancestral *E. coli* K-12 strains, has been lost in certain strains such as W3110 (Hayashi *et al.*, 2006). Thus, it is possible that the ability of certain, but not all, *E. coli* strains to grow on ethanolamine as carbon source (Scarlet and Turner, 1976) depends on the absence of this insertion element.

The results obtained with *S. enterica* EutD and Pta, indicate that although these enzymes catalyze the same reaction, they

* For correspondence. E-mail: drincovich@cefobi-conicet.gov.ar; Tel: +54-341-437-1955; Fax: +54-341-437-0044

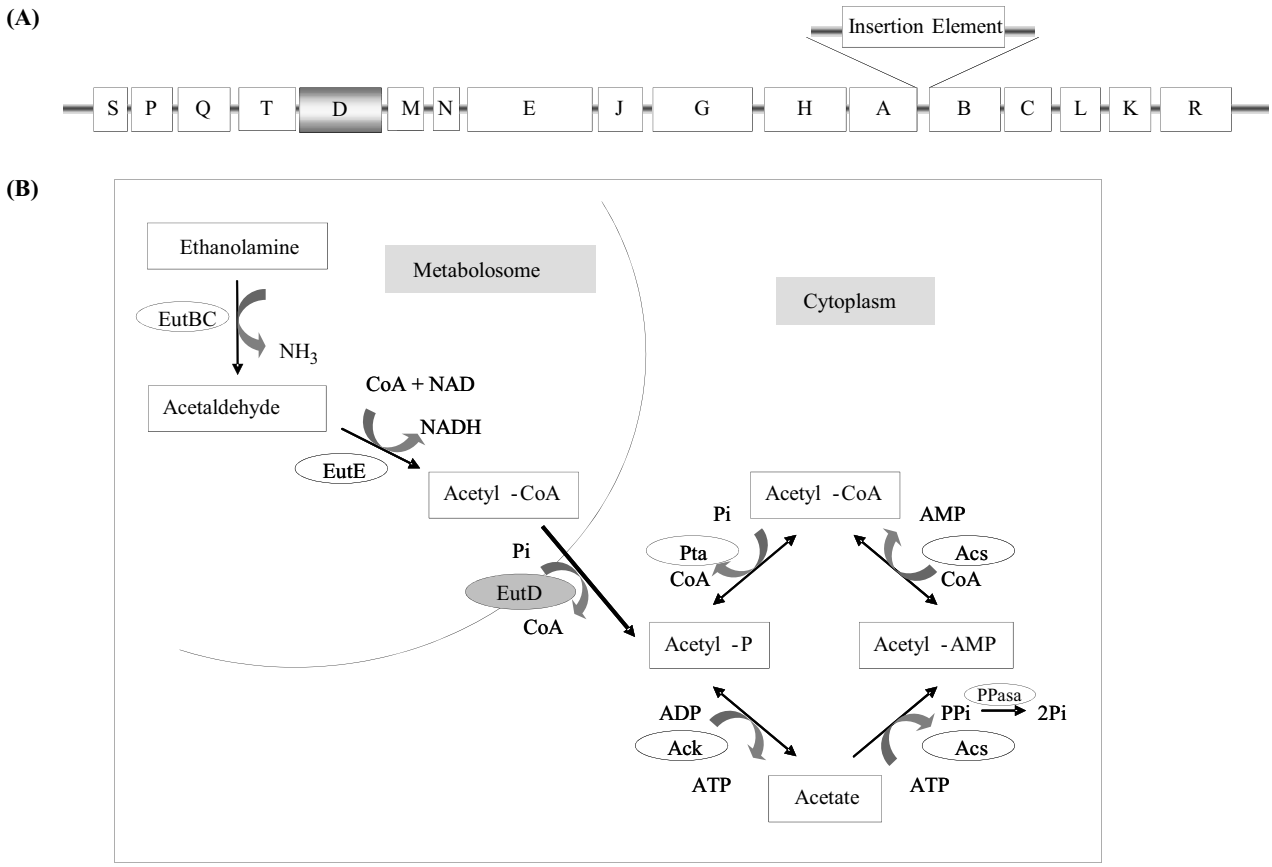


Fig. 1. (A) Gene organization of the *E. coli eut* operon. Genes of the *eut* operon are directly or indirectly involved in ethanolamine catabolism: e*utB* and *eutC* encode the large and small subunits of ethanolamine ammonia lyase (EutBC), respectively, and *eutE* codes for an acetaldehyde dehydrogenase (EutE). Genes *eutK*, *eutL*, *eutM*, *eutN*, and *eutS* are thought to be involved in the assembly of the metabolosome. An insertion element that disrupts genes of the *eut* operon in certain *E. coli* strains is also shown (Blattner *et al.*, 1997; Hayashi *et al.*, 2006). The product of the *eutD* gene (in grey) is characterized in the present work. (B) Enzymes involved in ethanolamine and acetate utilization in *E. coli*. EutBC and EutE enzymes participate in ethanolamine degradation and are located in the metabolosome. Two cytosolic pathways of acetate activation and production can be found in *E. coli***:** Acs catalyzes an irreversible pathway for high-affinity acetate activation, while AckA and Pta catalyze a reversible pathway involved in acetate production or assimilation at high acetate concentration. EutD (in grey) could be located in the metabolosome or in the cytosol.

do not fulfill equal roles in *S. enterica* metabolism (Brisnmade *et al*., 2005; Starai *et al*., 2005). It has been postulated that the selective pressure for the evolution of *S. enterica* EutD was the need to respond to low levels of intermediates, as *S. enterica* EutD has much higher catalytic efficiency than the house-keeping *S. enterica* Pta (Brinsmade *et al*., 2005). In the present paper, we report the biochemical characterization of the product of *E. coli eutD*, and compare its properties to EcPta (Campos-Bermudez *et al*., 2010). Moreover, growth complementation analysis indicate that in *E. coli*, EutD was able to complement a *pta acs* mutant strain growing on acetate. Studies on the kinetic and structural properties of EutD, as well as growth and complementation analysis of mutant strains, are expected to help in the elucidation of the role of EutD in *E. coli* metabolism.

Materials and Methods

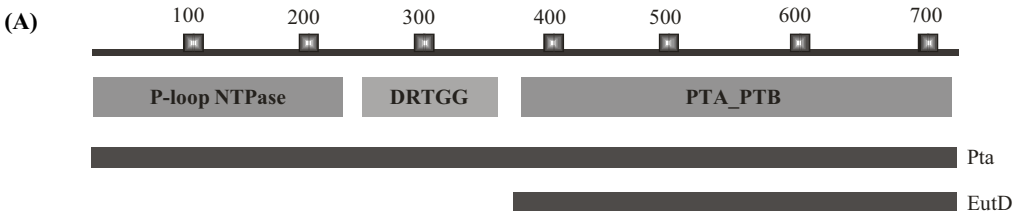
Bacterial strains and growth conditions

E. coli K-12 strain AG1, containing the plasmid pCA24N-eutD (ASKA clone JW2442, p-EutD), was obtained from the ASKA library (Kitagawa *et al.*, 2005). These strains were routinely cultured aerobically in Luria Bertani broth (LB).

For complementation analysis, *E. coli* strains were aerobically grown at 30°C in an M9 minimal medium supplemented with acetate as carbon and energy source. For expression of the chromosomal EutD protein, *E. coli* strains were aerobically grown at 30°C in minimal medium containing 10 mM ethanolamine plus 150 μM vitamin B12 (Scarlett and Turner, 1976). The medium was supplemented, when required, with the corresponding antibiotics (ampicillin or chloramphenicol).

Construction of *E. coli pta acs* deletion strain

E. coli pta acs deletion strain (FB22) was constructed using the *pta* single-gene deletion mutant JW2294, obtained from the NIG Collection (Baba *et al.*, 2006), as recipient strain. This collection is comprised of deletions of *E. coli* K-12 strain BW25113. The *acs* deletion in the JW2294 strain was performed as described by Datsenko and Wanner (2000). The cat^{+} cassette in plasmid pKD3 was amplified using 60 bp primers with perfect homology for the 5′ and 3′ ends of



(B)

```
Pta     SRIIMLIPTGTSVGLTSVSLGVIRAMERKGVRLSVFKPIAQPRTGGDAPDQTTTIVRANS 60
EutD    ------------------------------------------------------------

Pta     STTTAAEPLKMSYVEGLLSSNQKDVLMEEIVANYHANTKDAEVVLVEGLVPTRKHQFAQS 120
EutD    ------------------------------------------------------------

Pta     LNYEIAKTLNAEIVFVMSQGTDTPEQLKERIELTRNSFGGAKNTNITGVIVNKLNAPVDE 180
EutD    ------------------------------------------------------------

Pta     QGRTRPDLSEIFDDSSKAKVNNVDPAKLQESSPLPVLGAVPWSFDLIATRAIDMARHLNA 240
EutD    ------------------------------------------------------------

Pta     TIINEGDINTRRVKSVTFCARSIPHMLEHFRAGSLLVTSADRPDVLVAACLAAMNGVEIG 300
EutD    ------------------------------------------------------------

Pta     ALLLTGGYEMDARISKLCERAFATGLPVFMVNTNTWQTSLSLQSFNLEVPVDDHERIEKV 360
EutD    ------------------------------------------------------------

Pta     QEYVANYINADWIESLTATSERSRRLSPPAFRYQLTELARKAGKRIVLPEGDEPRTVKAA 420
EutD    -----------------MIIERCR------------ELALRAPARVVFPDALDQRVLKAA 31
                            **.*            *** :*  *:*:*:. : *.:***

Pta     AICAERGIATCVLLGNPAEINRVAASQGVELGAGIEIVDPE---VVRESYVGRLVELRKN 477
EutD    QYLHQQGLATPILVANPFELRQFALSHGVAMDG-LQVIDPHGNLAMREEFAHRWLARAGE 90
            ::*:** :*:.** *:.:.* *:** :.. ::::**.   .:**.:. * :    :

Pta     KGMTETVAREQLEDNVVLGTLMLEQDEVDGLVSGAVHTTANTIRPPLQLIKTAPGSSLVS 537
EutD    K--TPPDALEKLTDPLMFAAAMVSAGKADVCIAGNLSSTANVLRAGLRIIGLQPGCKTLS 148
        *  * . * *:* * :::.: *:. .:.*  ::* : :***.:*. *::*   **.. :*

Pta     SVFFMLLP--EQVYVYGDCAINPDPTAEQLAEIAIQSADS-AAAFGIEPRVAMLSYSTGT 594
EutD    SIFLMLPQYSGPALGFADCSVVPQPTAAQLADIALASAETWRAITGEEPRVAMLSFSSNG 208
        *:*:**      .  :.**:: *:*** ***:*** **::  *  * ********:*:.

Pta     SGAGSDVEKVREATRLAQEKRPDLMIDGPLQYDAAVMADVAKSKAPNSPVAGRATVFIFP 654
EutD    SARHPCVANVQQATEIVRERAPKLVVDGELQFDAAFVPEVAAQKAPASPLQGKANVMVFP 268
        *.  . * :*::**.:.:*: *.*::** **:***.:.:** .*** **: *:*.*::**

Pta     DLNTGNTTYKAVQRSADLISIGPMLQGMRKPVNDLSRGALVDDIVYTIALTAIQSAQQQ- 713
EutD    SLEAGNIGYKIAQRLGGYRAVGPLIQGLAAPMHDLSRGCSVQEIIELALVAAVPRQTEVN 328
        .*::**  ** .** ..  ::**::**:  *::*****. *::*:    ::*:    :

Pta     ----------
EutD    RESSLQTLVE 338
```

Fig. 2. Amino acid sequence comparison of EcPta and EcEutD. (A) Numerals at the top indicate the length and domain location for each protein. In boxes, the putative conserved domains (CDD, conserved domain database for protein classification, Marchler-Bauer *et al*., 2009) in EcPta: P-loop NTPase domain; DRTGG domain and PTA_PTB domain (Campos-Bermudez *et al*., 2010). EutD is composed only by the PTA_PTB domain. (B) EcPta and EcEutD sequences were aligned using CLUSTAL W (1.82). Results indicate similarity between them when comparing a stretch of approximately 400 amino acids in the C-terminus of Pta and the complete EutD sequence.

acs: delacs P1(Fw) 5′-GAGAACAAAAGCATGAGCCAAATTCACA AACACACCATTGTGTAGGCTGGAGCTGCTTCG-3′ and delacs P2(Rv) 5′-GGCAATTGTGGGTTACGATGGCATCGCGATAGCCT GCTTCATATGAATATCCTCCTTA-3′. The presence of the *pta acs* deletion was confirmed by sequencing. The mutated *pta acs E. coli* strain, called FB22, was transformed with plasmids pCA24N-eutD (p-EutD) or pCA24N-Pta (p-Pta) for complementation analysis. The introduced plasmids were induced by the addition of 0.1 mM isopropyl-β-D-thiogalactoside (IPTG).

Protein expression and purification

EcEutD was produced in *E. coli* strain AG1 by inducing the expression vector p-EutD. The systems used allow high-level expression of the recombinant protein sequence fused to a N-terminal His-Tag sequence. All chromatographic steps were performed on an ÄKTA purifier (General Electric).

Optimal induction conditions for EutD expression were achieved using IPTG as inducing agent, and different temperatures of induction were tested. Optimal overexpression of the fusion protein was achieved by inducing an AG1/p-EutD *E. coli* culture at OD_{600} 0.4-0.6 with 0.1 mM IPTG and incubation for 16 h at 16°C. In a typical protein preparation, AG1/p-EutD was grown in 500 ml LB medium and induced as described above. Bacteria were harvested by centrifugation and resuspended in 50 mM Tris-HCl pH 8.0, 1 mM phenylmethanesulfonyl fluoride, 0.01 mg/ml DNAse and 5 mM $MgCl_2$. Sonication was performed four times for 30 sec followed by centrifugation at 7,000×g for 10 min at 4°C. The bacterial lysate was applied to a Ni-agarose column (QIAGEN, Germany). After washing with 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole, the fusion protein was eluted with 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 200 mM imidazole. Final diafiltration of the fusion protein was performed in an Amicon concentrator using 50 mM Tris-HCl pH 7.8, 10% glycerol as exchange buffer.

Protein concentration

Protein concentration was determined by Sedmak and Grossberg (1977) using bovine serum albumin (BSA) as standard.

Enzyme activity measurements

EutD activity in the direction of acetyl-CoA synthesis was assayed at 30°C by monitoring the thioester bond formation of acetyl-CoA at 233 nm (ε_{233}=5.55 mM^{-1} cm^{-1}). The assay mixture contained 50 mM Tris-HCl, pH 7.8, 20 mM KCl, 10 mM lithium acetyl-P, 0.2 mM lithium CoA, and 2 mM dithiothreitol.

The acetyl-P synthesis activity of EutD was monitored by measuring the Pi-dependent CoA release from acetyl-CoA with Ellman's thiol reagent, 5′-5-dithiobis (2-nitrobenzoic acid), as the formation of the thiophenolate anion at 412 nm (ε_{412}=13,600 $M^{-1}cm^{-1}$). The assay mixture contained 50 mM Hepes (hydroxyethyl piperazineethanesulfonic acid) pH 7.8, 20 mM KCl, 0.1 mM 5′-5-dithiobis (2-nitrobenzoic acid), 0.1 mM acetyl-CoA, and 5 mM KH_2PO_4.

Steady-state kinetic parameters were determined both for the forward and the reverse reaction measuring the initial rates of formation of acetyl-CoA or CoA, respectively. Measurements were performed at least in triplicate. Kinetic constants were determined by fitting initial rate data to the Michaelis-Menten equation using non-linear regression (Bologna *et al*., 2007). Initial rates were fitted to the Hill equation when sigmoidal curves were observed (Bologna *et al*., 2007).

The optimal pH for reactions catalyzed by recombinant EutD was determined using different buffers: 50 mM MES (morpholineethanesulfonic acid) (pH 5.5 to 6.5), 50 mM Tricine-MOPS (morpholinopropanesulfonic acid) (pH 7.0 to 7.5), and 50 mM Tris-HCl (pH 7.5 to 8.5).

A variety of compounds were tested as potential inhibitors or activators of the EutD reverse acetyl-P forming activity. EutD activity was measured in the absence or presence of 0.5, 2, 5 or 10 mM of each effector (NADH, ATP, PEP, pyruvate, and aspartate) while substrate concentration was maintained at the K_m value (Table 1).

Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10 or 12% (w/v) polyacrylamide gels according to the method of Laemmli (1970). Electrophoresis was run at 150 V and 10°C and protein bands were visualized by Coomassie staining.

Gel filtration chromatography

The molecular mass of recombinant native EutD was determined by gel filtration chromatography on a fast protein liquid chromatography system using a Biosep-Sec S3000 (Phenomenex). The column was equilibrated with 100 mM Phosphate buffer at pH 7.4 and calibrated using molecular mass standards (Sigma, USA). The sample and the standards were applied separately in a final volume of 50 µl at a constant flow rate of 1 ml/min. All chromatographic steps were performed on an ÄKTA purifier (General Electric).

Results

Biochemical characterization of EcEutD

At least two genes encoding putative phosphotranscetylases can be found in *E. coli*: *pta* and *eutD*. Three conserved domains can be detected in EcPta (Fig. 2A; Campos-Bermudez *et al*., 2010). The C-terminal domain (CDD cl00390: PTA_PTB, Fig. 2A) is found in phosphate acetyltransferases and butaryltransferases, and it is the only domain found in EcEutD (Fig. 2A). These two proteins share 37% protein identity, as well as several conserved regions of the primary structure (Fig. 2B).

EcEutD was expressed as a His-Tagged fusion protein and purified to homogeneity using a Ni-agarose resin. The typical level of expression for EutD was 300 mg protein/liter of culture. The purified protein has a monomer molecular mass of 36 kDa (Fig. 3A), which corresponds to the predicted molecular mass of EutD by sequence analysis.

The native molecular mass of EutD was calculated by size

Table 1. Kinetic parameters for the acetyl-CoA and acetyl-P forming reactions of *E. coli* EutD (*Ec*EutD).
Kinetic values are given as Mean±SD. Each value is averaged over at least two different enzyme preparations. Ac-P: acetyl-P.
*The values obtained for EcPta (EcPta; Campos-Bermudez *et al*., 2010) are included for comparison.

Acetyl-CoA forming reaction						
	$K_{m\,\text{Ac-P}}$ (µM)	$K_{m\,\text{CoA}}$ (µM)	V_{max} (UI/mg)	k_{cat} (sec^{-1})	$k_{cat}/K_{m\text{Ac-P}}$ ($sec^{-1}\mu M^{-1}$)	$k_{cat}/K_{m\text{CoA}}$ ($sec^{-1}\mu M^{-1}$)
*Ec*EutD	311.7±4.7	32.7±1.9	656.4±20.1	415.5±12.7	1.3	12.6
*Ec*Pta*	900±0.4	67.2±5.3[a]	177.4±6.2	227.6±9.3	0.25	3.4
Acetyl-P forming reaction						
	$K_{m\,\text{Ac-CoA}}$ (µM)		V_{max} (UI/mg)	k_{cat} (sec^{-1})	$k_{cat}/K_{m\text{Ac-CoA}}$ ($sec^{-1}\mu M^{-1}$)	
*Ec*EutD	9.5±1.4		189.2±4.6	119.8±2.9	12.6	
*Ec*Pta*	44.9±4.1[b]		23.1±2.1	29.6±2.3	0.7	

[a] Sigmoidal kinetic reaction (Hill coefficient: 1.7; Campos-Bermudez *et al*., 2010).
[b] Sigmoidal kinetic reaction (Hill coefficient: 1.3; Campos-Bermudez *et al*., 2010)

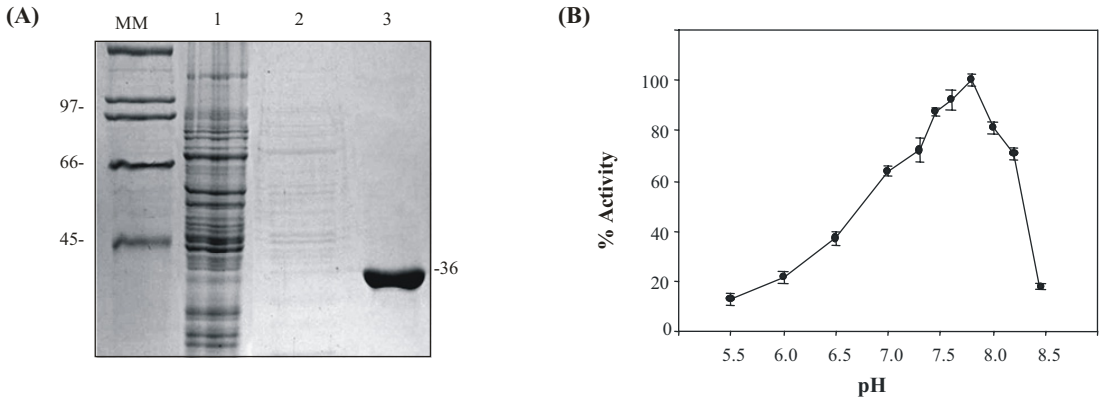


Fig. 3. (A) Coomassie Blue-stained SDS polyacrylamide gel of total protein extracts from induced AG1/p-EutD *E. coli* strain (Lane 1, 10 μg), Ni-NTA column wash fraction (Lane 2, 5 μg) and purified recombinant *E. coli* EutD (Lane 3, 5 μg). The estimated molecular mass of EutD is indicated on the right. Molecular mass markers (MM) were run on the left. (B) pH-dependent phosphotransacetylase activity of purified EutD. Activity is shown as a % of the maximum, obtained at pH 7.8.

exclusion chromatography. The value obtained (79.5±2.7 kDa) indicates that EutD assembles as dimer (36 kDa per subunit). Purified EcEutD displayed phosphotransacetylase activity. A detailed kinetic characterization of the purified EutD was performed in both directions of the enzymatic reaction (i.e. acetyl-CoA synthesis or acetyl-P synthesis). All kinetic parameters for EutD were determined at pH 7.8, since it was optimal for enzyme activity determinations (Fig. 3B).

Kinetic parameters for the acetyl-CoA-forming reaction catalyzed by EutD

The kinetic plot of the acetyl-CoA forming reaction catalyzed by EcEutD was hyperbolic for both substrates, acetyl-P and CoA (Table 1). The enzyme displayed 10-times higher affinity for CoA than for acetyl-P (Table 1). EcEutD affinity for CoA was higher than those of *S. enterica* EutD (46 μM, Brinsmade and Escalante-Semerena, 2004) and EcPta (Table 1, Campos-Bermudez *et al*., 2010). However, its affinity value for acetyl-P ranked between those obtained for *S. enterica* EutD (129 μM) and for EcPta (Table 1). The k_{cat} for EcEutD was nearly 5-times lower than the k_{cat} for *S. enterica* EutD (1927 sec^{-1}) and nearly 2-times higher than the k_{cat} for EcPta (Table 1). Thus, the catalytic efficiency of EcEutD for both substrates was significantly higher than the one obtained with EcPta (Table 1).

Kinetic parameters for the acetyl-P forming reaction

With regard to the acetyl-P forming reaction catalyzed by EcEutD, the results obtained indicate that EutD catalyzes the reverse reaction at rates nearly 4-times lower than those for the acetyl-CoA forming reaction (Table 1). However, the affinity for acetyl-CoA was 4-times higher than that for CoA, rendering similar catalytic efficiencies for both directions of the EutD reaction (Table 1).

EcEutD affinity for CoA was nearly 5-times higher than that obtained for EcPta (Table 1). Similarly, the k_{cat} value estimated for EutD was 4-times higher than the one obtained for Pta (Table 1). Thus, the catalytic efficiency for the acetyl-P forming reaction catalyzed by EutD was more than 18-times higher than the efficiency observed when using EcPta (Table 1).

Regulation of EcEutD activity by metabolic effectors

The effect of several metabolites already known as metabolic effectors of different Ptas was analyzed for EcEutD in the acetyl-P-forming reaction. Pyruvate activated EutD in a concentration-dependent manner, reaching 156% of activation at 5 mM (Fig. 4). On the other hand, the acetyl-P forming activity of EcEutD was not significantly modified in the presence of NADH, ATP, PEP and aspartate, even at concentrations as high as 10 mM (Fig. 4). It is worth mentioning that equal concentrations of these compounds significantly modified enzymatic activity when tested on EcPta (Campos-Bermudez *et al.*, 2010).

Complementation of acetate utilization by EcEutD

E. coli employs two different pathways for conversion of acetate into acetyl-CoA (Wolfe, 2005; El-Mansi *et al*., 2006; Kumari *et al*., 2007). The high-affinity pathway involves acetyl-

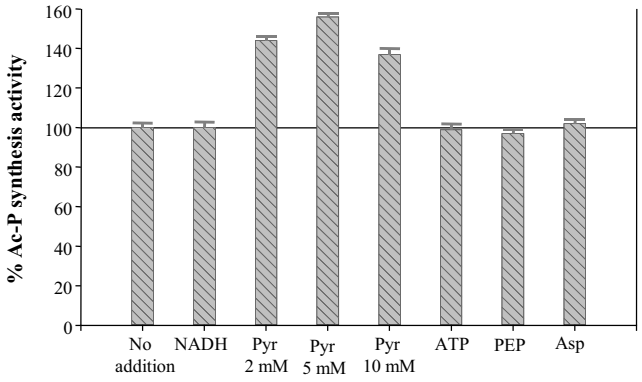


Fig. 4. Regulatory properties of recombinant EcEutD in the acetyl-P forming direction. The acetyl-P forming activity of EcEutD (Fig. 1B) was monitored in the absence or presence of 5 mM NADH, ATP, Asp or PEP and different pyruvate concentrations. Substrate concentrations were maintained at the Km value (Table 1). Results are presented as the ratio between % of activity in the presence of the effectors relative to the activity measured in their absence. Assays were performed at least in triplicate, and error bars indicate SD.

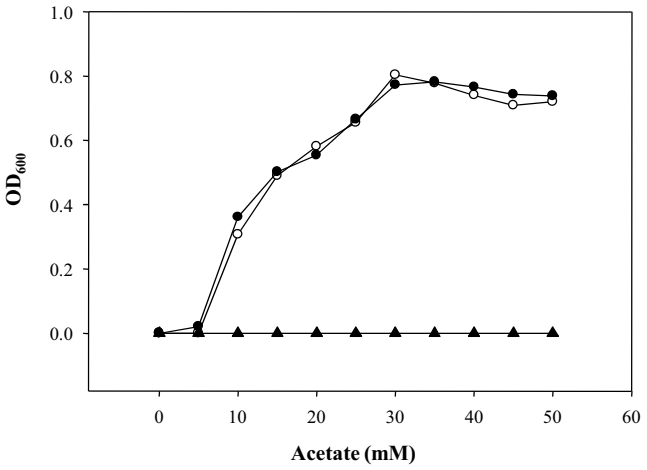


Fig. 5. EutD can restore growth of a *pta acs* mutant strain on acetate as sole carbon source. Growth of *pta acs* mutant strain (▲), *pta acs* transformed with p-Pta (●), and *pta acs* transformed with p-EutD (○) on minimal medium with the addition of the indicated acetate concentration and in the presence of IPTG. OD_{600} was measured after a 40 h growth at 30°C.

CoA synthetase (Acs) which activates acetate to acetyl-CoA when acetate is present at a low concentration in the environment (Starai and Escalante Semerena, 2004; Fig. 1B). The other pathway, which is reversible, involves acetate kinase (Ack) and Pta, and operates at high acetate concentration (Fig. 1B). Inactivation of both of these pathways renders a strain unable to use acetate as a carbon and energy source.

To investigate whether EcEutD is able to compensate for the lack of Pta activity during growth on acetate, the *eutD* gene was introduced into a *pta acs* strain (FB22) unable to grow on acetate (Campos-Bermudez *et al*., 2010). IPTG-induced expression of *eutD* in the *pta acs* strain transformed with p-EutD, restored its capability to grow on minimal medium with acetate as sole carbon source (Fig. 5). The growth profile obtained for *pta acs*/p-EutD at different concentrations of acetate was similar to the profile obtained when FB22 was complemented with EcPta (*pta acs*/p-Pta; Fig. 5), indicating that EutD can fulfill the role of Pta under this growth conditions.

Growth of a *pta acs* mutant strain on acetate using endogenous EutD

The mutant *pta acs* strain (FB22) failed to grow on acetate as carbon source (Fig. 4). However, this is surprising given that this mutant strain contains the *eutD* gene that encodes a protein with phosphotransacetylase activity (Table 1).

In this way, we wondered if the induction of the expression of the endogenous EutD would circumvent the need for the Pta enzyme during growth of the *pta acs* strain on acetate. Thus, the *pta acs* strain was grown on 30 mM acetate, with the addition of ethanolamine (10 mM) and vitamin B12 (150 μM) as inducers of endogenous EutD expression (Scarlet and Turner, 1976). In this case, the *pta acs* strain culture reached a cell density comparable to that measured for the wild type strain (Table 2). As a control, in the presence of 10 mM ethanolamine and vitamin B12, the *pta acs* strain was not able to grow (Table 2). Moreover, the wt strain (BW25113) was also unable to grow on ethanolamine as sole carbon source (Table 2). Since it has been shown that certain *E. coli* strains have an insertion element that disrupts genes of the *eut* operon (Fig. 1A; Blattner *et al.*, 1997; Hayashi *et al.*, 2006), it is possible that this element could avoid the expression of crucial genes for ethanolamine utilization. However, EutD is induced in the presence of ethanolamine (Table 2) since the insertion element is found downstream of *eut*D (Fig. 1A).

Table 2. Growth on acetate of *E. coli pta acs* double mutant (FB22) in the presence of ethanolamine and vitamin B12

Culture medium contained M9 salts supplemented with 30 mM acetate, 10 mM ethanolamine or 150 μM vitamin B12 (vitB12) as indicated. Final OD values obtained after a 40 h growth at 30°C are shown. Results are the mean of at least three independent experiments.

Strain	Growth condition	Final OD_{600}
wt[a]	acetate	1.14 ± 0.11
wt[a]	ethanolamine, vitB12	0.006 ± 0.01
pta acs	acetate	0.005 ± 0.01
pta acs	ethanolamine, vitB12	0.004 ± 0.01
pta acs	acetate, ethanolamine, vitB12	0.95 ± 0.08
pta acs/p-Pta	acetate	0.77 ± 0.08
pta acs/p-EutD	acetate	0.79 ± 0.11

[a] strain BW25113 obtained from the NIG Collection (Baba *et al*., 2006)

Phosphotransacetylase activity (acetyl-CoA forming activity) was assayed in the *pta acs* strain growing on acetate in the absence (0.01±0.005 U/mg) and presence (3.68±0.08 U/mg) of ethanolamine and vitamin B12 (Table 2). The results confirm the induction by ethanolamine and vitamin B12 of a protein with phosphotransacetylase activity in the *pta acs* strain.

Discussion

EcEutD: a highly active dimeric phosphotransacetylase regulated by pyruvate

In the present work, we performed a detailed kinetic characterization of EcEutD. The enzyme behaves as a phosphotransacetylase, and parameters for both the acetyl-CoA and acetyl-P forming reactions were estimated (Table 1). EcEutD displays similar catalytic efficiency when either CoA or acetyl-CoA are used as substrates in the two directions of the reaction (Table 1). When compared to EcPta (Campos-Bermudez *et al*., 2010), EcEutD was significantly more efficient for all the substrates analyzed in both reactions, *i.e.* acetyl-CoA or acetyl-P synthesis (Table 1). With regard to *S. enterica* EutD, which was characterized only in the acetyl-CoA forming reaction (Brinsmade *et al*., 2004), EcEutD displays lower k_{cat} and affinity for acetyl-P, but higher affinity for CoA (Table 1).

Among different compounds tested as metabolic effectors for the acetyl-P forming reaction catalyzed by EcEutD, pyruvate was the only one that modified this activity (Fig. 4), and the activation observed resembles that of EcPta (Campos-Bermudez *et al*., 2010). Interestingly, while EcEutD was unaffected by NADH, ATP and PEP, EcPta showed activation by PEP and inhibition by NADH and ATP (Campos-Bermudez *et al*., 2010). This distinct regulation may be related to differential physiological roles for the two phosphotransacetylases, being EcPta activity linked to acetate excretion and to the operation of the Tricarboxylic Acid cycle (Campos-Bermudez *et al*.,

2010).

Recently, the analysis of EcPta truncated proteins indicated that the PTA_PTB domain (Fig. 2A) is the catalytic one (Campos-Bermudez *et al*., 2010). However, this PTA_PTB domain expressed as a recombinant protein, displayed only a low phosphotransacetylase activity which was not regulated at all by any of the effectors of EcPta (pyruvate, PEP, NADH and ATP; Campos-Bermudez *et al*., 2010). Moreover, it was also found that this domain alone did not exhibit a stable native assembly, unlike the complete EcPta (Campos-Bermudez *et al*., 2010). On the other hand, a highly active dimeric phosphotransacetylase (EcEutD) lacking the N-terminal domain of Pta (Fig. 2A) was characterized in the present work. Thus, the results obtained in the present work in comparison to the characterization of the truncated EcPta fragments (Campos-Bermudez *et al*., 2010), indicate that other regions of EcEutD primary structure, not found in the C-terminal region of EcPta, may be involved in the stabilization of the native state and phosphotranscetylase catalytic efficiency. Examination of the protein sequence alignment between EcEutD and Pta, shows an extra domain at the C-terminal end of EutD, not found in Pta (Fig. 2B). This, and other divergent regions between the two proteins (Fig. 2B), could be related to the ability of EcEutD to display a stable native assembly. Moreover, it is possible that the high activity (Table 1) and pyruvate regulation of EcEutD are both related to its capacity to generate a stable native dimeric state. On the other hand, since EutD is not regulated by NADH, ATP and PEP, the allosteric binding sites for these compounds are most probably located at the N-terminal domain of EcPta (Campos-Bermudez *et al*., 2010).

EutD is able to compensate for the lack of phosphotransacetylase activity to sustain growth on acetate

E. coli pta acs double mutant strain is unable to grow on minimal medium with acetate as a sole carbon source (Fig. 5). However, in the present work, we found that this deficiency can be corrected by complementation with EcEutD. This complementation was obtained by either expressing EcEutD from the introduced p-EutD plasmid (Fig. 5) or by inducing the *eut* operon with ethanolamine plus vitamin B12 (Table 2). In this case, measurable phosphotransacetylase activity was assayed in the *E. coli pta acs* strain (Table 2), confirming the expression of a phosphotransacetylase enzyme different from Pta. As a whole, these results indicate that EutD and Pta are equivalent in function under these growth conditions, and that the growth characteristics of *pta acs*/p-Pta and *pta acs*/p-EutD depends on the low affinity of the Ack enzyme for acetate (Brown *et al*., 1977; Kumari *et al*., 1995) and not on differences in kinetics between the two phosphotransacetylases.

Do the two *E. coli* phosphotransacetylases Pta and EutD differ in localization?

The results obtained in the present work, in correlation to the recent characterization of EcPta (Campos-Bermudez *et al*., 2010) indicate that, in this bacteria, there are two phosphotransacetylases that differ in their catalytic efficiency (Table 1), activity regulation (Fig. 4), and protein structure (Fig. 2). Although these two enzymes fulfill equivalent functions under certain growth conditions (Table 2), a strictly-dependent expression on ethanolamine and vitamin B12 is observed for EutD (Table 2), while EcPta is expressed as a housekeeping enzyme.

Other potential difference between EcEutD and EcPta is their localization in *E. coli*. As *eutD* belongs to the *eut* operon, it is possible that this enzyme is located within the ethanolamine metabolosome, although this has not been elucidated yet (Fig. 1B; Brinsmade *et al.*, 2005). However, our current results about the growth complementation of the *pta acs* strain by the induction of the *eut* operon (Table 2) suggest a cytosolic localization of EutD. Further experiments are needed to elucidate EutD localization in *E. coli*, taking into account the possibility that the formation of the metabolosome may be disrupted in certain strains due to the presence of the insertion element in the *eut* operon (Fig. 1A).

Acknowledgements

This work was funded by grants from CONICET and Agencia Nacional de Promoción Científica y Tecnológica. MFD and CSA are members of the Researcher Career of CONICET and FPB, VCB and DDS are fellows of the same institution.

References

Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. Datsenko, M. Tomita, B. Wanner, and H. Mori. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Sys. Biol.* 2, 2006.0008.

Blackwell, C.M., F.A. Scarlett, and J.M. Turner. 1977. Microbial metabolism of amino alcohols: control of formation and stability of partially purified ethanolamine ammonia-lyase in *Escherichia coli*. *J. Gen. Microbiol.* 98, 133-139.

Blackwell, C.M. and J.M. Turner. 1978. Microbial metabolism of amino alcohols: purification and properties of coenzyme B12-dependent ethanolamine ammonia-lyase of *Escherichia coli. Biochem. J.* 175, 555-563.

Blattner, F.R., G. Plunkett, C.A. Bloch, N.T. Perna, V. Burland, M. Riley, J. Collado-Vides, and *et al*. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* 277, 1453-1462.

Bologna, F.P., C.S. Andreo, and M.F. Drincovich. 2007. *Escherichia coli* malic enzymes: two isoforms with substantial differences in kinetic properties, metabolic regulation and structure. *J. Bacteriol*. 189, 5937-5946.

Brinsmade, S.R. and J.C. Escalante-Semerena. 2004. The *eutD* gene of *Salmonella enterica* encodes a protein with phosphotransacetylase enzyme activity. *J. Bacteriol.* 186, 1890-1892.

Brinsmade, S.R. and J.C. Escalante-Semerena. 2007. *In vivo* and *in vitro* analyses of single-amino acid variants of the *Salmonella enterica* phosphotransacetylase enzyme provide insights into the function of its N-terminal domain. *J. Biol. Chem.* 282, 12629-12640.

Brinsmade, S.R., T. Paldon, and J.C. Escalante-Semerena. 2005. Minimal functions and physiological condictions required for growth of *Salmonella enterica* on ethanolamine in the absence of the metabolosome. *J. Bacteriol*. 187, 8039-8046.

Brown, T.D.K., M.C. Jones-Mortimer, and H.L. Kornberg. 1977. The enzymic interconversion of acetate and acetyl-coenzyme A in *Escherichia coli*. *J. Gen. Microbiol.* 102, 327-336.

Campos-Bermudez, V.A., F.P. Bologna, C.S. Andreo, and M.F. Drincovich. 2010. Functional dissection of *Escherichia coli* phosphotransacetylase structural domains and analysis of key compounds involved in activity regulation. *FEBS J.* 277, 1957-1966.

Datsenko, K.A. and B.L. Wanner. 2000. One-step inactivation of

chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 97, 6640-6645.

El-Mansi, M., A.J. Cozzone, J. Shiloach, and B.J. Eikmanns. 2006. Control of carbon flux through enzymes of central and intermediary metabolism during growth of *Escherichia coli* on acetate. *Curr. Opin. Microbiol.* 9, 173-179

Hayashi, K., N. Morooka, Y. Yamamoto, K. Fujita, K. Isono, S. Choi, E. Ohtsubo, T. Baba, B.L. Wanner, H. Mori, and T. Horiuchi. 2006. Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. *Mol. Syst. Biol*. 2, 2006 0007.

Kitagawa, M., T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, and H. Mori. 2005. Complete set of ORF clones of *Escherichia coli* ASKA library (A Complete Set of *E. coli* K-12 ORF Archive): Unique resources for biological research. *DNA Res*. 12, 291-299.

Kofoid, E., C. Rappleye, I. Stojiljkovic, and J. Roth. 1999. The 17-gene ethanolamine (*eut*) operon of *Salmonella typhimurium* encodes five homologues of carboxysome shell proteins. *J. Bacteriol.* 181, 5317-5329.

Kumari, S., C.M. Beatty, D.F. Browing, S.J.W. Busby, E.J. Simel, G. Hovel-Miner, and A.J. Wolfe. 2007. The intracellular concentration of acetyl phosphate in *Escherichia coli* is sufficient for direct phosphorylation of two-component response regulators. *J. Bacteriol*. 189, 5574-5581.

Kumari, S., R. Tishel, M. Eisenbach, and A.J. Wolfe. 1995. Cloning, characterization, and functional expression of *acs*, the gene which encodes acetyl Coenzyme A synthetase in *Escherichia coli*. *J. Bacteriol.* 177, 2878-2886

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Marchler-Bauer, A., J.B. Anderson, F. Chitsaz, M. Derbyshire, C. DeWeese-Scott, J.H. Fong, L.Y. Geer, and *et al*. 2009. CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Res*. 37, D205-D210.

Mori, K., R. Bando, N. Hieda, and T. Toraya . 2004. Identification of a reactivating factor for adenosylcobalamin-dependent ethanolamine ammonia lyase. *J. Bacteriol*. 186, 6845-6854.

Sagermann, M., A. Ohtaki, and K. Nikolakakis. 2009. Crystal structure of the EutL shell protein of the ethanolamine ammonia lyase microcompartment. *Proc. Natl. Acad. Sci. USA* 106, 8883-8887.

Scarlett, F.A. and J.M. Turner. 1976. Microbial metabolism of amino alcohols. Ethanolamine catabolism mediated by coenzyme B12-dependent ethanolamine ammonia-lyase in *Escherichia coli* and *Klebsiella aerogenes*. *J. Gen. Microbiol.* 95, 173-176.

Sedmak, J.J. and S.E. Grossberg. 1977. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Anal. Biochem.* 79, 544-552.

Sheppard, D.E. and J.R. Roth. 1994. A rationale for autoinduction of a transcriptional activator: ethanolamine ammonia-lyase (EutBC) and the operon activator (EutR) compete for adenosyl-cobalamine in *Salmonella typhimurium*. *J. Bacteriol.* 176, 1287-1296.

Starai, V.J. and J.C. Escalante-Semerena. 2004. Acetyl-coenzyme A synthetase (AMP forming). *Cell Mol. Sci*. 61, 2020-2030.

Starai, V.J., J. Garrity, and J.C. Escalante-Semerena. 2005. Acetate excretion during growth of *Salmonella enterica* on ethanolamine requires phosphotransacetilase (EutD) activity, and acetate recapture requires acetyl-CoA synthetase (Acs) and phosphotransacetylase (Pta) activities. *Microbiology* 151, 3793-3801.

Wolfe, A.J. 2005. The acetate switch. *Microbiol. Mol. Biol. Rev.* 69, 12-50.