Control of carbon flux through enzymes of central and intermediary metabolism during growth of *Escherichia coli* on acetate

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During aerobic growth of *Escherichia coli* on acetate, the component parts of the 'acetate switch' are turned-on as a consequence of direct competition, on the one hand, between phosphotransacetylase (PTA) and α-ketoglutarate dehydrogenase (α-KGDH) for their common co-factor free-CoA (HS-CoA) and, on the other hand, between isocitrate lyase (ICL) and isocitrate dehydrogenase (ICDH) for their common substrate isocitrate. Flux analysis revealed that competitions at both junctions in central metabolism are resolved in a precise way, so that the fraction of HS-CoA flux processed through PTA for biosynthesis relative to that processed through α-KGDH for energy generation, matches that observed for isocitrate flux through ICL relative to ICDH at the junction of isocitrate. Whereas the mechanism involved in the partition of carbon flux at the level of HS-CoA in central metabolism remains to be unravelled, the competition at the junction of isocitrate is resolved by the reversible phosphorylation/inactivation of ICDH and the operation of the glyoxylate bypass, the expression of which is subject to regulation at the transcriptional and translational levels as well as being dependent on growth rate.

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Introduction

The successful adaptation of *Escherichia coli*, and other organisms, to constant nutritional changes, which range from feast to famine, relies primarily on the organism's ability to 'switch off' carbon flux (see Glossary) to acetate excretion and to 'turn on' acetate utilisation. The realisation that acetyl phosphate, an energy rich substrate, might act as a global signal [1,2] in the regulation of cell division [3], pathogenicity [4], biofilm formation [5•] and the partition of free-CoA (HS-CoA) flux between phosphotransacetylase (PTA) and α-ketoglutarate dehydrogenase (α-KGDH) [6•] triggered renewed and vigorous interest in the 'acetate switch' in general [7••], and in the molecular mechanisms involved in the control and partition of carbon flux among various enzymes of central and intermediary metabolism in particular [8••,9••]. The latter aspect, the subject of this review, is complex, not only because of the presence of duplicate but differently regulated isoenzymes but also because of competition at metabolic junctions between catabolic and anabolic enzymes [10]. Such competition is resolved through operation of a wide range of regulatory control mechanisms, some of which are global, whereas others are specific [11,12•].

Metabolic inter-conversion of central and intermediary metabolism between acetogenesis and acetate utilization: the acetate switch

During growth of *E. coli* on acetogenic substrates, flux to acetate excretion is primarily achieved through the activities of PTA and acetate kinase (AK) (Figure 1). In the absence of these enzymes, the organism employs pyruvate oxidase (PO) for the excretion of acetate [12•,13,14•]. Unlike *E. coli*, the halophilic archaea do not employ the PTA–AK or PO pathways for the excretion of acetate; instead they use an ADP-forming acetyl CoA synthetase [15•,16•].

Recently, the acetate switch has been defined as the moment at which acetate excretion levels equal acetate assimilation levels [7••]. In this article, we define the acetate switch as the point at which the organism switches, or inter-converts, its enzymatic machinery from acetate excretion to acetate utilization. The point at which the acetate switch is triggered depends on growth conditions; for example, during growth of *E. coli* ML308 in batch culture on acetogenic substrates — those, such as glucose, that support flux to acetate excretion — the acetate switch is triggered following the onset of stationary phase, at which point the primary carbon source is fully consumed (Figure 2), oxygen uptake and carbon dioxide evolution cease and the intracellular level of acetyl CoA diminishes significantly. The acetate switch

Glossary

Metabolic flux: Metabolic flux through a given enzyme is usually expressed in terms of μmol mg^{-1} dry weight per unit time (Table 1) [30].

Carbon flux: Carbon flux can be calculated by multiplying the throughput of carbon through a given enzyme by the specific growth rate (μ) [30].

Isocitrate junction: During growth on acetate, the glyoxylate bypass enzyme, isocitrate lyase (ICL), must compete directly with the Krebs cycle enzyme (ICDH) for their common substrate thus creating a junction at the level of isocitrate in central metabolism [9••].

Moiety conserved cycle: This is a cycle that has no sinks for metabolites — that is, no output to biosynthesis [23]. For example, the Krebs cycle is not a moiety conserved cycle because it has at least two sinks at the level of α-ketoglutarate and oxaloacetate from which metabolites are withdrawn for biosynthesis. An example of a moiety conserved cycle, however, is the cycle in which ICDH is inter-converted from being catalytically active to catalytically inactive (phosphorylated), catalysed through the bifunctional regulatory enzyme ICDH kinase/phosphatase [9••].

Turnover of metabolites: The turnover of a given metabolite is a function of its intracellular concentration and the total flux of this metabolite [30]. For example in this paper we reported the turn over of isocitrate as a function of its concentration and the total (combined) flux of isocitrate through ICDH and ICL, thus working out how fast or how a slow the isocitrate pool is used up and, in turn, regenerated.

Ultrasensitivity: The theory of ultrasensitivity [24] unravelled a significant feature in cellular regulation. It states that the higher the concentration of the substrate (the enzyme subject to regulation) the more effective the regulation. For example, we see that in the acetate phenotype, the intracellular concentration of ICDH is nearly twice as much as that observed in the glycerol phenotype. This results in more effective inactivation of ICDH by phosphorylation, catalysed by ICDH kinase/phosphatase in the acetate phenotype [36].

Central and intermediary metabolism: Central metabolism is primarily composed of three different pathways, namely: glycolysis, the pentose phosphate pathway and the Krebs cycle. The primary function of central metabolism is to generate reducing power (NADH, NADPH and $FADH_2$), ATP and biosynthetic precursors. Intermediary metabolism on the other hand focuses on the biosynthetic routes that emerge from central metabolism (personal view of EMT El-Mansi).

is then turned on in order to facilitate the utilization of the excreted acetate through the expression of acetyl CoA synthetase (ACS) and PTA–AK (Figure 1) [17,18•]. In sharp contrast to the wild type, *E. coli* EM1017, the strain deficient in isocitrate dehydrogenase (ICDH), was unable to utilize the excreted acetate (Figure 2) [19,20•], suggesting that flux through ICDH is essential for the operation of the acetate switch. During growth in continuous cultures (chemostats), however, the acetate switch appears to be triggered when the growth rate (μ) diminishes to 0.35 h^{-1} or less, as evidenced by the detection of isocitrate lyase (ICL) activity — 50% of that observed in acetate phenotype (Figure 3) — as well as its corresponding mRNA transcripts in cells grown on the acetogenic substrate pyruvate (Figure 3) (J-N Phue, M El-Mansi and J Shiloach, unpublished).

Another central feature of the events leading to the activation of the acetate switch is the inactivation of a large fraction of ICDH following exhaustion of glucose and onset of stationary phase [21]. Once the excreted acetate is fully consumed by *E. coli*, ICDH is activated, and the acetate switch is turned off. Therefore, the phosphorylation and dephosphorylation of ICDH, which results in its inactivation and activation, respectively, appear to be equated, in turn, with the turning on and turning off of the acetate switch. This reversible inactivation of ICDH is catalysed by ICDH kinase/phosphatase, through reversible phosphorylation [22]; ICDH kinase/phosphatase is a bifunctional regulatory enzyme, the catalytic activities of which constitute a moiety-conserved cycle [23], require ATP and exhibit 'zero-order ultrasensitivity' [24].

In sharp contrast to *E. coli*, *Corynebacterium glutamicum* does not excrete acetate during growth on glucose or on other carbohydrates except under oxygen limitation [25,26••]. This suggests that the acetate switch does not operate in *C. glutamicum* under aerobic conditions; however, it remains to be established whether the acetate switch in *C. glutamicum* is subject to global anaerobic regulators such as FNR (fumarate and nitrate reduction).

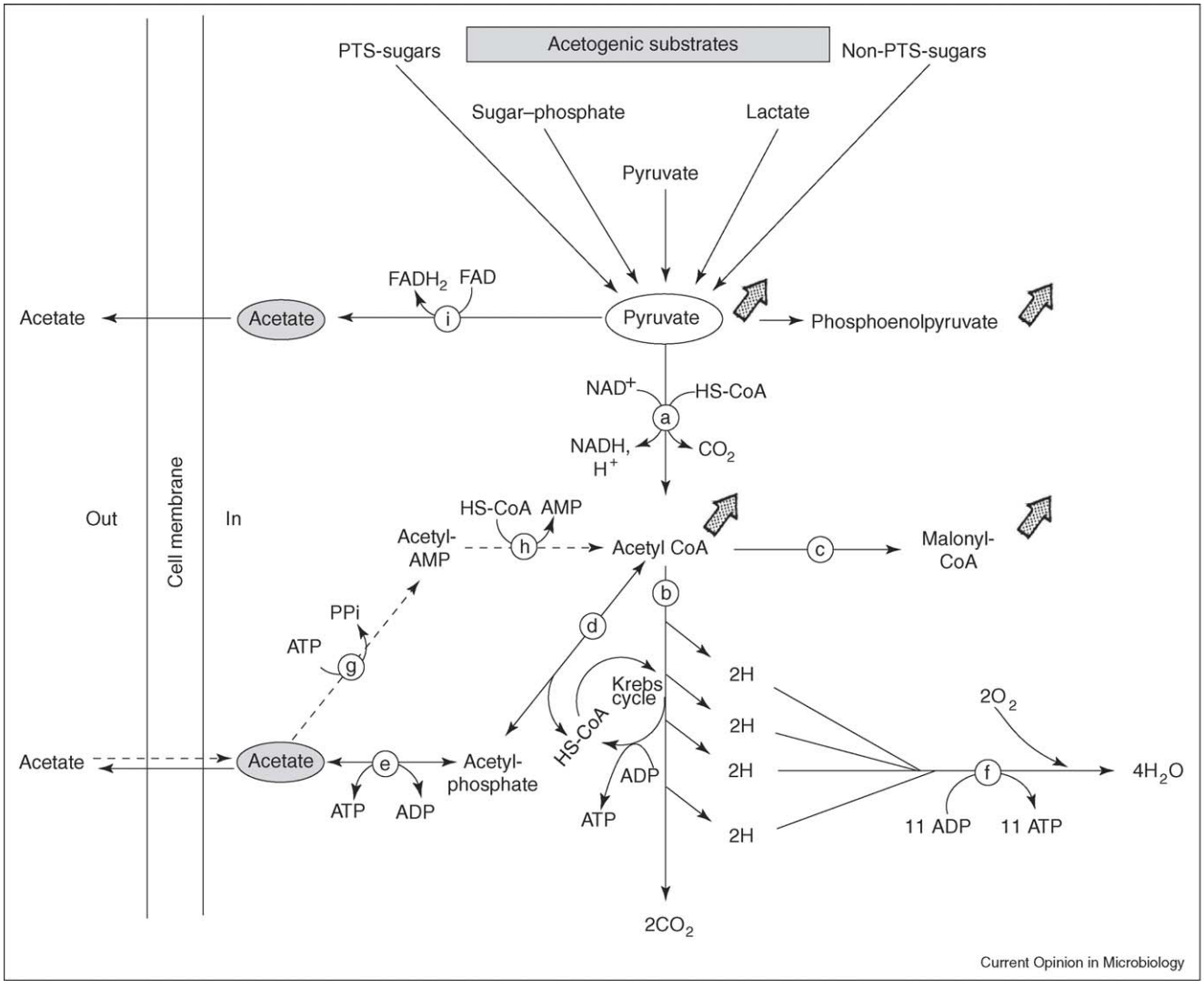
Acetate utilization

As the acetate switch is turned on, acetate is initially activated and converted to acetyl CoA either directly through the activity of ACS or in a two-step pathway involving PTA and AK (Figure 1). Subsequent oxidation of acetyl CoA through the Krebs cycle requires the operation of the anaplerotic sequence of the glyoxylate bypass [21,22], which consists of two enzymes, isocitrate lyase (ICL, EC 4.1.3.1) and malate synthase (MS, EC 2.3.3.9). Whereas ICL catalyses the aldole cleavage of isocitrate to succinate and glyoxylate — this bypasses the two steps in the Krebs cycle in which carbon is lost, in the form of carbon dioxide — MS catalyses the condensation of acetyl CoA and glyoxylate to give malate and HS-CoA, thus fulfilling its anaplerotic function [10,12•].

Partition of carbon flux at the junction of isocitrate

Although measurement of carbon flux is complex, it is made possible by the use of radioactive isotopes, GC-MS (gas chromatography-mass spectrophotometry) and 2D-NMR (two-dimensional nuclear magnetic resonance), as well as computer software technologies, such as Gepasi and MMT2 metabolic modelling programmes [27]. Analysis by Zhao *et al.* [28•] revealed that the switch from glucose metabolism to acetate metabolism was accompanied by a decrease in CO_2 evolution and an increase in the efficiency of carbon conversion to biomass. From measurements of the pool size of isocitrate (1.54 μmol mg^{-1} dry weight) [29] and the total flux through ICDH and ICL (14.43 μmol mg^{-1} h^{-1}; Figure 4), it has been shown that the pool of isocitrate 'turns over' 2.6 times per second (i.e. the whole pool is used and replenished) during growth on acetate, which is much slower than those reported during growth on acetogenic substrates (18.7 and 26.0 for glucose and glycerol

Figure 1



An overview of the central metabolic pathways of acetogenic metabolism in *E. coli*, highlighting the routes to acetate excretion and utilization as well as the central role of HS-CoA in the operation of the Krebs cycle and in energy generation. Dashed lines indicate the routes for acetate utilization, whereas solid lines indicate the routes for acetate excretion. Large grey arrows indicate flux to biosyntheses (intermediary metabolism). Enzymes are as follows: a, pyruvate dehydrogenase complex; **b**, citrate synthase; **c**, acetyl CoA carboxylase; **d**, PTA; **e**, AK; **f**, the electron transport chain; **g** and **h**, ACS (AMP-forming); **i**, PO.

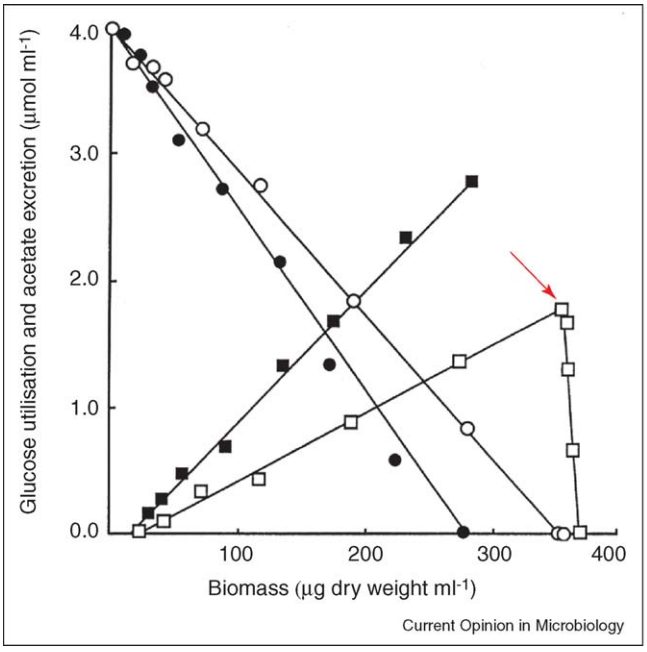
phenotypes, respectively [30]). Similarly, from measurements of the pool sizes of HS-CoA and acetyl CoA [31], and the total flux of each of these (Figure 4), the turn-over value for HS-CoA was found to vary, depending on the strain, ranging from 4.6–7.4 times per second, whereas the acetyl CoA pool turn-over value ranged from 8.5–9.5 times per second.

Interestingly, metabolic fluxes through central metabolism in *C. glutamicum* are higher than those in *E. coli* (Table 1) [32], but flux through PTA in relation to flux through α-KGDH (1.7), does not match that for ICDH in relation to ICL (3.17), thus reflecting the different regulatory-control mechanisms employed by both organisms for the control of metabolic flux [25,33•].

Impact of ICDH phosphorylation on the partition of carbon flux at the junction of isocitrate

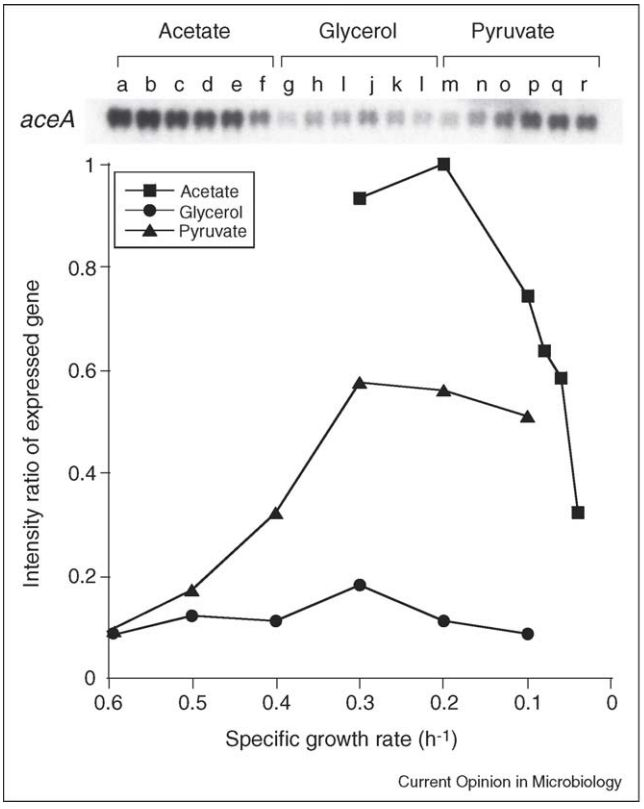
The catalytic activities of ICDH kinase/phosphatase constitute a moiety-conserved cycle [23] and facilitate the metabolic inter-conversion of ICDH between the catalytically active and inactive forms [22]. It is possible, therefore, that such inter-conversion coincides with the turning on and turning off of the acetate switch. Although phosphorylation/inactivation of ICDH has now been shown to be a prerequisite for growth of *E. coli* on

Figure 2



Patterns of glucose utilization (○,●) and acetate excretion (□, ■) during growth of *E. coli* ML308 (white shapes) and ICDH-deficient *E. coli* strain EM1017 (black shapes) in batch cultures at 37 °C. The arrow indicates the point at which the acetate switch is triggered in the wild type but not in the mutant following the exhaustion of glucose.

Figure 3



Transcriptional control of the expression of the glyoxylate bypass operon, as a function of growth rate (μ), during growth of *E. coli* ML308 in continuous culture on pyruvate (▲) and acetate (■) at 37 °C. The levels of mRNA transcripts (inset) were determined by Phue *et al.* [14•,20•] during growth in continuous culture on pyruvate, as previously described, and plotted as a function of growth rate.

acetate, the ability of some ICDH kinase/phosphatase-deficient mutants to grow on acetate was found to be because of low levels of ICDH activity in these mutants, which mimics the impact of phosphorylation [34]. Interestingly, *in silico* simulation studies revealed that successful partitioning of carbon flux at the junction of isocitrate, in the absence of ICDH phosphorylation/inactivation, can also be achieved by increasing the concentration of ICL above a certain threshold [35]. According to the theory of ultrasensitivity [24], the impact of *in vivo* phosphorylation on the inactivation of ICDH is enhanced, because the intracellular ICDH protein concentration in the acetate phenotype is nearly twice as high as that reported for *E. coli* growing on acetogenic substrates [36].

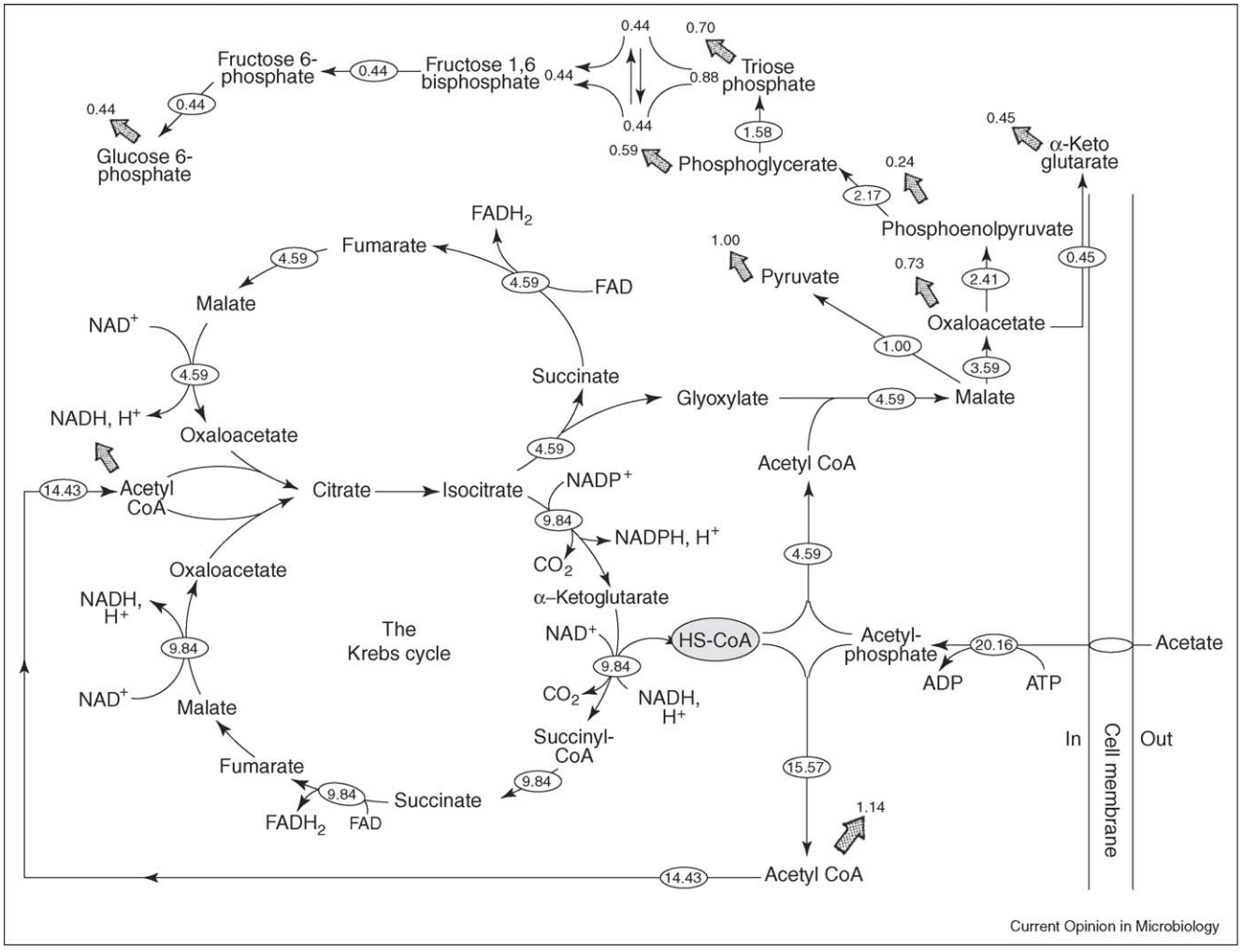
Control of expression of the glyoxylate bypass operon

The structural organization of the glyoxylate bypass operon *(aceBAK)* has been thoroughly investigated [22]. Significantly, unlike the intergenic region between *aceB* and *aceA*, the intergenic region between *aceA* and *aceK* is palindromic, which, in turn, leads to the formation of a stable stem-and-loop structure, thus eliciting a decrease in *aceK* transcription [37]. Inefficient translation and premature transcriptional-termination of mRNA might also contribute to the observed decrease in the expression of *aceK*. Differential expression among the enzymes of the glyoxylate bypass operon is physiologically advantageous because the concentration of each enzyme is sufficient to meet the metabolic demands of central and intermediary metabolism. Furthermore, unlike IclR and FadR repressors, FruR, a pleiotropic transcriptional regulator, stimulates the synthesis of the *aceBAK* operon [20,38].

The signal that triggers the acetate switch in preparation for adaptation to acetate

Although the nature of the primary signal that triggers the acetate switch has been studied intensively, it remains the subject of much speculation. *In vitro* studies revealed that the binding of IclR to the operator or promoter region of the *aceBAK* operon was not sensitive to either acetate or acetyl CoA [39] suggesting that acetate, per se, is not this signal. Similarly, *in vivo* studies revealed that phosphorylation/inactivation of ICDH was not directly related to acetate, but rather that it was dependent on the bacteria's need to maintain high intracellular levels of isocitrate and HS-CoA [36]. Unlike pyruvate and oxaloacetate, which have been ruled out as possible signals because neither were capable of influencing IclR binding,

Figure 4



The distribution and partition of carbon flux in *E. coli* ML308 among various enzymes of central and intermediary metabolism, during growth on acetate as the sole carbon and energy source in batch culture at 37 °C. The metabolic network and fluxes portrayed above; drawn to highlight the competition at metabolic junctions in general and for isocitrate and HS-CoA in particular, are based on the monomeric composition of *E. coli* [41], the data given by Holms [30] and the pathways portrayed for acetate metabolism [8••]. Values are given as μmol min^{-1} mg^{-1} dry weight. Large grey arrows indicate flux to biosyntheses (intermediary metabolism).

Table 1

Metabolic fluxes *in vivo*, through various enzymes of central metabolism in *E. coli* and of *C. glutamicum* during growth on acetate minimal medium

	Net flux (μmol min^{-1} mg^{-1} dry weight)	
Pathway or reaction	*E. coli*	*C. glutamicum*
AK-PTA	20.16	64.80
ICL-MS	4.59	11.88
ICD-α-KGDH	9.84	37.68
Succinate dehydrogenase	14.43	48.24
PEP carboxykinase	2.41	8.64
Phosphoglycerate kinase	1.58	1.92
Citrate synthase	14.43	49.56

Data from [30,32].

phosphoenolpyruvate (PEP) severely impaired the binding of IclR and, as such, might act as an inducer [39].

The role of HS-CoA and its thioester derivatives

The intracellular concentrations of HS-CoA and its thioester derivatives (acetyl CoA, succinyl CoA and malonyl CoA) are tightly regulated, with the acetyl CoA:HS-CoA ratio changing in response to growth conditions [31]. The metabolic inter-conversion between acetyl CoA and HS-CoA through PTA is, therefore, central to successful adaptation and to growth on acetate. A recently proposed hypothesis, on the one hand, implicates direct competition between PTA and α-KGDH for their common cofactor, HS-CoA, and, on the other hand, between ICDH and ICL for their common substrate, isocitrate, as being essential for successful adaptation to growth on

acetate [8••]. In this hypothesis, it is proposed that PTA out-competes α-KGDH for HS-CoA, by virtue of its high-affinity for HS-CoA or by another, hitherto unknown, mechanism, thus α-KGDH is a rate-controlling enzyme in the Krebs cycle. This, in turn, leads to the accumulation of α-ketoglutarate and isocitrate. Consequently, the acetate switch turns on the expression of the glyoxylate bypass operon, thus rendering a large fraction (75%) of ICDH inactive [40] and in turn, facilitating flux through the anaplerotic sequence of the glyoxylate bypass. The recent finding that loss of pyruvate oxidase PoxB was accompanied by a drop in growth rate and in efficacy of carbon conversion to biomass [12] lends further support to the above hypothesis [8••].

Conclusions and perspectives

In *E. coli*, the activation of acetate to acetyl CoA directly by ACS or indirectly by acetyl phosphate through the activities of AK and PTA means that α-KGDH is rate-controlling in the Krebs cycle, thus leading to accumulation of isocitrate and triggering of the acetate switch, which is equated with slow growth rate, a low turn over of the isocitrate pool and the phosphorylation/inactivation of ICDH. There are, however, indications that this mechanism is not ubiquitous among all microorganisms, for example, *C. glutamicum* and *Acinetobacter calcoaceticus* appear to have a different mechanism.

Flux analysis in *E. coli* revealed that the fraction of HS-CoA flux processed through PTA for biosynthesis relative to that processed through α-KGDH for energy generation, matches that observed for isocitrate flux through ICL relative to ICDH, thus implicating a global regulatory control mechanism for the partition of free-CoA among various enzymes of central and intermediary metabolism.

In future research it will be necessary to over come two major challenges: understanding the mechanism by which acetyl phosphate exerts influence through its ability to act as a phosphoryl donor, directly or indirectly, to various members of the two-component signal transduction pathways; together with addressing the question of whether PTA acts as a sensor and/or response regulator for the intracellular concentrations and concentration ratio of acetyl CoA:HS-CoA. This will significantly enhance our understanding of cellular metabolism, with respect to virulence and pathogenicity.

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