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The EutQ and EutP Proteins are Novel Acetate Kinases Involved in Ethanolamine Catabolism: Physiological Implications for the Function of the Ethanolamine Metabolosome in *Salmonella enterica*

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Summary

Salmonella enterica catabolizes ethanolamine inside a compartment known as the metabolosome. The *e*thanolamine *ut*ilization (*eut*) operon of this bacterium encodes all functions needed for the assembly and function of this structure. To date, the roles of the EutQ and EutP were not known. Herein we show that both proteins have acetate kinase activity, and that EutQ is required during anoxic growth of *S. enterica* on ethanolamine and tetrathionate. EutP and EutQ-dependent ATP synthesis occurred when enzymes were incubated with ADP, Mg(II) ions and acetyl-phosphate. EutQ and EutP also synthesized acetyl-phosphate from ATP and acetate. Although EutP had acetate kinase activity, *eutP* strains lacked discernable phenotypes under the conditions where *eutQ* strains displayed clear phenotypes. The kinetic parameters indicate that EutP is a faster enzyme than EutQ. Our evidence supports the conclusions that EutQ and EutP represent novel classes of acetate kinases. We propose that EutQ is necessary to drive flux through the pathway under physiological conditions, preventing a buildup of acetaldehyde. We also suggest that ATP generated by these enzymes may be used as a substrate for EutT, the ATP-dependent corrinoid adenosyltransferase, and for the EutA ethanolamine ammonia-lyase reactivase.

Keywords

bacterial metabolism; ethanolamine catabolism; acetate kinases; metabolosome; bacterial microcompartments; acetate activation; substrate-level phosphorylation

Introduction

Salmonella enterica serovar Typhimurium strain LT2 (hereafter *S.* Typhimurium) is a Gamma-proteobacterium that can generate energy via fermentation or oxidative phosphorylation. The latter mechanism can use molecular oxygen or, under anoxic conditions, this bacterium can respire to diverse terminal electron acceptors, such as nitrate, fumarate, timethylamine-*N*-oxide, dimethyl sulfoxide, and tetrathionate (Escalante-

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Semerena & Roth, 1987, Barrett & Clark, 1987). *S.* Typhimurium can use ethanolamine as a carbon, nitrogen and energy source (Chang & Chang, 1975, Roof & Roth, 1988b, Roof & Roth, 1989), and the breakdown of this two-carbon amino alcohol occurs within a metabolosome, also known as a bacterial microcompartment (BMC[1] or MCP) (Bobik, 2006, Cheng *et al.*, 2008, Yeates *et al.*, 2011) (Fig. 1A). The metabolosome is a proteinaceous polyhedron that is assembled by proteins encoded by the 17-gene *e*thanolamine *ut*ilization (*eut*) operon (Fig. 1B), whose expression is induced by the presence of ethanolamine and coenzyme B_{12} (Kofoid *et al.*, 1999, Buan *et al.*, 2004, Held *et al.*, 2013, Roof & Roth, 1988a). It is thought that the metabolosome serves several functions: i) to prevent cell damage by containing the acetaldehyde product of ethanolamine ammonia-lyase (Brinsmade *et al.*, 2005); ii) to prevent carbon loss from volatilization of acetaldehyde (Penrod & Roth, 2006); and iii) to conserve and recycle cofactors used in ethanolamine catabolism (Huseby & Roth, 2013). All of the proteins encoded by the *eut* operon have assigned functions except for EutQ, EutP and EutJ.

In the intestine, ethanolamine is derived from phosphatidylethanolamine (Larson *et al.*, 1983, Fung & Proulx, 1969), a major component of eukaryotic membranes (Cotton, 1972). Although ethanolamine is an abundant source of carbon and nitrogen in the intestinal environment occupied by *S.* Typhimurium, not all microbes present in it can ferment or respire ethanolamine. Most microbes require molecular oxygen to respire aminoalcohol, but *S.* Typhimurium sidesteps this need by using tetrathionate as terminal electron acceptor (Thiennimitr *et al.*, 2012, Price-Carter *et al.*, 2001). When *S.* Typhimurium triggers intestinal inflammation and white blood cells are recruited to the infection site, the reactive oxygen species released generate tetrathionate from thiosulfate present in the gut (Winter *et al.*, 2010). Access to tetrathionate gives *S.* Typhimurium a competitive advantage over other intestinal microbes (Thiennimitr *et al.*, 2012). Generation of tetrathionate via the inflammatory response is therefore important for *S.* Typhimurium pathogenesis (Baumler *et al.*, 2011).

Previous studies did not assign roles to the proteins encoded by *eutP, eutQ,* and *eutJ.* Strains carrying deletions in these genes grew as well as the wild-type strain on ethanolamine as a carbon and energy source (Kofoid *et al.*, 1999, Stojiljkovic *et al.*, 1995, Buan & Escalante-Semerena, 2006). This paper reports the functions of EutP and Eut, but not the function of EutJ (a putative DnaK-like chaperone). Relevant to the work reported here is the observation by Penrod and Roth (Penrod & Roth, 2006), who showed that *eutP* and *eutQ* strains excreted more acetaldehyde than the wild-type strain when grown on glycerol with ethanolamine as a nitrogen source. From a structural standpoint, EutQ belongs to the cupin superfamily (SSF51182, PF06249), which includes a wide variety of enzymes such as carbohydrate-binding isomerases, flavonoid-binding dioxygenases and nuclear factors (Dunwell *et al.*, 2004). The structure of EutP has not been reported, but bioinformatics analyses show that EutP is a member of the P-loop containing nucleoside triphosphate hydrolase superfamily (SSF52504, PF10662). In light of scant experimental and bioinformatics data, the functions of these proteins remained unknown.

Given that *S.* Typhimurium respires ethanolamine to tetrathionate under anoxic conditions, we decided to investigate possible functions of EutP and EutQ proteins during anoxic

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growth of this bacterium with ethanolamine as the sole source of carbon and energy and tetrathionate as terminal electron acceptor. Our data show that EutQ is required under such conditions, yet it is dispensable when *S.* Typhimurium is growing on ethanolamine in the presence of oxygen. Results of *in vitro* activity assays and complementation studies support the conclusion that EutQ is an acetate kinase. The physiological roles of EutP and EutQ under oxic and anoxic conditions are discussed.

Results

Efforts in our laboratory looking for growth defects caused by the absence of the EutP or EutQ protein of *S.* Typhimurium during growth on ethanolamine under oxic conditions were not fruitful. However, when we shifted our attention to anoxic growth conditions, it became clear that, at least the EutQ function was needed for growth of this bacterium on ethanolamine and tetrathionate.

Phenotypes of eutP and eutQ strains growing under anoxic conditions on ethanolamine and tetrathionate

To determine whether or not *eutP* and *eutQ* strains had a growth phenotype on ethanolamine as the sole source of carbon and energy under anoxic conditions, we grew each strain on ethanolamine (30 mM) as carbon and energy source and tetrathionate (40 mM) as terminal electron acceptor. After consuming the small amount of glycerol (0.5 mM) present in the medium to stimulate growth, the *eutQ* strain stopped growing at an OD_{600} ~0.2 (Fig. 2, triangles), whereas the $eutQ^{+}$ strain grew to an OD_{600} ~1 (Fig. 2, squares). Induction of a plasmid-encoded $eutQ^{+}$ allele using low concentrations of inducer (arabinose, 0.05 mM) restored growth (Fig. 2, inverted triangles). In contrast, the *eutP* strain grew as well as the $eutP^{+}$ strain under the same conditions (Fig. S1).

Excess EutQ has a negative effect on growth on ethanolamine and tetrathionate, but the effect is avoided when the medium is supplemented with pantothenate

When we increased the expression of plasmid-borne $eutQ^{+}$ we observed a dramatic negative effect on the ability of *S.* Typhimurium to grow on ethanolamine and tetrathionate. As shown in figure 3A, the growth behavior of the *eutQ*/p$eutQ^{+}$ strain in ethanolamine/tetrathionate medium containing 1 mM arabinose was indistinguishable from that of the *eutQ* strain carrying the empty cloning vector pBAD24 (compare triangles vs inverted triangles). Surprisingly, this effect was avoided by the addition of pantothenate, a precursor to coenzyme A (CoA) (Fig. 3B, inverted triangles). Our interpretation of these results is discussed below.

Inhibitory levels of acetaldehyde are responsible for the EutQ phenotype

As mentioned above, Penrod and Roth reported that a *S.* Typhimurium *eutQ* strain released acetaldehyde into the medium during growth on glycerol glycerol and ethanolamine (Penrod & Roth, 2006). Germane to this observation are results from previous work from our laboratory, which implicated acetaldehyde as a strong inhibitor of *S.* Typhimurium growth on ethanolamine (Brinsmade *et al.*, 2005). The correlation between aldehyde accumulation and growth inhibition prompted us to directly test the idea that acetaldehyde caused the

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EutQ phenotype. For this purpose we introduced into a *eutQ* strain individual plasmids carrying wild-type alleles of genes encoding *bona fide* acetaldehyde dehydrogenases, such as *S.* Typhimurium EutE (Zhu *et al.*, 2011), *S.* Typhimurium PduP (Leal *et al.*, 2003), and *S.* Typhimurium AdhE (Burton & Stadtman, 1953). Data presented in figure 4A show that *eutQ* strains that synthesized acetaldehyde dehydrogenases grew at similar rates and reached full density. In contrast, the control *eutQ* strain carrying the empty cloning vector failed to grow (Fig. 4A, inverted triangles). We obtained additional experimental evidence to support the hypothesis that acetaldehyde accumulation was responsible for the EutQ phenotype. The experiment we performed was based on the previously reported beneficial effect of glutathione on cells growing on ethanolamine (Rondon *et al.*, 1995, Brinsmade *et al.*, 2005). As seen in figure 4B (open triangles), the addition of glutathione restored growth of the alluded strain at a rate similar to that of the *eutQ*/p*eutQ*$^+$ strain, and allowed the culture to reach full density.

In vivo evidence suggests that EutQ has acetate kinase activity

Although the above results could be interpreted to mean that EutQ had acetaldehyde dehydrogenase activity, we considered that such a possibility was unlikely, given the fact that EutE has been identified as the acetaldehyde dehydrogenase encoded by the *eut* operon (Zhu *et al.*, 2011). Instead, we hypothesized that the synthesis of acetyl-CoA from acetaldehyde and CoA was, for some reason, an unfavorable reaction and that EutQ played a key role in pulling the reaction forward with the eventual release of acetate. Given that EutD is a phosphotransacetylase (Brinsmade & Escalante-Semerena, 2004), we posited that EutQ had acetate kinase activity. To test this idea we introduced into the *eutQ* strain wild-type alleles encoding the housekeeping acetate kinase AckA (EC 2.7.2.1), or propionate/acetate kinases (EC 2.7.2.15) such as PduW and TdcD (Wolfe, 2005, Palacios *et al.*, 2003, Hesslinger *et al.*, 1998, Bobik *et al.*, 1999). Remarkably, synthesis of AckA, PduW or TdcD restored anoxic growth of the *eutQ* strain on ethanolamine and tetrathionate (Fig. 5). These results strongly suggested that EutQ had acetate kinase activity. To obtain additional *in vivo* evidence to support this conclusion we performed a reciprocal experiment where EutQ would be expected to compensate for the absence of the AckA acetate kinase. A *ackA* strain had a significant growth defect on ethanolamine and tetrathionate, which was efficiently corrected by ectopically expressed *eutQ*$^+$ or *ackA*$^+$ (Fig. 6, squares, diamonds). Our interpretation of these results is presented in the Discussion section.

EutQ and EutP have acetate kinase activity *in vitro*

Highly purified preparations of EutP and EutQ (Fig. S2) were assayed for acetate kinase activity *in vitro* using HPLC to resolve substrates from products. Due to the lability of AcP and the difficulty in detecting low amounts of this compound by HPLC, we performed the EutQ reaction in the opposite direction, providing AcP and ADP as substrates for the enzyme to make ATP (Fig. 7). Panels B, C of figure 7 show that EutP and EutQ synthesized ATP from AcP and ADP, relative to the results obtained in the absence of either enzyme (panel A). The presence of AMP in the no-enzyme control indicates that it might have been an impurity of the stock solution. The formation of ATP was confirmed using ^{31}P-NMR spectroscopy (Fig. 8). Fig. 8A shows the chemical shifts of phosphates associated with commercially available ADP and ATP. Panel B shows the spectrum of a reaction mixture

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devoid of enzyme, and panel C shows the spectrum of the sample containing EutQ, which contained all the peaks expected for ATP. Similar results were obtained with EutP (data not shown).

EutQ and EutP were also assayed for acetate kinase activity in a continuous spectrophotometric assay that measured the oxidation of NADH. The reaction rate was dependent on the concentration of protein (Figure S3), and both enzymes failed to use the non-hydrolyzable ATP analogue, AMP-PCP, as substrate (data not shown). To test for the presence of divalent ions, the EutQ acetate kinase was dialyzed against buffer A containing EDTA (2 mM), but lacking imidazole (three buffer changes, 4°C for 2 h each). EutQ dialyzed under such conditions did not lose any activity compared to untreated enzyme (data not shown). EutQ used guanosine triphosphate (GTP) as substrate (870 μM/min/mg), but did it at a 45% slower rate than the same reaction containing an equimolar ATP concentration (1300 μM/min/mg). Neither EutQ nor EutP displayed acetate kinase activity when cytidine triphosphate (CTP) or thymidine triphosphate (TTP) was used in lieu of ATP.

The *eutP* gene is immediately upstream of *eutQ* in the *eut* operon (Fig. 1B), and its 3′ end overlaps with the start codon of *eutQ*. Although we have been unable to find a phenotype for *eutP* strains under conditions in which a *eutQ* strain has a phenotype, we considered the possibility that EutP and EutQ may interact. Surprisingly, EutP demonstrated acetate kinase activity independently of EutQ, and generated ATP from ADP and acetyl-phosphate, and acetyl-phosphate from ATP and acetate (Figs. 8, 9). Similarly to EutQ, EutP did not have activity with AMP-PCP, CTP, or TTP, but had a 28% lower specific activity with GTP (1750 μmol/min/mg) compared to ATP (2400 μmol/min/mg). Dialysis of EutP against EDTA did not have an effect on its specific activity, and its kinetic parameters indicated that EutP was a slightly faster enzyme than EutQ, with nearly identical K_m values, and k_{cat} values that were approximately 30–40% higher than those for EutQ for either acetate or ATP (Table 2).

The EutP- and EutQ-catalyzed reactions can be coupled to a physiologically relevant phosphotransacetylase

EutP and EutQ can make AcP from acetate and ATP, or can catalyze the substrate-level phosphorylation of ADP using AcP as the phosphate donor. These conclusions were reached on the basis of data presented in figure 9. The reaction mixtures used to obtain the results shown in panels A–D used the phosphotransacetylase EutD to generate AcP from AcCoA and *ortho*-phosphate. When EutQ, EutP or both were present in the reaction mixture, ATP was formed at the expense of AcCoA (panels B–D); in the absence of EutP or EutQ ATP was not formed (panel A). The reaction was driven in reverse, with AcP generated by EutQ or EutP. The resulting AcP was used by EutD to yield AcCoA from CoA present in the mixture (panels F–H); again, in the absence of EutQ or EutP, AcCoA was not formed (panel E). Notably, the amount of acetyl-CoA converted to CoA by EutD increased upon co-incubation of EutQ (by 15%) or EutP (by 44%).

Kinetic analysis of EutQ and EutP

Kinetic parameters of EutQ and EutP were determined using the lactate dehydrogenase/ pyruvate kinase coupled assay describe under *Experimental procedures*. Our results differ

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slightly from published values for the housekeeping *S. enterica* acetate kinase AckA (Chittori *et al.*, 2012). In comparison, EutQ had a ten-fold higher K_m for ATP (0.70 mM) than that of AckA (0.07 mM), but a two-fold lower K_m for acetate (0.7 mM) than that of AckA (1.2 mM).

As mentioned above, EutQ belongs to the cupin superfamily of proteins, and many members of this superfamily bind transition metals to catalyze a variety of reactions. However, EutQ belongs to a class of cupins that lack a metal-binding motif (Dunwell *et al.*, 2004). In place of one of the conserved histidines of the metal binding motif is an aspartate residue (D175), which has been proposed to form an acidic pair with a nearby glutamate (E173) (Fig. S4). These two residues are located deep within a negatively charged cleft, as seen in the three-dimensional crystal structure of EutQ from *Clostridium difficile* (PDB 4AXO) (Pitts *et al.*, 2012). This cleft was proposed to be the active site, and remarkably, the authors modeled an acetyl moiety deep within the site, demonstrating the feasibility of this enzyme binding acetate (Pitts *et al.*, 2012). However, changing either of these two residues to alanine did not impact the ability of the EutQ variants to restore growth of the *eutQ* strain on ethanolamine and tetrathionate. Changing both of the residues to alanine resulted in a slight growth defect, compared to complementation by the *eutQ*$^+$ allele (Fig. S5)

EutP and EutQ have no similarity to known acetate kinases

Comparisons of the EutP (159 aa) and EutQ (229 aa) sequences to the *S.* Typhimurium AckA enzyme (400 aa) (Figs. S6B, C) clearly show marked differences in size and sequence, placing EutP and EutQ in a different class of acetate kinases. In spite of these differences, there are several residues conserved in EutP, EutQ and AckA. *Methanosarcina thermophila* AckA acetate kinase was crystallized with a variety of nucleotide and acetyl substrates (PDB 1G99, 1TUY, 1TUU) (Ingram-Smith *et al.*, 2005, Gorrell *et al.*, 2005, Miles *et al.*, 2002, Gorrell & Ferry, 2007) and from its comparison to the *S.* Typhimurium AckA, we identified residues able to make polar contact with the substrates (Fig. S5A). From this analysis, it appears that ADP-binding residues D285 and N337 of *S.* Typhimurium AckA align with E147 and N170 of EutQ, and the acetyl-binding residue H94 aligns to H14 of EutQ. These residues were identified using ClustalW2, which is not ideal for alignment of only two proteins. The more appropriate algorithm, BLAST, did not identify significant similarity between EutQ and AckA (E value: 1.3, coverage: 11%, identity: 35%). Future site-directed mutagenesis studies will determine if these residues contribute to EutQ function.

Discussion

EutQ is required for ethanolamine catabolism in *S.* Typhimurium under ecologically relevant conditions

Our data (Fig. 2) show for the first time that EutQ activity is required for growth *S.* Typhimurium on ethanolamine in an anoxic environment where the electron acceptor tetrathionate is present. Surprisingly, the absence of EutQ did not affect growth of this bacterium on ethanolamine in an oxic environment, a behavior that is discussed below under a separate context. The conditional need for EutQ highlights the importance of ethanolamine

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respiration to tetrathionate in the anoxic environment in the intestine of its host. In addition, the need for EutQ also raises questions regarding metabolite flux through the pathway and about the mechanisms of import and egress of enzyme substrates and products between the metabolosome lumen and the cytosol by as-yet-unidentified means.

EutQ and EutP are novel acetate kinases

In vivo and *in vitro* evidence reported here (Figs. 5–9) strongly support the conclusion that EutQ and EutP have acetate kinase activity. Unfortunately, the available crystal structure of the *S.* Typhimurium EutQ apo-protein (PDB 2PYT) does not provide insights into the identity of the active site. Interestingly, the investigators that reported the crystal structure of the *Clostridum difficile* EutQ homologue did model an acetyl moiety in it, without knowing that the enzyme was an acetate kinase (Pitts *et al.*, 2012). This putative binding site for acetate, however, is not yet supported by experimental evidence. The authors proposed that the active site of EutQ was located deep within a negative cleft of each beta-barrel in the cupin-like structure, and that a conserved aspartate (D175) and glutamate (E173) residue were important for activity (Pitts *et al.*, 2012) (Fig. S3). In our hands, *S.* Typhimurium EutQ acetate kinase activity did not require divalent metal cofactors, as dialysis with EDTA did not affect its activity. Consistent with the idea that EutQ is not a metalloprotein, we note that the His residues involved in metal binding among cupins are not conserved. Changing either D175 or E173 to alanine did not affect the ability of the resulting EutQ variants to restore growth of a *eutQ* strain on ethanolamine/tetrathionate under anoxic conditions, which suggests that these residues do not play a significant role in substrate binding or catalysis.

Do EutP and EutQ work together or independently, and are they needed for tetrathionate respiration?

Bioinformatics information available from databases suggests that EutP binds ATP near its N terminus (Protein Feature View, RSCB PDB). Our data show that EutP has acetate kinase *in vitro* activity, and its kinetic parameters indicate that EutP is a slightly more efficient enzyme than EutQ, including a ~40% higher k_{cat} with respect to ATP than EutQ (Table 2). From a physiologic standpoint it is not clear why *S.* Typhimurium has retained two genes encoding acetate kinases within the *eut* operon. One possibility could be that EutP and EutQ may be monomers of an acetate-kinase hetero-oligomer. If this were the case, the lack of phenotypes for *eutP* strains (Fig. S1) would indicate that EutQ is the critical monomer of such a putative hetero-oligomer. As mentioned above, EutQ has been proposed to have an acetate-binding site, and EutP contains a nucleotide-binding P-loop. These two domains may interact to enhance acetate kinase activity. It is also possible that in addition to its enzymatic activity, EutQ may interact with other Eut proteins, including EutD or the proteins comprising the shell of the metabolosome, and that such roles are critical to the function of the putative hetero-oligomer. Another possibility is that *eutP* is transcribed very minimally compared to *eutQ* under the conditions tested. At present, data to support these ideas are lacking.

The presence of EutQ and EutP is not linked to the ability of *S.* Typhimurium to use tetrathionate as an electron acceptor. Notably, the genome of many ethanolamine-catabolizing organisms such as *Klebsiella pneumoniae, Escherichia coli* and *Shigella*

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flexneri encode copies of *eutQ* and *eutP*, yet these bacteria cannot respire to tetrathionate (Barrett & Clark, 1987). Therefore, EutQ and EutP are not directly involved in tetrathionate respiration in *S.* Typhimurium. A requirement for *eutQ* or *eutP* has yet to be tested in other organisms.

Are EutP and EutQ functions required in other ethanolamine catabolizing bacteria?

Across organisms with *eut* operons, the *eutP* and *eutQ* genes are not always adjacent, and in some cases one, the other or both are missing. For example, the *Nocardioides, Listeriaceae*, and *Entercoccaceae eut* operons only have *eutQ*, and the *Clostridiaciae* and *Fusobacterium nucleatum* do not have *eutP* and *eutQ* located next to one another (Tsoy *et al.*, 2009). Numerous other genomes do not have either *eutP* or *eutQ*, although these organisms often have minimal *eut* operons lacking the shell and most other *eut* proteins except for EutBC, the small and large subunits of the ethanolamine ammonia-lyase central to ethanolamine catabolism.

Despite the lack of a phenotype for *eutP* strains, there may be a link between EutP activity and pathogenesis. Of the 24 strains encoding *eutP*, 54% are known to cause food poisoning (Tsoy et al., 2009). Of the 24 strains carrying *eutQ,* 38% are pathogenic. In comparison, of the 85 strains carrying *eutBC*, the critical genes for ethanolamine catabolism, only 17% can cause disease. Although it is unclear if all these genes are active, the high percentage of pathogenic ethanolamine-respiring organisms encoding *eutP* may indicate a function for EutP activity in virulence.

Acetate kinase activity may be important for increasing flux through the EutD phosphotransacetylase

The growth inhibition caused by overexpression of *eutQ*$^{+}$ was intriguing, but even more so was the relief of such inhibition by exogenous pantothenate (Fig. 3). Although the toxicity could be due to EutQ aggregation caused by non-physiological protein levels casued by overexpression, such an argument would not explain the effect of pantothenate. One possible explanation for both observations is that high levels of EutQ deprive the cell of AcCoA needed for anabolic purposes by increasing the conversion of AcCoA to AcP by EutD. We propose that pantothenate reverses this problem by increasing the pool of free CoA, which in turn is used by the CoA-consuming acetaldehyde dehydrogenase (EutE) enzyme to generate excess AcCoA, some of which can be diverted to central metabolism and promote cell growth. This idea is supported by results from *in vitro* experiments in which EutD was coupled to EutQ and EutP. EutQ, EutP, or both in combination enhanced the EutD-catalyzed conversion of AcCoA to CoA (Fig. 9E–H).

The above hypothesis can also help us explain the increased excretion of acetaldehyde in *eutQ* strains. If in fact EutQ and EutP increase flux through the EutD enzyme as proposed above, their absence apparently results in less active EutD, which in turn would lead to less active EutE aldehyde dehydrogenase. With less flux through EutE, the acetaldehyde produced by ethanolamine ammonia-lyase (EutBC) would accumulate. This hypothesis is consistent with an earlier observation that *eutQ* and *eutP* mutant strains excrete more acetaldehyde than the wild-type strain (Penrod & Roth, 2006). If this hypothesis were

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correct, expressing aldehyde dehydrogenases in *eutQ* strains should correct the phenotype, a prediction that was experimentally validated (Fig. 4). An alternative hypothesis suggesting that EutQ activity is more important for energy charge maintenance is discussed below.

EutQ may play a role in increasing the energy charge and ATP recycling inside the metabolosome

The standard oxidation/reduction potential of tetrathionate/thiosulfate is low, +170 mV, relative to that of the oxygen/water couple (+815 mV) (Kapralek, 1972, Thauer *et al.*, 1977). When the cell respires ethanolamine to tetrathionate a G = −94.6 kJ/mol are available for ATP synthesis, compared to a G = −219 kJ/mol available when molecular oxygen is the electron acceptor. This substantial difference in free energy likely results in a lower energy charge when tetrathionate is the electron acceptor, hence placing a greater importance on ATP synthesis via substrate-level phosphorylation by EutP and EutQ.

EutQ may be important for energy generation via substrate-level phosphorylation, which would explain why *eutQ* strains and *ackA* strains grow poorly on ethanolamine/tetrathionate. Complementation by alternative acetate kinases in a *eutQ* strain could be due to the restoration of ATP levels, which supports the substrate-level phosphorylation hypothesis. However, if EutQ activity is only important for generation of ATP, why is overexpression of EutQ toxic? and why can this toxicity be rescued by pantothenate? We suggest that if EutQ is necessary for substrate-level phosphorylation, and to ensure flux through EutD, as proposed above.

It is also possible that EutQ is required to recycle ATP within the metabolosome, although it is not known for certain if EutQ is localized inside or outside the compartment. Cofactor recycling within the metabolosome is an important aspect of the functionality of metabolosomes. Several enzymes have been shown to be involved in cofactor recycling within both the *eut* and *pdu* metabolosomes of *S.* Typhimurium. For example, EutD, EutE and EutG are needed to recycle CoA and NAD^+ within the *eut* metabolosome, while PduP, PduQ and PduL fulfill the same role in the *pdu* metabolosome (Huseby & Roth, 2013, Liu *et al.*, 2015, Cheng *et al.*, 2012). The cobalamin-recycling enzyme EutT (Johnson *et al.*, 2004, Sheppard *et al.*, 2004, Buan & Escalante-Semerena, 2006, Mera *et al.*, 2007, Moore *et al.*, 2014) and the ethanolamine ammonia-lyase reactivase EutA (Aaron *et al.*, 2007, Mori *et al.*, 2004) are ATP-consuming enzymes, hence ATP is either generated inside, is transported into the metabolosome or both. Notably, ATP is the only co-substrate whose regeneration inside the metabolosome has not been accounted for. On the basis of our data, we propose that EutQ, and possibly EutP, fulfill this role. EutA is the only Eut enzyme that generates ADP as reaction product, but whether EutA is inside or outside of the metabolosome remains an open question. If EutA were located inside the metabolosome, there would be a need for ATP-recycling inside the structure or ATP flux into it.

If cofactor recycling is a role of EutQ, then why do *eutQ* strains grow on ethanolamine under oxic conditions? One explanation would be that the flow of ATP into the metabolosome increases during oxic growth on ethanolamine due to the high-energy charge generated under such conditions.

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Other ideas for why oxic growth conditions may enable a *eutQ* strain to tolerate higher acetaldehyde concentrations

If the absence of EutQ slows the activity of EutE as proposed above, under oxic growth conditions a strain lacking EutQ may tolerate the resulting increased concentration of acetaldehyde for several reasons. First, the concentration of the detoxifying tripeptide glutathione (GSH) is four times higher during oxic growth than under anoxic growth (Fahey *et al.*, 1978). Additionally, during oxic growth other proteins known to consume acetaldehyde, such as aldehyde dehydrogenase (AdhE) may be present at higher levels. Such a combination of detoxifying factors would prevent acetaldehyde from damaging proteins and DNA.

Why do cytosolic acetate kinases compensate for the absence of EutQ?

It is unlikely that the acetate/propionate kinases AckA, PduW or TdcD ever enter the metabolosome, yet they can compensate for the absence of EutQ during growth of a *eutQ* strain on ethanolamine/tetrathionate (Fig. 5). One intepretation is that EutQ is located outside the metabolosome, and that AcP diffuses outside the shell and can be equally accessed by any acetate kinase. However, if this hypothesis is correct, why is there a need for specialized *eut* acetate kinases at all? We suggest it is more likely that EutQ, and perhaps EutP, are located within the metabolosome. Inside the metabolosome, they can interact immediately with their upstream product, which is likely AcP from the EutD phosphotransacetylase. Our interpretation for the complementation by alternative acetate kinases in a *eutQ* strain is that AcP generated by EutD inside the metabolosome accumulates without being consumed by EutQ, and escapes into the cytosol through pores proposed to be small molecule transporters (Chowdhury *et al.*, 2015). Once in the cytosol, AcP is consumed by AckA, PduW or TdcD, reducing the concentration of AcP inside the metabolosome with the concomitant acceleration of AcCoA and acetaldehyde by EutD and EutE, respectively. Additionally, the ATP produced by these enzymes may increase the energy charge of the bacterium to a level sufficient to overcome growth arrest. Future work will address these possibilities.

Conclusion

This work provides the first experimental evidence that the *S.* Typhimurium EutQ and EutP proteins have acetate kinase activity. Bioinformatics analyses support the conclusion that EutP and EutQ belong to new classes of acetate kinases. We conclude that acetaldehyde accumulation is responsible for the observed lack of growth of *eutQ* strains under growth conditions that lower the energy charge of the cell and the concentration of aldehyde quenching biomolecules. We also suggest that substrate-level phosphoylation via the EutQ acetate kinase plays a significant role in cellular growth during ethanolamine respiration. Our findings are significant, not only because we have identified new classes of acetate kinases, but because of the need for these enzymes during respiration of ethanolamine to tetrathionate, a condition known to be critical to *S.* Typhimurium in the context of the disease-causing capabilities of this bacterium. *In vivo* evidence supporting a role for the EutP enzyme in ethanolamine catabolism is yet to be obtained.

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Experimental procedures

Construction of expression and complementation vectors

To generate a recombinant construct of EutQ with a cleavable *N*-terminal hexahistidine (H_6) tag, the *S.* Typhimurium *eutQ*$^+$ allele was PCR amplified from strain JE6583 (Table 1). The primers used for the amplification were 5′-NNGCTCTTCNTTCGTGAAAAAACTTATCACAGCTAACGA-3′ (forward), and 5′-NNGCTCTTCNTTATCATACGGATTGCCAGTTTG-3′ (reverse). The PCR fragment and the vector pTEV18 were cut with restriction enzyme BspQI and ligated, yielding plasmid pEUT112. The latter directed the synthesis of a recombinant H_6-EutQ whose tag was removed using recombinant tobacco etch virus (rTEV) protease (Blommel & Fox, 2007). Tagless EutQ contained two residues (Gly-Thr) upstream of the *N*-terminal Met residue; these residues did not affect enzyme function. This cloning strategy was also used to generate pTEV18 constructs containing *eutP*$^+$ (plasmid pEUT110) and *eutP*$^+$ *eutQ*$^+$ (plasmid pEUT129). For *eutP*$^+$, the following primers were used: 5′-NNGCTCTTCNTTCATGAAACGTATTGCTTTTGTCG-3′ (forward), and 5′-NNGCTCTTCNTTATTAGCTGTGATAAGTTTTTTCACCTG-3′ (reverse). Plasmid pEUT129 was generated using the *eutP*$^+$ forward primer and the *eutQ*$^+$ reverse primer. *eutQ* alleles encoding variant EutQ proteins were generated using the QuikChange XL site-directed mutagenesis kit (Stratagene). The pEUT112 and the pEUT56 (lab collection) plasmids were used as templates for the PCR-based site-directed mutagenesis according to the manufacturer's instructions. Mutations in the *eutQ* sequence were confirmed by DNA sequencing using BigDye® protocols (ABI-Prism). DNA sequences were determined at the Georgia Genomics Facility at the University of Georgia-Athens.

Protein overproduction and purification

Plasmids expressing *eutP*$^+$ or *eutQ*$^+$ were transformed into *Escherichia coli* BL21(λDE3) using a described heat shock protocol (Maniatis *et al.*, 1982). Overnight cultures were inoculated 1:100 (v/v) into 2L of Terrific Broth (http://openwetware.org/wiki/Terrific_Broth) supplemented with ampicillin (100 μg/mL). Cultures were grown to an OD_{600} ~0.7 at 37°C with shaking. Protein expression was induced with isopropyl-β-D-1 thiogalactopyranoside (IPTG, 0.5 mM) and shaken overnight at 10°C. Cell pellets were harvested at 12,000 × *g* using a Beckman/Coulter Avanti J-25I centrifuge equipped with a JLA-16.250 rotor. Cell pellets were frozen at −80°C until used. Cell pellets were re-suspended in buffer A (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 50 mM, pH 7) containing sodium chloride (NaCl, 500 mM), imidazole (70 mM) and *tris*-(2-carboxyethyl)phosphine (TCEP, 0.25 mM)), at a ratio of 5 mL per gram of cell paste. Cells were broken using a French pressure cell under 103 MPa and 4°C. To ensure cell breakage, the procedure was performed twice. Cellular debris was removed from solution by centrifugation (45,000 × *g* for 45 min) followed by filtration through a 0.45-μm-pore filter.

His-tagged proteins were separated from crude cell-free extracts using an AKTA*purifier* fast protein liquid chromatograph equipped with a 5-mL HisTrap column (GE Healthcare). The column was washed with 40 mL of buffer A, and tagged protein was eluted using a 50-mL linear gradient to 100% buffer B (buffer A containing 500 mM imidazole). H_7-rTEV

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protease (1 mg/mL) was added to the elutate at a 1:50 ratio (v/v) to cleave the H_6-tag from the protein of interest. H_7-rTEV was prepared as described (Rocco *et al.*, 2008). The tagged protein/protease mixture was dialyzed three times into buffer A (1L each), once for 1 h, once overnight, and once for 3 h, all at 4°C.

The protein of interest was loaded onto a fresh HisTrap column and eluted with 25 mL of buffer A to remove the cleaved His-tag and H_7-rTEV. Tagless protein was dialyzed into buffer C (buffer A with no imidazole and 10%(v/v) glycerol) thrice as described above. Proteins were flash-frozen in liquid N_2 and stored at −80°C until use. Protein purity was assessed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), followed by staining with Coomassie Brilliant Blue R (Sigma) (Sasse, 1991).

In vivo assessment of function

To assess the role of EutQ *in vivo*, a strain (JE8816, *metE205 ara*-9 *eutQ1182*) was tested for phenotypes when grown on minimal medium. No-carbon essential (NCE) minimal medium (Vogel & Bonner, 1956, Berkowitz *et al.*, 1968) was supplemented with ethanolamine (30 mM), methionine (0.5 mM), NH_4Cl (30 mM), $MgSO_4$ (1 mM) L-(+)-arabinose (500 μM), glycerol (0.5 mM), and Wolfe's trace minerals (Balch & Wolfe, 1976). The small amount of glycerol added to all cultures greatly reduced the length of the lag phase. The growth rate in the early stages of growth observed in all curves was due to the use of glycerol as the source of carbon and energy. All reported growth rates were calculated after glycerol depletion. When growth curves were performed under anoxic conditions, tetrathionate (40 mM) was supplied as electron acceptor. Anoxic medium was degassed in an anoxic chamber (Coy) for 24 h, and stirred for 1 h to remove all oxygen prior to inoculation. Growth under the stated conditions was monitored for 24 h or less due to precipitation of sulfur granules after prolonged growth (Price-Carter *et al.*, 2005). Plasmids used for *in vivo* analyses were constructed as described above and in Table 1. Plasmids carrying *eutQ* wild type or mutant alleles were under the control of the arabinose-inducible P_{araBAD} promoter in plasmid pBAD24 (Guzman *et al.*, 1995).

Spectrophotometric acetate kinase assay

To determine the kinetic parameters of homogeneous EutQ, we used a modified version of a described acetate kinase coupled assay (Bergmeyer *et al.*, 1984). Briefly, the assay measures the rate at which an acetate kinase phosphorylates acetate at the expense of ATP. The resulting ADP is consumed by pyruvate kinase, which reacts it with PEP to make ATP and pyruvate. Pyruvate is reduced to lactate by lactic dehydrogenase at the expense of NADH. NADH consumption was monitored spectrophotometrically. Each reaction contained HEPES buffer (84 mM, pH adjusted at 25°C), sodium acetate (NaOAc, made fresh, variable concentration), $MgCl_2$ (6.6 mM), NADH (1.1 mM), pyruvate kinase/lactate dehydrogenase (1–1.6/1.6–2.3 U, respectively), myokinase (0.66 U), phophoenolpyruvate (PEP, 1.9 mM, made fresh) and ATP (variable concentration). Reactions were set up in a 96-well plate and initiated by the addition of EutQ or EutP (2 μM). The reaction was monitored at 340 nm on a Spectramax Plus (Molecular Devices). Reactions were performed at 40°C.

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HPLC determination of EutQ and EutP reaction products

EutP and EutQ can make AcP at the expense of acetate and ATP yielding ADP as a byproduct. The reaction can be reversed by providing ADP and AcP to the enzymes. We used HPLC to show that the acetate kinase activities of EutP and EutQ are reversible. For this purpose, the chromatograph was equipped with a Partisil-10 SAX column (250 × 4.6 mm, Phenomenex) and an NH_2 SecurityGuard cartridge (Phenomenex). A gradient of potassium phosphate (monobasic, 25°C) from 0.05 (pH 4.9) to 1 M (pH 4.0) was applied to the column over a 35-min period at a flow rate of 1 mL/min at 25°C. Reaction products were detected at 254 nm. Prior to HPLC analysis, reaction mixtures were incubated at 37°C for 1 h (when using AcP and ADP as substrates) or 2 h (when coupled to EutD), and filtered through Spin-X columns (Costar, 0.45 μm). To test the back reaction (*i.e.* AcP + ADP ➔ acetate + ATP), we mixed EutP or EutQ (0.7 μM each), ADP (1 mM), AcP (1mM) and $MgCl_2$ (1 mM) in PIPES buffer (20 mM, pH7) in a 500 μl final volume. In some cases we used the EutD phosphotransacetylase to generate AcP *in situ* from AcCoA and *ortho*-phosphate (P_i). In such cases, the reaction mixtures contained EutD (0.7 μM), AcCoA (0.5 mM), and KH_2PO_4 (1 mM) *in lieu* of AcP. The forward reaction (*i.e* acetate + ATP ➔ AcP + ADP) was demonstrated by coupling the production of AcP by EutP or EutQ to the synthesis of AcCoA by EutD. For this purpose, the reaction mixtures contained EutD (0.7 μM), EutP or EutQ (0.7 μM), ATP (1 mM), acetate (1 mM), $MgCl_2$ (1 mM), and coenzyme A (0.5 mM).

^{31}P-NMR spectroscopy

For ^{31}P-NMR analysis, reaction mixtures were prepared as described for HPLC analysis. Reaction mixtures (500 μl) were brought up to a final volume of 600 μL in D_2O (17%, v/v). Proton-decoupled ^{31}P-NMR spectra were obtained using a Varian Unity Inova500 500 mHz spectrometer in the Chemical Sciences Magnetic Resonance Facility fo the University of Georgia. A total of 1024 scans were performed per sample. Chemical shifts were referenced to H_3PO_4 (85%) set to 0.0 ppm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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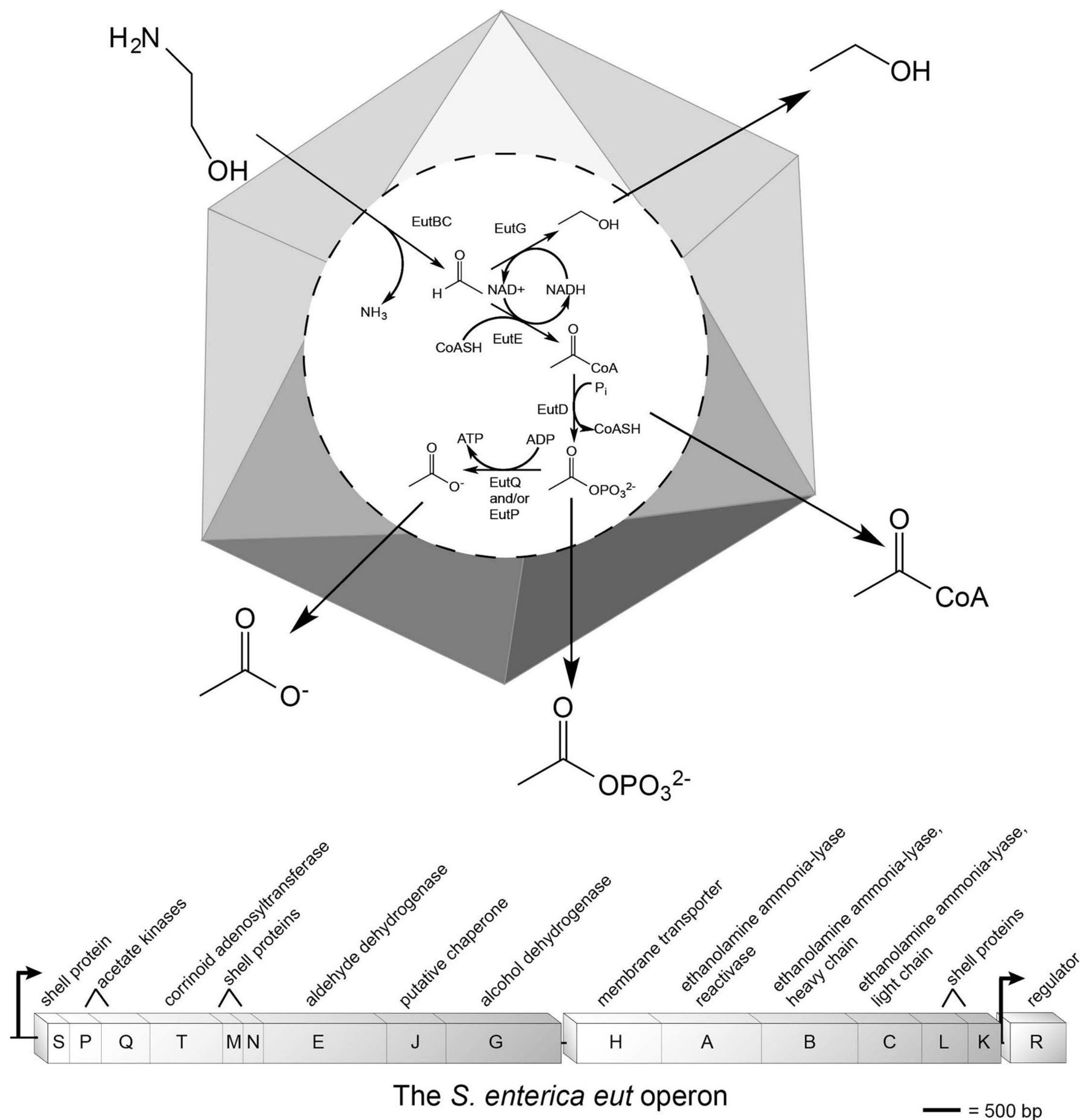

Figure 1. The ethanolamine catabolic pathway

(A) The ethanolamine metabolosome, made of EutSMNLK, is represented as a polyhedral shell. The scheme for the breakdown of ethanolamine is shown as occurring within the shell; products of the pathway are shown outside the shell. (B) The *e*thanolamine *ut*ilization (*eut*) operon of *S.* Typhimurium.

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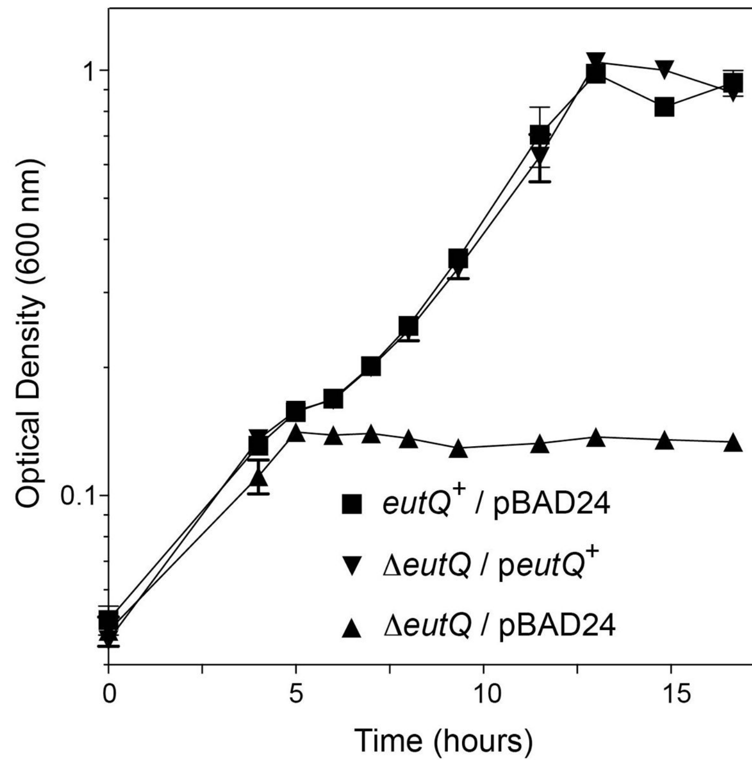

Figure 2. Growth phenotype of a ***eutQ*** **strain, and correction of it by ectopic expression of** ***eutQ*****+**

Strains expressing *eutQ*+ from a plasmid under anoxic growth conditions in minimal medium supplemented with ethanolamine (30 mM), NH_4Cl (30 mM), tetrathionate (40 mM) and L(+)-arabinose (0.05 mM). Growth rates were calculated for each culture during the logarithmic phase of growth. Oxidized tetrathionate can precipitate, making standard doubling times based on optical density difficult to calculate. Therefore, we normalized the mean percent change of optical density of each strain (triplicate) to the linear portion of the log phase in lieu of a standard doubling time calculation (mean OD% • h^{-1}): *eutQ*+/pBAD24, 14.0; *eutQ*/pBAD24, no growth; *eutQ*/p*eutQ*+, 13.5.

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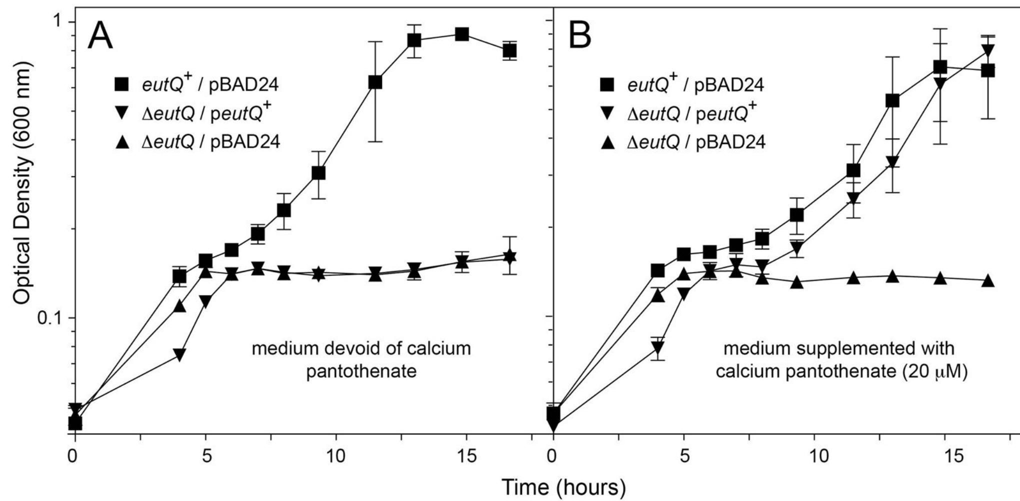

Figure 3. Elevated expression of *eutQ*$^+$ is deleterious for growth, but the effect is counteracted by exogenous pantothenate

A. Strains expressing *eutQ*$^+$ from a plasmid under anoxic growth conditions in minimal medium supplemented with ethanolamine (30 mM), NH_4Cl (30 mM), tetrathionate (40 mM) and L(+)-arabinose (1 mM). Growth rates were calculated for each culture during the logarithmic phase of growth (mean OD% • h^{-1}): *eutQ*$^+$/pBAD24, 14; *eutQ*/pBAD24, 2; *eutQ*/p*eutQ*$^+$, 1. B. Same conditions as in panel A, but with the addition of calcium pantothenate (20 μM). Growth rates: *eutQ*$^+$/pBAD24, 15; *eutQ*/pBAD24, 1; *eutQ*/p*eutQ*$^+$, 13.

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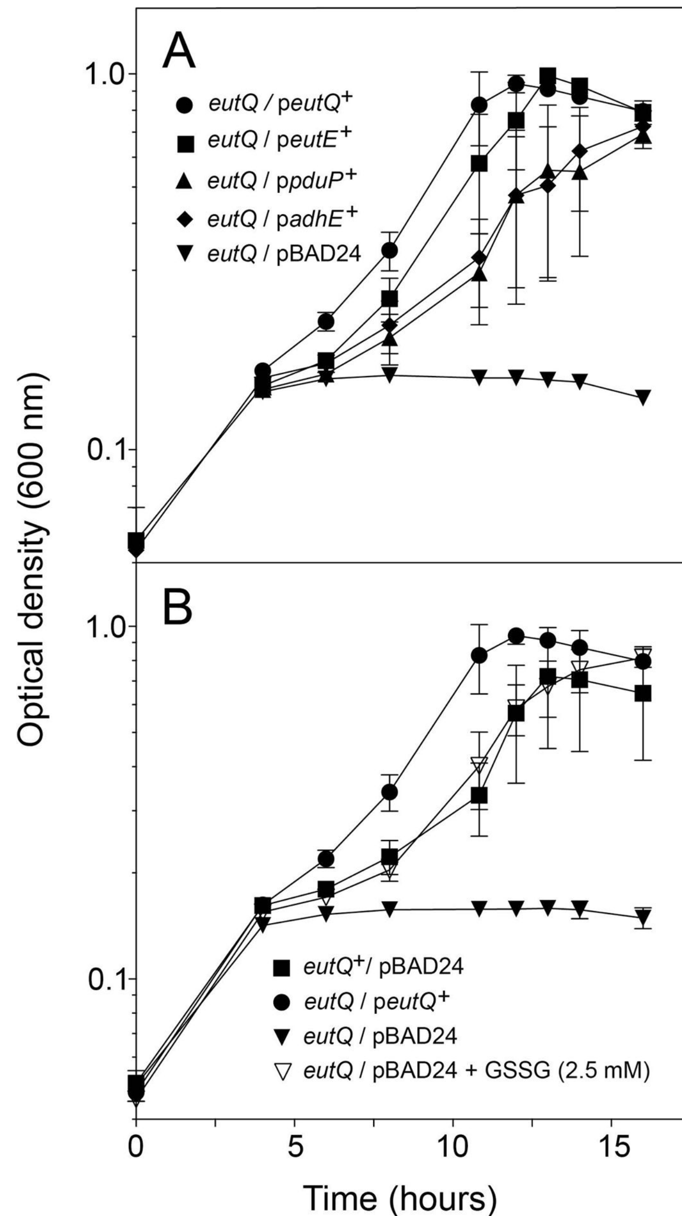

Figure 4. The EutQ phenotype is caused by acetaldehyde, and can be corrected by aldehyde dehydrogenases or glutathione

Strains were grown anoxically on minimal medium supplemented with ethanolamine (30 mM), NH_4Cl (30 mM), and tetrathionate (40 mM) at 37°C. A. In all cases, ectopic gene expression was induced using arabinose (0.5 mM), except for *eutQ*$^+$, which was induced using a 10-fold lower concentration of arabinose (0.05 mM). Growth rates were calculated at the log phase of the cultures (mean OD% • h^{-1}): *eutQ/peutQ*$^+$, 15; *eutQ/peutE*$^+$, 16; *eutQ/ppduP*$^+$, 9; *eutQ/padhE*$^+$, 9; *eutQ*/pBAD24, no growth. B. All strains were induced with 0.05 mM arabinose. *eutQ*/pBAD24 was supplemented with oxidized glutathione (GSSG, 2.5 mM) where indicated. Growth rates: *eutQ*$^+$/pBAD24, 12; *eutQ/peutQ*$^+$,16; *eutQ/*pBAD24, 1; *eutQ/*pBAD24 + GSSG, 14.

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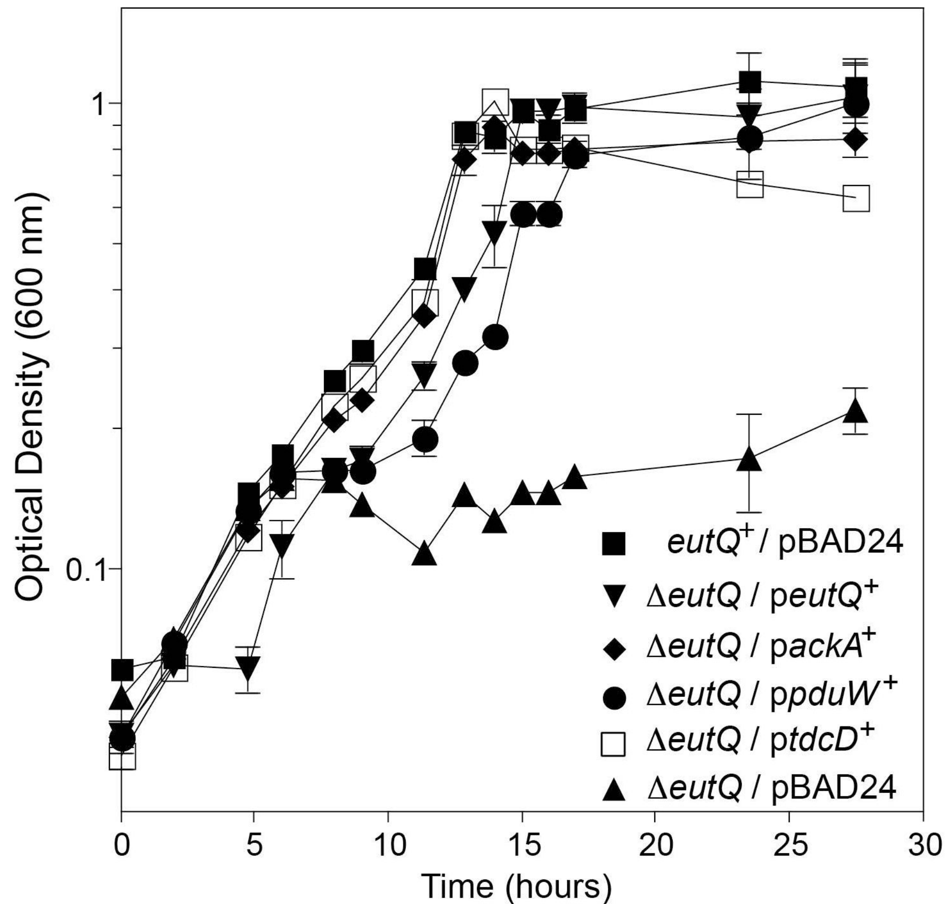

Figure 5. Complementation of the EutQ phenotype by acetate kinases

Strains were grown anaerobically on minimal medium supplemented with ethanolamine (30 mM), NH_4Cl (30 mM), tetrathionate (40 mM) and L(+)-arabinose (0.05 mM) at 37°C. Growth rates were calculated for each culture during the logarithmic phase of growth (mean OD% • h^{-1}): *eutQ*$^+$/pBAD24, 12; *eutQ*/p*eutQ*$^+$, 14; *eutQ*/p*ackA*$^+$, 15; *eutQ*/p*pduW*$^+$, 18; *eutQ*/p*tdcD*$^+$, 16; *eutQ*/p*eutQ*$^+$, 14; *eutQ*/pBAD24, 1.

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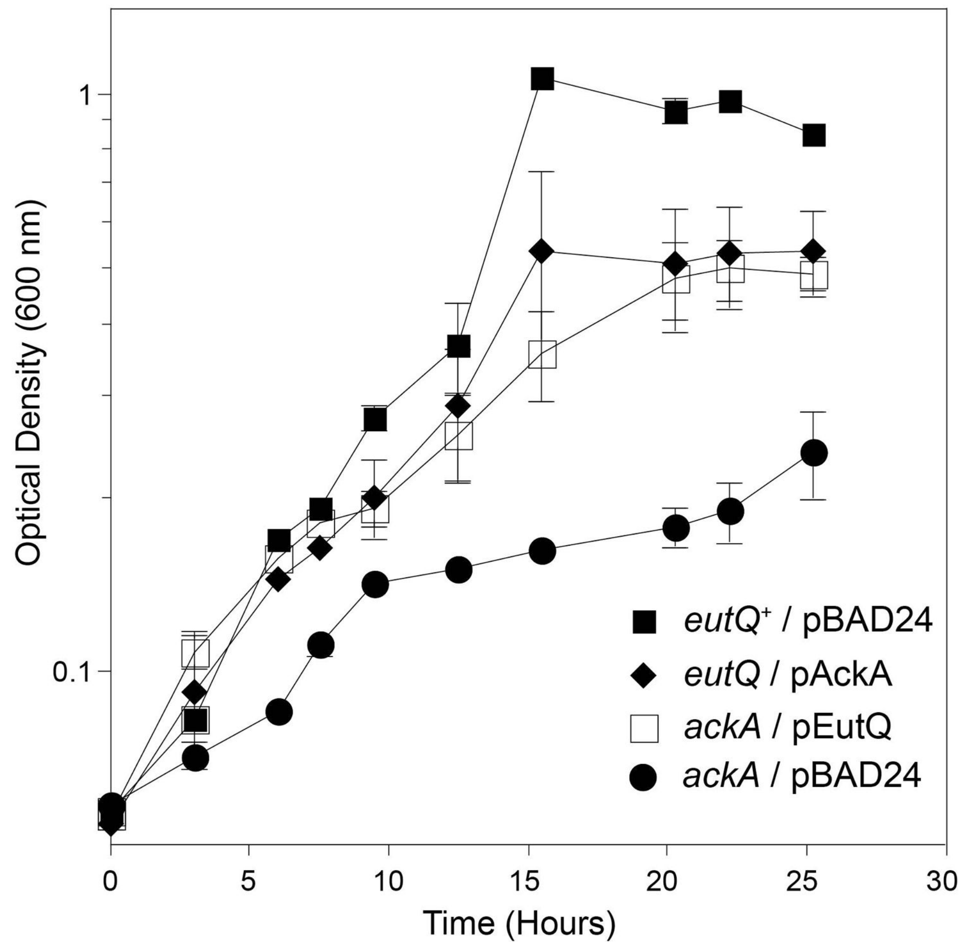

Figure 6. Complementation of the AckA phenotype on 30 mM ethanolamine by EutQ

Strains were grown anaerobically on minimal medium supplemented with ethanolamine (30 mM), tetrathionate (40 mM), and L(+)-arabinose (0.05 mM). Growth rates were calculated for each culture during the logarithmic phase of growth (mean OD% • h^{-1}): *eutQ*$^+$/pBAD24, 11; *eutQ*/p*ackA*$^+$, 11; *ackA*/pBAD24 6; *ackA*/p*eutQ*$^+$, 9.

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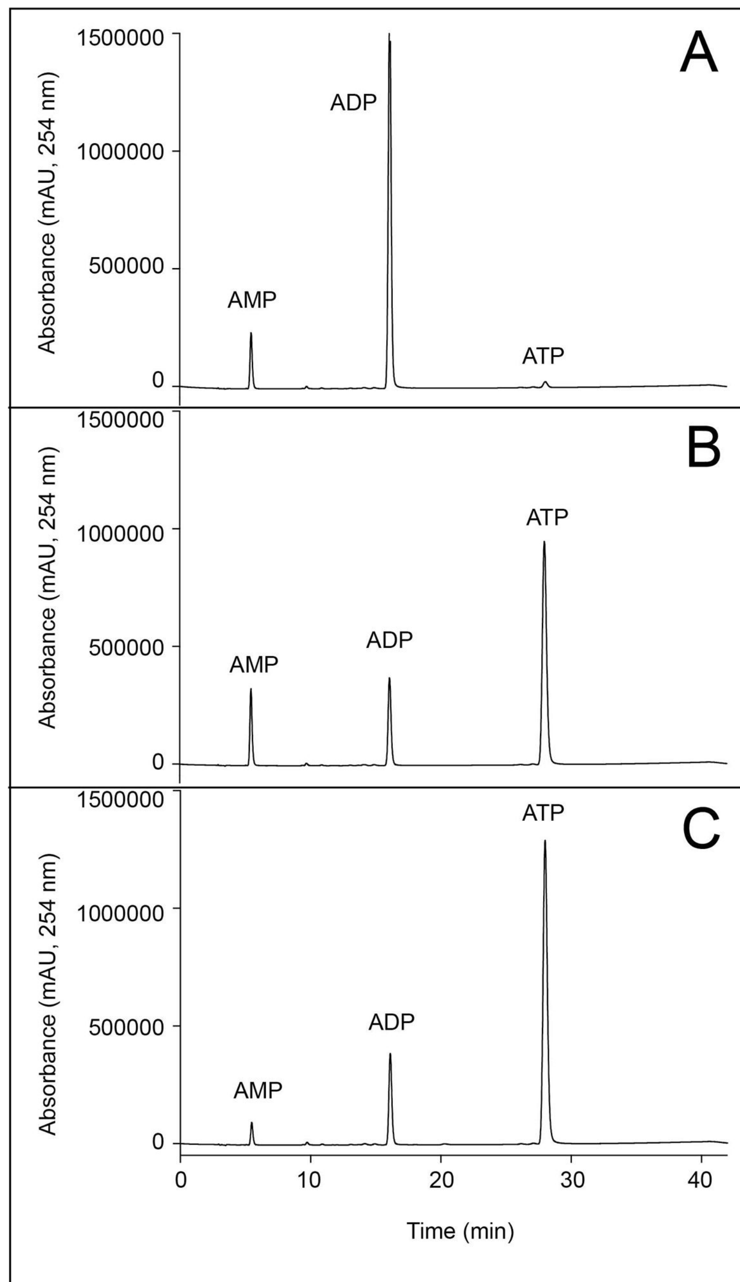

Figure 7. EutQ and EutP generate ATP *in vitro* via phosphorylation of ADP

All reaction mixtures contained AcP and ADP (1 mM each), and were incubated for 1 hour at 37°C. A. No-enzyme control; B. EutQ (0.7 μM). C) EutP (0.7 μM). Peaks were labeled in comparison to authentic standards. All reactions were monitored at 254 nm.

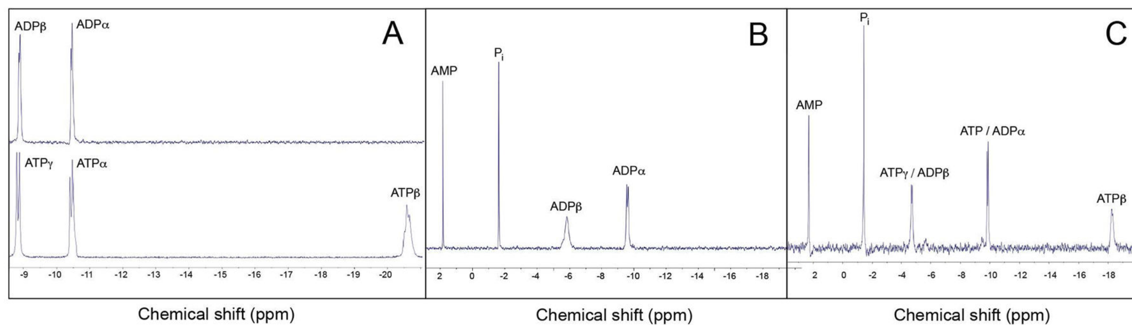

Figure 8. EutQ dependent synthesis of ATP from ADP and acetyl-phosphate monitored by ^{31}P-NMR spectroscopy

Reaction mixtures (1 ml final volume) containing EutQ enzyme (3.5 μg), ADP (5 mM), AcP (5 mM), and $MgCl_2$ (5 mM) were prepared for ^{31}P-NMR analysis as described under *Experimental procedures*. Panel A shows proton-decoupled ^{31}P-NMR spectra of commercially available ADP (top) and ADP (bottom), both at 10 mM. B. No-enzyme control; C. Products of the EutQ reaction. Note that the shifts of ATP and ADP are shifted in the positive direction because of complexation with the Mg^{2+} ions in the EutQ reaction, a shift that has been described in the literature (Bock, 1980, Szabo, 2008).

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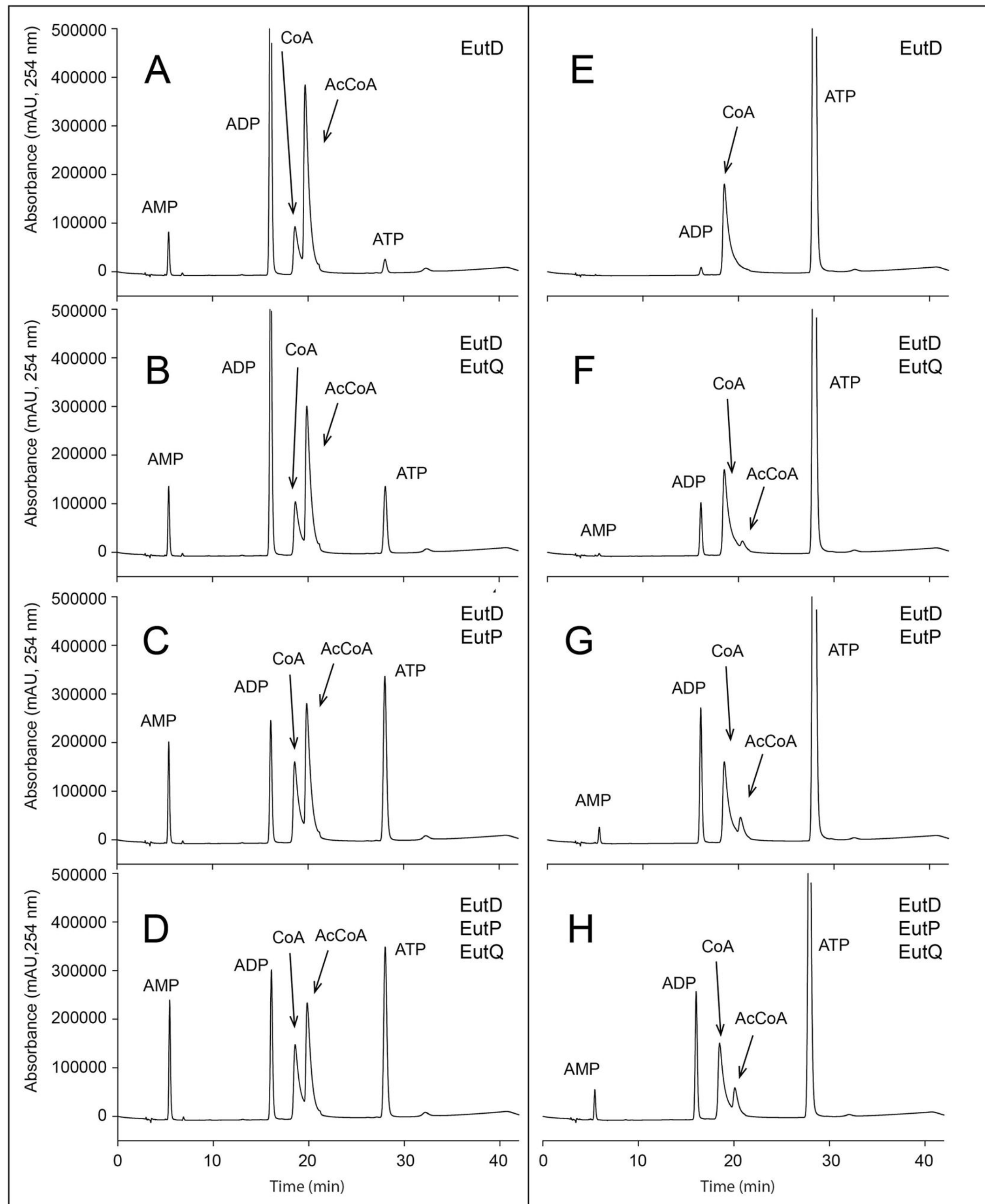

Figure 9. Use of HPLC to monitor the reversibility of the EutP- and EutQ-catalyzed reactions coupled to the EutD phosphotransacetylase

Reaction mixtures included enzymes shown in the upper right of each panel. Panels A–D. In these experiments all reaction mixtures contained P_i + ADP + AcCoA. Under these conditions *bona fide* acetate kinases synthesized ATP at the expense of AcP generated by EutD. Panels E–H. In these experiments EutQ and/or EutP converted acetate to acetyl-phosphate at the expense of ATP. EutD was added to the reaction mixture for the *in situ*

generation of AcCoA from AcP + CoA. All reactions were monitored at 254 nm, and all proteins used in these experiments were >95% homogeneous (Fig. S2).

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Table 1

Strains and plasmids used over the course of this study

Strains	Genotype	Cloning vector	Plasmid	Protein encoded	Source, reference
***E. coli* strains**					
JE3892	BL21 (λ*DE3*)				Lab collection
JE8833	XL10 Gold	pBAD30	pEUT56	$EutQ^{WT}$	Lab collection
JE19891 (DH5α)	*fhuA2 (argF-lacZ)U169 phoA glnV44 Φ80 (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	pBAD24	pACK7	$AckA^{WT}$	Lab collection
JE8066	DH5α		pADH3	$AdhE^{WT}$	Lab collection
JE8111	DH5α	pBAD30	pPDU18	$PduP^{WT}$	Lab collection
JE7994	DH5α		pEUT30	$EutE^{WT}$	Lab collection
***S.* Typhimurium strains**[1]					
JE6583	*metE205 ara-9*				Lab collection
Derivatives of strain JE6583					
JE6814	*prpC114*::MudJ[2]		pTDCD1	$TdcD^{WT}$	Lab collection
JE8749	*ack103*				Lab collection
JE8816	*eutQ1183*				Lab collection
JE21128	*ack103*	pBAD24	Control		This work
JE21130	*ack103*	pBAD30	pEUT56		This work
JE5304	*hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilv-452 rpsL120 xyl-404 galE719 H1-b H2-e, n,x) fla-66 nm*				Formerly JR501 (Ryu & Hartin, 1990)
Derivatives of JE5304					
JE20867			pEUT110	$EutP^{WT}$	This work
JE20869		pTEV18	pEUT112	$EutQ^{WT}$	This work
JE5912		pBAD30	pPDU1	$PduW^{WT}$	Lab collection
Derivatives of JE8816					
JE14190		pBAD24	Control	None	Lab collection
JE14116		pBAD24	pEUT56	$EutQ^{WT}$	This work
JE21131		pBAD24	pACK7	$AckA^{WT}$	This work
JE21167		pBAD30	pPDU1	$PduW^{WT}$	This work

Strains	Genotype	Cloning vector	Plasmid	Protein encoded	Source, reference
JE21168		pBAD18s	pTDCD1	$TdcD^{WT}$	This work
JE21381		pTAC-85	pADH3	$AdhE^{WT}$	Lab colection
JE21382		pBAD30	pPDU18	$PduP^{WT}$	Lab collection
JE21383		pBAD30	pEUT30	$EutE^{WT}$	Lab collection

[1] *S.* Typhimurium strains were derivatives of *S. enterica* sv Typhimurium strain LT2

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Table 2

Kinetic constants for EutQ and EutP

Enzyme	Substrate:	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
EutQ	Acetate	0.7 ± 0.2	318	454 ± 101
	ATP	0.5 ± 0.1	434	868 ± 145
EutP	Acetate	0.7 ±0.2	545	778 ± 173
	ATP	0.5 ± 0.2	657	1314 ± 377

[a]Data were obtained using the lactate dehydrogenase/pyruvate kinase coupled assay described under *Experimental procedures*. Assays were run in triplicate. Values (means ± standard deviations) were calculated using nonlinear regression in Prism4 (GraphPad Software, v. 4.0a, 2003)