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ORIGINAL PAPER

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Flux to acetate and lactate excretions in industrial fermentations: physiological and biochemical implications

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Abstract The efficiency of carbon conversion to biomass and desirable end products in industrial fermentations is diminished by the diversion of carbon to acetate and lactate excretions. In this study, the use of prototrophic and mutant strains of *Escherichia coli*, as well as enzyme active site directed inhibitors, revealed that flux to acetate excretion is physiologically advantageous to the organism as it facilitates a faster growth rate (μ) and permits growth to high cell densities. Moreover, the abolition of flux to acetate excretion was balanced by the excretion of lactate as well as 2-oxoglutarate, isocitrate and citrate, suggesting a 'bottle-neck' effect at the level of 2-oxoglutarate in the Krebs cycle. It is proposed that the acetate excreting enzymes, phosphotransacetylase and acetate kinase, constitute an anaplerotic loop or by-pass, the primary function of which is to replenish the Krebs cycle with reduced CoA, thus relieving the bottle-neck effect at the level of 2-oxoglutarate dehydrogenase. Furthermore, flux to lactate excretion plays a central role in regenerating proton gradient and maintaining the redox balance within the cell. The long-held view that flux to acetate and lactate excretions is merely a function of an 'over-flow' in central metabolism should, therefore, be re-evaluated.

Keywords Acetate excretion · Phosphotransacetylase · Acetate kinase · Fluoroacetate resistance · Coenzyme A

Introduction

The excretion of acetate as well as other partially oxidised intermediates, the so-called 'over-flow' metabolites, during both batch and continuous fermentations is well documented [4, 8, 10, 11, 13, 19, 20, 21]. Such a diversion in carbon flow diminishes the efficiency of carbon conversion to biomass and desirable end-product(s) per unit volume [8, 10, 18]. A number of different strategies including the use of chemostats [10, 14], fed-batch and two-stage cultivations [22], and the use of mutation [2, 4] as well as metabolic engineering strategies [1, 6, 18, 21] have all been employed to control flux to acetate excretion. Although the pathways involved in the synthesis and excretion of acetate and lactate are well documented (Fig. 1), the physiological significance of their synthesis and excretion remains uncertain. In addressing this issue, this paper provides new insights into the biochemical and physiological roles of lactate dehydrogenase (pyruvate reductase) and the acetate-excreting enzymes, and argues against the current notion that flux to acetate and lactate excretion is merely a reflection of metabolite 'over-flow' in central metabolism.

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Materials and methods

Bacterial strains

Escherichia coli ATCC 15224 (ML308) and its fluoroacetate-resistant derivatives were used in this study. Apart from being constitutive for β-galactosidase (I^-, z^+, y^+ a^+), *E. coli* ML308, the parent strain, was considered as wild-type. Fluoroacetate-resistant strains were selected for their ability to grow on pyruvate in the presence of fluoroacetate as previously described [10]. Such colonies were further tested for their ability to excrete acetate.

Growth conditions

Batch culture and continuous cultures were carried out on minimal media supplemented with appropriate

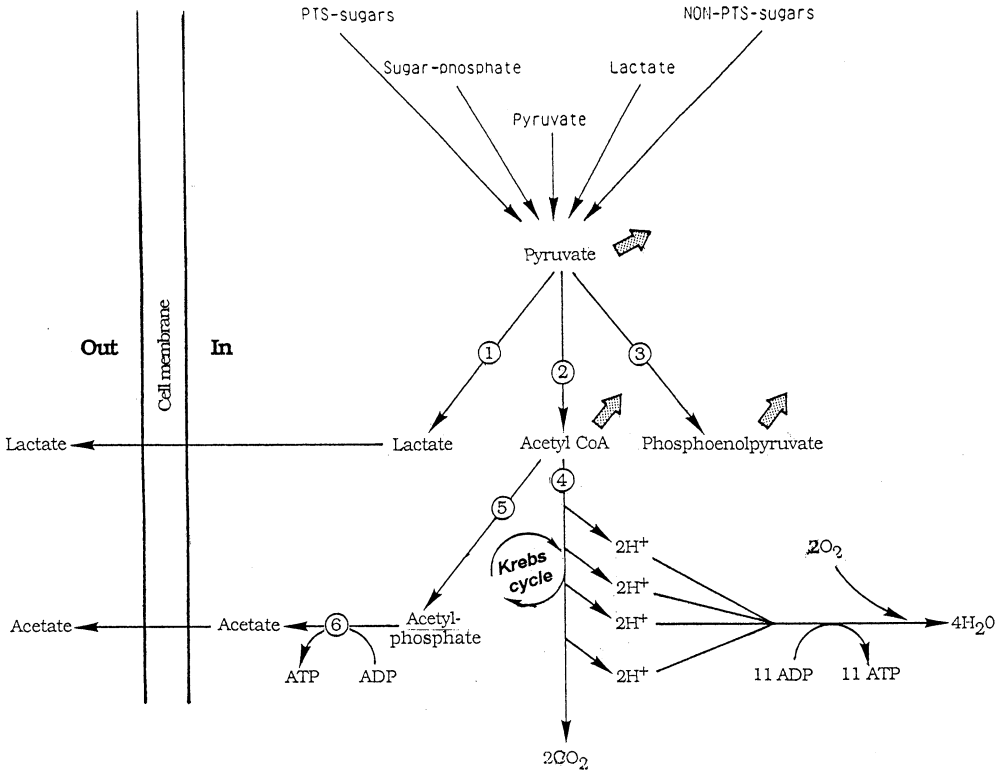

Fig. 1 Metabolic interrelationship among the amphibolic routes of central metabolism en route to acetate and lactate excretions in *Escherichia coli*. *Large shaded arrows* indicate flux to biosynthesis. Key enzymes: *1* lactate dehydrogenase (pyruvate reductase), *2* pyruvate dehydrogenase, *3* pyruvate kinase, *4* citrate synthase and the rest of the Krebs cycle enzymes, *5* phosphotransacetylase, *6* acetate kinase

carbon source (glucose, gluconate, fructose, acetate or pyruvate) as previously described [9, 10]. During growth in chemostats, the limiting factor was the carbon source as previously described [10].

Detection and measurements of acetate and primary carbon source

Detection and quantification of acetate, lactate and primary carbon source during the course of batch and continuous fermentations were achieved through HPLC and enzymatic analysis [10]. HPLC analysis of culture filtrates (22 μm filter; Millipore, Bedford, Mass.) was carried out at room temperature on an organic acid column (Amnix HPX-87H; Bio-Rad, Richmond, Calif.). Aliquots (100 μl) of culture filtrates were applied to the column and elution was carried out at a flow rate of 1 ml min^{-1} using 0.01 *N* H_2SO_4 as the mobile phase. Carbon source and excreted metabolites were monitored at 215 nm.

Measurements of enzymic activities

Acetate kinase and phosphotransacetylase activities were measured following the formation of acetylhydroxamate and NADH, respectively, as previously described [4, 9, 10].

Results

Acetate excretion as a function of growth rate

A relatively large number of substrates were tested for their ability to support flux to acetate excretion during growth of *E. coli* ML308 in batch and continuous cultures. With the sole exception of fructose [maximum growth rate(μ_{max}) = 0.60 h^{-1}] and 2-oxoglutarate (μ_{max} = 0.32 h^{-1}), all carbon sources supported flux to acetate excretion to varying degrees (Fig. 2). Among substrates tested, pyruvate sustained the highest flux to acetate excretion while glycerol (not shown) supported the least. The data shown in Fig. 2 also show that during growth in chemostats, flux to acetate excretion was demonstrated only at dilution rates ($D = \mu$) above 0.35 h^{-1}.

Effect of specific inhibitors on flux to acetate excretion during batch culture fermentation

A third-passage pyruvate-grown culture of *E. coli* ML308 was used to inoculate six flasks, each containing different concentrations (0.0, 10, 20, 30, 40 and 50 μM) of 3-bromopyruvate. The effect of various concentrations of bromopyruvate on growth rate (μ) and flux to acetate excretion was established (Table 1). The data clearly show that the higher the concentration of

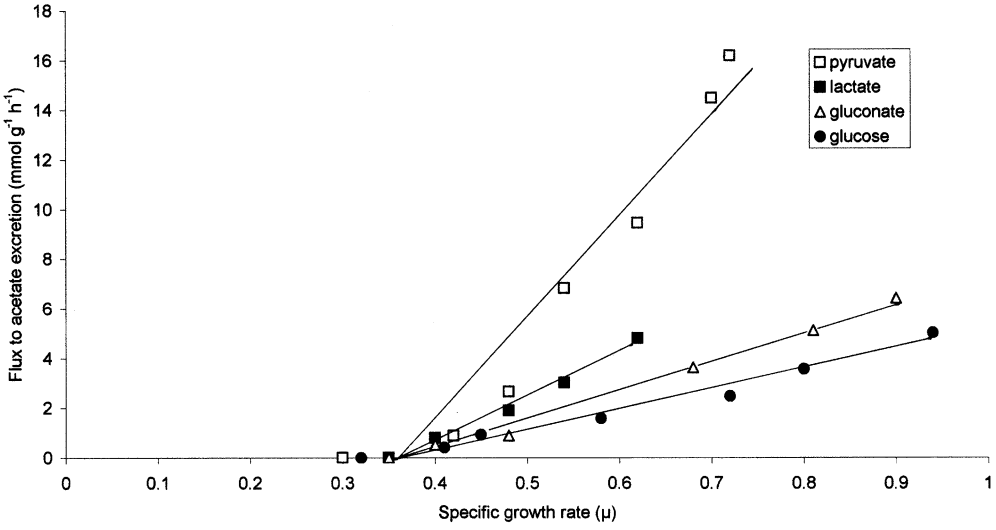

Fig. 2 Flux to acetate excretion as a function of dilution rate ($D = \mu$) during growth of *E. coli* in continuous cultures on pyruvate, lactate, gluconate and glucose. Growth conditions were as previously described [10]

bromopyruvate (an active-site-directed inhibitor of pyruvate dehydrogenase), the lower the growth rate and flux to acetate excretion. Interestingly, at a concentration of 50 μM, bromopyruvate abolishes flux to acetate excretion and diminishes μ to 0.35 h^{-1}, the same growth rate at which no flux to acetate excretion was observed during growth in chemostats (Fig. 2). It must be emphasised, however, that the drop in growth rate observed in the chemostats is of a totally different nature to that observed as a result of using active-site-directed inhibitors such as bromopyruvate.

Effect of mutations in phosphotransacetylase and acetate kinase on flux to acetate excretion

The activity of each of the above enzymes in the three fluoroacetate-resistant isolates, which were unable to excrete acetate, was established (Table 2). Interestingly, all three mutants exhibited a biphasic pattern of growth (Fig. 3). As the route to acetate excretion becomes unavailable, the fluoroacetate-resistant mutants divert excessive flux to lactate excretion as evidenced by the data shown for strain EM39 (Fig. 3). In this case, a flux to lactate excretion of 3.5 mmol (g dry weight)$^{-1}$ h^{-1} was observed, which is some 19-fold higher than that observed in the wild type. Furthermore, when the cultures were challenged with acetate (20 mM) at the end of growth, the cells were able to assimilate the added acetate and grow, albeit at a slower rate (Fig. 3). Lactate excretion during growth of the fluoroacetate-resistant mutants was detected following HPLC analysis on an organic acid column. The presence of lactate was further verified and quantified enzymatically using lactate dehydrogenase. In addition to diverting a large fraction of carbon source to lactate excretion, another consequence of abolishing flux to acetate excretion was a drop in growth rate (μ_{max}) from 0.72 h^{-1} for the wild type to 0.63 h^{-1} in the fluoroacetate-resistant strains.

Table 1 Effect of varying concentrations of 2-bromopyruvate on growth rate (μ) and flux to acetate excretion during growth of *Escherichia coli* ML308 on pyruvate in batch cultures at 37°C. Growth conditions were as previously described [10]. Data shown are the mean of four experiments. Data for growth rates are ±0.02, $n = 4$, while the data for acetate excretion are ±0.04, $n = 4$

Culture	2-Bromopyruvate concentration (μM)	μ (h^{-1})	Acetate excretion (mmol g^{-1} h^{-1})
1 Control	None	0.712	16.81
2	10	0.646	12.37
3	20	0.557	6.36
4	30	0.504	2.91
5	40	0.438	0.91
6	50	0.352	UD[a]

[a]Undetectable, i.e. less than 0.008 mmol g^{-1} h^{-1}

Discussion

Flux to acetate and lactate excretions are widely regarded by industrialists as wasteful because they diminish the efficiency of carbon conversion to desirable end products. With the sole exception of fructose, all

Table 2 Specific growth rate (μ) and specific activities of acetate kinase and phosphotransacetylase in pyruvate-grown cells of *E. coli* ML308 and its fluoroacetate-resistant mutants. Specific activities of acetate kinase and phosphotransacetylase were expressed as micromoles acetylhydroxamate and NADH, respectively, generated per milligram per minute at 30°C [4, 10]. Values given are the mean of three separate experiments and the range of results is expressed as a percentage of the mean

Bacterial strain	μ	Acetate kinase	Phospho transacetylase
E. coli ML308 (parental strain)	0.72 ± 0.09	4.1 ± 1.2%	2.8 ± 1.8%
E. coli ML308/EM26	0.63 ± 0.08	0.14 ± 1.8%[a]	0.09 ± 2.4%
E. coli ML308/EM39	0.63 ± 0.02	0.14 ± 1.1%[a]	0.05 ± 2.1%
E. coli ML308/EM40	0.61 ± 0.05	0.15 ± 1.5%[a]	0.05 ± 2.1%

[a]The very low acetate kinase activities recorded in the mutants are not due to acetate kinase itself but rather the presence of other ATPase activities within the cell extracts

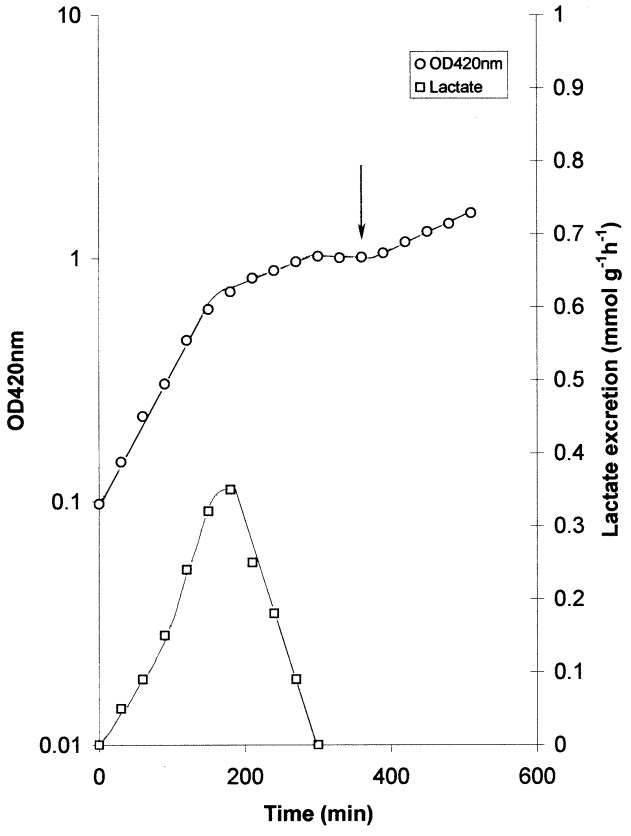

Fig. 3 Diauxic growth pattern and flux to lactate excretion, and it subsequent utilisation, during growth of the fluoroacetate-resistant strain, *E. coli* ML308/EM39 on pyruvate in batch culture. *Arrow* Point at which the culture was challenged with acetate (20 mM) at the end of growth. Growth conditions were as previously described [10]

carbon sources tested sustain a relatively high flux to acetate excretion (Fig. 2). As the carbon supply becomes growth-rate-limiting in chemostats as a result of decreasing the flow rate, flux to acetate excretion as well as growth rate diminished progressively. At a dilution rate ($D = \mu$) of 0.35 h^{-1} or less, no flux to acetate excretion was detected. Interestingly when μ was diminished through the use of specific inhibitors (Table 1), again no flux to acetate excretion was observed below 0.35 h^{-1}. This in turn suggests that a perfect balance between carbon input and output to biosynthesis and energy generation was achieved at this specific growth rate, i.e. 0.35 h^{-1}. Furthermore, one might be tempted to infer that acetate excretion is a function of μ rather than growth substrate, which is contrary to current understanding. Certainly, with the sole exception of fructose, all carbon sources that supported growth rates higher than 0.35 h^{-1}, supported flux to acetate excretion [13]. These results are in good agreement with earlier observations, which indicated that metabolic interventions leading to the abolition of flux to acetate excretion were accompanied by a decrease in growth rate [8, 10, 15]. The lack of flux to acetate excretion detected during growth on fructose was somewhat puzzling as fructose supports a relatively high growth rate ($\mu_{max} = 0.60$ h^{-1}). However, recent investigations revealed that the fructose repressor (FruR), which is expressed during growth on fructose, inhibits the synthesis of the acetate-excreting enzyme phosphotransacetylase, along with many other proteins involved in carbon and energy metabolism [3, 5, 16]. Such an effect is known as a pleiotropic effect, as phosphotransacetylase is not directly related to fructose metabolism. The inhibitory effect of FruR on the synthesis of phosphotransacetylase was further substantiated when no activity was detected for the enzyme in crude extracts of fructose-grown cultures of *E. coli* ML308. Unlike fructose-grown cultures, pyruvate-grown cultures showed ample phosphotransacetylase activity (Table 2).

The association between flux to acetate excretion on the one hand, and fast growth rate [4, 10] and high-cell-density growth [4] on the other, is now explicable in the light of the anaplerotic function proposed in this study for phosphotransacetylase (Fig. 4), which relieves the 'bottleneck' effect at the level of 2-oxoglutarate dehydrogenase.

In the light of the results obtained in this study, and the statement reiterating the Darwinian viewpoint that 'Nothing in biology makes sense except in the light of evolution' [7], one might be tempted to extrapolate by suggesting that the selective pressure that led to the evolution of the acetate-excreting enzymes was the apparent(desperate) need for higher intracellular levels of free CoA, a rate-controlling intermediate in central metabolism (E.M.T. El-Mansi, unpublished observation). The recent finding that increasing the intracellular concentration of free CoA through metabolic engineering was accompanied by higher yield of central metabolites [17] lends further support to the function proposed for phosphotransacetylase.

Assimilation of fluoroacetate gives rise to fluoroacetyl CoA, which is converted, by condensation with oxaloacetate, to fluorocitrate. Fluorocitrate is a potent inhibitor of the Krebs cycle enzyme aconitase (EC 4.2.1.3) and, as such, mutants that are resistant to fluoroacetate must be deficient in one or both of the enzymes involved in the conversion of fluoroacetate to fluoroacetyl CoA, namely acetate kinase and phosphotransacetylase. It is hardly surprising therefore, that such mutants are impaired in their ability to excrete acetate and display negligible enzymatic activities for the acetate-excreting enzymes (Table 2).

As the route to acetate excretion becomes unavailable in the fluoroacetate-resistant strains, the imbalance created at the junction of acetyl CoA appears to be addressed by diverting carbon to lactate excretion, which was fully consumed at the end of growth on the primary carbon source, thus displaying a diauxic pattern of growth (Fig. 3). In this case flux to lactate excretion appears to be advantageous to the cell as it regenerates transmembrane proton gradient and, in turn, ATP, as illustrated in Fig. 4. Furthermore, flux to lactate excretion in the fluoroacetate-resistant mutants, as well as in general terms in wild-type organisms, also plays a significant role in maintaining the redox balance within the

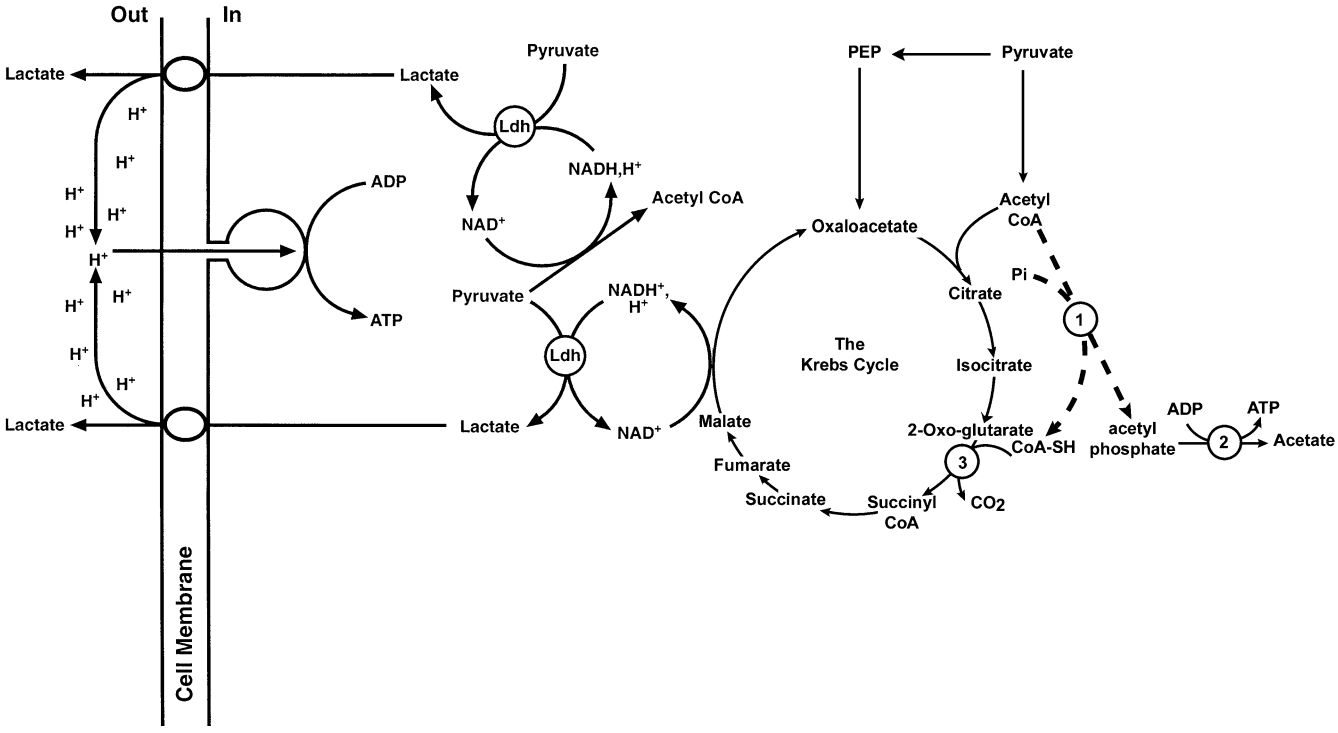

Fig. 4 A schematic representation highlighting the anaplerotic functions of phosphotransacetylase, replenishing the central metabolic pathways with free CoA (*dashed arrows*), and lactate dehydrogenase, generating proton gradient required for ATP generation and maintaining the redox balance, during growth of *E. coli* on pyruvate or substrates that converge on pyruvate. Key enzymes: *1* phosphotransacetylase, *2* acetate kinase, *3* 2-oxoglutarate dehydrogenase, *Ldh* lactate dehydrogenase

cell through the regeneration of NAD^+, the cofactor required for the activities of 2-oxoglutarate dehydrogenase, malate dehydrogenase and pyruvate dehydrogenase (Fig. 4).

Rather unexpectedly, pyruvate-grown cultures of fluoroacetate-resistant strains were able to metabolise acetate at the end of growth (Fig. 3), presumably via the activity of acetyl CoA synthetase (acetate: CoA ligase, AMP forming; EC 6.2.1.1), an irreversible enzyme that cannot be used for acetate excretion. The involvement of another, hitherto unknown, system for the uptake of acetate and its subsequent activation to acetyl CoA must not be ruled out.

It is noteworthy, however, that acetic acid in its undissociated form is capable of diffusing into the cell, thus reducing the intracellular pH [12] and this, in turn, may elicit the expression of several stress proteins including proteases, thus leading to a drop in yield of recombinant proteins (E.M.T. El-Mansi, unpublished observations).

Conclusions

This paper concludes that flux to acetate and lactate excretions are not merely a reflection of metabolite 'overflow' in central metabolism, but rather serve a specific metabolic function that facilitates faster growth rate and permits high-cell-density growth. While flux to lactate excretion serves to maintain the redox balance and regenerates proton gradient for ATP formation, flux to acetate excretion is physiologically advantageous, not only because the conversion of acetylphosphate to acetate is coupled to ADP phosphorylation but also, and more importantly, because it replenishes the central metabolic pathways with free CoA, thus relieving the 'bottleneck' effect at the level of 2-oxoglutarate dehydrogenase in the Krebs cycle. It is important, therefore, that we recognise the anaplerotic function of the acetate-excreting enzyme, phosphotransacetylase, in replenishing intermediary metabolism with free CoA, a function, hitherto unrecognised, for which phosphotransacetylase evolved.

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