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In *Salmonella enterica*, the sirtuin-dependent protein acylation/deacylation system (SDPADS) maintains energy homeostasis during growth on low concentrations of acetate

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Abstract

Acetyl-coenzyme A synthetase (Acs) activates acetate into acetyl-coenzyme A (Ac-CoA) in most cells. In *Salmonella enterica*, *acs*⁺ expression and Acs activity are controlled. It is unclear why the sirtuin-dependent protein acylation/deacylation system (SDPADS) controls the activity of Acs. Here we show that, during growth on 10 mM acetate, *acs*⁺ induction in a *S. enterica* strain that cannot acetylate (*i.e.* inactivate) Acs leads to growth arrest, a condition that correlates with a drop in energy charge (0.17) in the acetylation-deficient strain, relative to the energy charge in the acetylation-proficient strain (0.71). Growth arrest was caused by elevated Acs activity, a conclusion supported by the isolation of a single-amino acid variant (Acs^{G266S}), whose overproduction did not arrest growth. Acs-dependent depletion of ATP, coupled with the rise in AMP levels prevented the synthesis of ADP needed to replenish the pool of ATP. Consistent with this idea, overproduction of ADP-forming Ac-CoA synthesizing systems did not affect the growth behavior of Pat-deficient or Pat-proficient strains. The Acs^{G266S} variant was >2 orders of magnitude less efficient than the Acs^{WT} enzyme, but still supported growth on 10 mM acetate. This work provides the first evidence that SDPADS function helps cells maintain energy homeostasis during growth on acetate.

INTRODUCTION

Acetyl coenzyme A (Ac-CoA) plays a central role in the carbon and energy metabolism of all cells. Ac-CoA is a critical metabolite that is generated via the degradation of larger molecules, or directly from acetate by AMP-forming Ac-CoA synthetases (Acs, aka acetate-CoA ligase, EC 6.2.1.1), by ADP-forming Acs (EC 6.2.1.13), or by the combined activities of acetate kinase (Ack, EC 2.7.2.1) and phosphate acetyltransferase (Pta, EC 2.3.1.8, aka phosphotransacetylase) (Starai & Escalante-Semerena, 2004a, Ingram-Smith *et al.*, 2006, Brown *et al.*, 1977) (Fig. S1).

Of relevance to the work reported here is the AMP-forming Acs enzyme. Acs is a member of the broadly distributed AMP-forming acyl-CoA synthetase family of enzymes (PF00501) that activates weak organic acids into their corresponding acyl-CoA derivatives via an acyl-AMP intermediate (Starai & Escalante-Semerena, 2004a).

The activation of weak organic acids by acyl-CoA synthetases is costly to cells, since it requires two moles of ATP per mole of substrate; one mol of ATP is consumed to activate the organic acid, while the second mol of ATP is needed to convert AMP to ADP, the

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immediate precursor of ATP. Further loss of energy resources during the course of the Acs reaction is caused by the hydrolysis of pyrophosphate to orthophosphate.

Reactions 1 through 5 (shown below) summarize the energetic cost to *S. enterica* during growth on low concentrations of acetate. Growth on 10 mM acetate depends on the activity of acetyl-CoA synthetase (Acs, EC 6.2.1.1). Reactions 1 and 2, are catalyzed by Acs, reaction 3 is catalyzed by pyrophosphate phosphohydrolase (Ppa, aka inorganic pyrophosphatase, EC 3.6.1.1), reaction 4 is catalyzed by adenylate kinase (EC 2.7.4.3), and reaction 5 is catalyzed by ATP :AMP transphosphorylase (Adk, aka adenylate kinase, EC 2.7.4.3).

In addition to its anabolic and catabolic roles, Ac-CoA is also used as a co-substrate by diverse acetyltransferases that modify proteins, tRNA, or small molecules in response to specific metabolic needs (Starai *et al.*, 2002, Garrity *et al.*, 2007, Ikeuchi *et al.*, 2008, Vetting *et al.*, 2005, Vetting *et al.*, 2004, Crosby *et al.*, 2010). At any given time, the intracellular CoA pool consists of a mixture of free CoA and, primarily, acetyl- malonyl- and succinyl-CoA thioesters (Bennett *et al.*, 2009), with the total amount and ratio of these compounds changing in response to the carbon source available, the growth phase of the culture, and environmental stresses (Vallari *et al.*, 1987, Chohnan *et al.*, 1997, Chohnan *et al.*, 1998).

Unlike CoA and its thioesters, adenine nucleotides are held at a constant ratio in growing cells, regardless of the carbon source (Chohnan *et al.*, 1998, Bennett *et al.*, 2009). Energy charge represents the availability of energy in terms of the ratios between the three adenine nucleotides in the cell, and is defined by the ratio: $[\text{ATP}] + 1/2[\text{ADP}] / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$ (Atkinson, 1968).

During exponential growth of *E. coli*, the energy charge is maintained between 0.8 and 0.9, with cells losing viability when the energy charge drops below 0.5 (Chapman *et al.*, 1971). The total adenine nucleotide pool size changes in response to the culture medium, but the energy charge remains constant (Chapman *et al.*, 1971).

The regulation of *acs*⁺ expression has been best studied in *E. coli*. In this bacterium, *acs*⁺ expression is controlled by a complex interplay of local and global regulatory proteins (CRP, FNR, IclR), sigma factors (σ^{70} , σ^S), and nucleoid-associated proteins (Fis, IHF), which together modulate *acs*⁺ transcription from two promoters (Beatty *et al.*, 2003, Browning *et al.*, 2004, Browning *et al.*, 2002, Kumari *et al.*, 2000a, Kumari *et al.*, 2000b, Shin *et al.*, 1997). In bacteria such as *Vibrio fischeri*, *acs*⁺ expression is further complicated by the involvement of the AinS quorum-sensing system (Studer *et al.*, 2008). Together,

these factors induce *acs*⁺ transcription in response to increased cAMP levels, decreased oxygen partial pressure, increased carbon flux through the glyoxylate bypass, and moderate cell density, as well as at the transition between exponential and stationary phase.

As mentioned above, the activity of Acs is also regulated at the posttranslational level by the sirtuin-dependent protein acylation/deacylation system (SDPADS) (Starai et al., 2002, Starai & Escalante-Semerena, 2004b). SDPADS control of Acs activity has been reported in bacteria (e.g. *S. enterica*), lower eukaryotes (e.g. *Saccharomyces cerevisiae*), and mammals, including humans (Starai et al., 2002, Starai et al., 2003, Hallows et al., 2006, Schwer et al., 2006).

The SDPADS of *S. enterica* consists of Pat, a Gcn5-like protein N-acetyltransferase (GNAT), and CobB, an NAD⁺-dependent, class-III protein deacetylase, a sirtuin (Starai et al., 2002, Starai et al., 2003, Starai & Escalante-Semerena, 2004b, Starai et al., 2004, Blander & Guarente, 2004, North & Verdin, 2004, Zhao et al., 2004). The Pat enzyme uses Ac-CoA to acetylate residue Lys609 of Acs (Starai & Escalante-Semerena, 2004b); the resulting Acs^{Ac} enzyme cannot catalyze the synthesis of Ac-AMP from acetate and ATP (reaction 1), but it can convert Ac-AMP to Ac-CoA (reaction 2) (Starai & Escalante-Semerena, 2004b). The NAD⁺-dependent CobB sirtuin deacetylates Acs^{Ac} to restore its activity (Starai et al., 2002). Thus the SDPADS is a feedback system that appears to control Acs activity in response to indicators of the carbon and energy charge of the cell (i.e., Ac-CoA and NAD⁺). Although the SDPADS is needed to control the activities of enzymes and transcription factors in bacteria (Starai et al., 2002, Garrity et al., 2007, Crosby et al., 2010, Thao et al., 2010, Wang et al., 2010), inactivation of the SDPADS is not lethal.

The layer of posttranslational regulation of Acs activity is intriguing, given that, as mentioned above, *acs*⁺ expression is tightly controlled. To further understand why cells need the SDPADS to modulate Acs activity, we uncoupled transcriptional and posttranslational controls of Acs by placing the wild-type *acs*⁺ allele under the control of an L-(+)-arabinose-inducible promoter. We analyzed the growth behavior of Pat-deficient and Pat-proficient strains as a function of *acs*⁺ expression level during growth on 10 mM acetate. When *acs*⁺ was expressed above chromosomal levels, Pat acetyltransferase activity was essential for growth. Without Pat-mediated inactivation of Acs, elevated Acs activity led to ATP depletion, a rise in AMP, and growth arrest.

RESULTS

Elevated Acs activity arrests growth on acetate in a *pat* strain that cannot acetylate Acs

Salmonella enterica requires Acs activity for growth on 10 mM acetate (Starai et al., 2003). A strain carrying a chromosomal deletion of *acs*⁺ (Δ *acs*) grew well on 10 mM acetate when the *acs*⁺ allele was provided in trans. In the experiments described below, the expression of *acs*⁺ from plasmid pACS7 was induced by addition of L-(+)-arabinose (Guzman et al., 1995). The level of *acs*⁺ induction was of no consequence when cells carried the *pat*⁺ allele (Fig. 1A; Table 1, line 1). In contrast, when *pat*⁺ was inactivated, growth on acetate occurred only when *acs*⁺ expression was low; moderate to high levels of *acs*⁺ induction completely arrested growth (Fig. 1B; Table 1, line 2). Growth arrest was observed within 2 h after induction of *acs*⁺ expression (Fig. 1B).

To confirm that the growth defect was caused by elevated Acs activity, we quantified Acs activity in cell-free extracts of *pat*⁺ and *pat* strains before and after L-(+)-arabinose induction. Before induction, Acs activity in the *pat* strain was 2–3 fold above the level of Acs activity in the *pat*⁺ strain before or after induction (Fig. 2). The specific activity of Acs, expressed in μ mol AMP/min/mg of protein, in strain JE9960 (Δ *acs pat* / *pacs*⁺) was $0.3 \pm$

0.06 when *acs*⁺ expression was induced, and 0.02 ± 0.01 when *acs*⁺ was not induced; a 15-fold increase in Acs specific activity. In strain JE9912 ($\Delta acs / pac$ ⁺), the specific activity of Acs was below the limit of detection of our assay, even in the presence of inducer. Western blotting analysis revealed equivalent levels of Acs in the *pat*⁺ and *pat* strains (Fig. 2), indicating that the differences in activity were not attributable to lower levels of Acs protein.

Pat acetyltransferase activity does not prevent growth arrest caused by the overproduction of an acetylation-resistant Acs variant

To confirm that the observed growth defect was caused by elevated Acs activity, we analyzed the growth behavior of strains overproducing the acetylation-resistant Acs^{L641P} variant protein (Starai *et al.*, 2005a). In the absence of inducer, residual synthesis of Acs^{L641P} in a *pat*⁺ strain supported poor growth; the same level of expression failed to support growth of the *pat* strain. These results suggested that the activity of Acs^{L641P} was lower than that of Acs^{WT}. This possibility was confirmed in vitro. The K_m of Acs^{L641P} for acetate was one order of magnitude higher than that of Acs^{WT}, while the k_{cat} was approximately six-fold slower, *i.e.* a 64-fold less efficient enzyme than Acs^{WT} (Table 2, lines 1, 2).

In contrast, synthesis of Acs^{L641P} protein in response to 25 μ M L-(+)-arabinose yielded nearly identical growth phenotypes in *pat*⁺ and *pat* strains (Table 1, lines 3, 4), suggesting that the level of activity of Acs^{L641P} synthesized under these conditions was not enough to compromise growth. However, a 10-fold increase in the concentration of inducer (250 μ M) resulted in a clear growth defect of *pat*⁺ and *pat* strain. These results were consistent with the inability of Pat to acetylate (thus inactivate) Acs^{L641P}.

Overproduction of an Acs variant with reduced activity does not lead to growth arrest

To further support the argument that elevated Acs activity caused growth arrest in the absence of Pat, we sought to isolate a variant of Acs with reduced enzymatic activity. For this purpose, we mutagenized plasmid pACS7 (*acs*⁺) in vivo using the mutator *E. coli* XL1-Red strain. Mutagenized plasmids were transformed into the Δacs *pat* strain, and plated on minimal medium + acetate (10 mM) + L-(+)-arabinose (250 μ M) to select for a plasmid encoding a variant of Acs that did not arrest growth of the *pat* strain. This approach yielded plasmid pACS21. DNA sequencing of the *acs* allele identified a mutation that resulted in a Gly-to-Ser substitution at position 266; hereafter we refer to the variant encoded by this allele as Acs^{G266S}. To confirm that the effect observed was caused by the single G266S substitution, the causative mutation was re-introduced into the *acs*⁺ allele (in plasmid pACS7) by site-directed mutagenesis. The reconstructed plasmid, named pACS23, was used to confirm the phenotype originally observed with plasmid pACS21. That is, when cells synthesized low levels of Acs^{G266S} protein (*i.e.*, no inducer in the medium), *pat*⁺ and *pat* strains grew poorly and slowly (Table 1, lines 5, 6). This result was consistent with insufficient levels of Acs activity needed for growth on 10 mM acetate. Notably, growth of the *pat* strain was not compromised when the concentration of inducer in the medium was high [250 μ M L-(+)-arabinose]; in fact, the doubling time of the *pat* strain was slightly shorter than that of the *pat*⁺ strain (Table 1, lines 5, 6).

Evidence that the Acs^{G266S} variant was less active than the Acs^{WT} enzyme was obtained in vitro. The kinetic parameters of the Acs^{G266S} (K_m for acetate, k_{cat} , and k_{cat}/K_m) were substantially different than those of the Acs^{WT} enzyme (Table 2, lines 1, 3). The most notable effect of the G266S substitution was on the K_m of the enzyme for acetate, which was 268-fold higher than that of the Acs^{WT} enzyme. The effect on the K_m for acetate was not the only change, however, the k_{cat} was 3-fold slower than Acs^{WT}. Under substrate saturation

conditions, the catalytic efficiency (k_{cat}/K_m) of the Acs^{G266S} enzyme was reduced approximately 790-fold, relative to the catalytic efficiency of the Acs^{WT} enzyme.

Control experiments were performed with the Acs^{K609A} variant enzyme, which lacks the site of acetylation modified by Pat, and cannot catalyze the synthesis of Ac-AMP (Starai & Escalante-Semerena, 2004b, Reger *et al.*, 2007). Consistent with previously reported data, Acs^{K609A} did not allow growth on acetate (10 mM) at any level of induction in either *pat*⁺ or *pat* strains (Table 1, lines 7, 8), and had no detectable in vitro activity.

Why is elevated Acs activity a problem?

To determine why elevated Acs activity inhibited cell growth, we focused on two parameters that can be directly affected by Acs activity, CoA homeostasis and energy charge. The rationale for looking at CoA homeostasis was that uncontrolled Acs activity could deplete the pool of free CoA at a rate that exceeded the rate of Ac-CoA consumption, or Ac-CoA hydrolysis, thus leaving the cell with insufficient free CoA levels. On the other hand, excessive Acs activity could unbalance the energy charge of the cell leading to a low-energy state that could not support growth.

CoA homeostasis is maintained

To investigate the possibility that elevated Acs activity disrupted CoA homeostasis, we determined the level of CoA derivatives over the course of 90 min after induction of *acs*⁺ at mid-exponential phase; these studies were performed in strains JE11864 (Δ *acs panD*) and JE11865 (Δ *acs pat*). The strategy was to inactivate the *panD*⁺ gene (encoding aspartate decarboxylase) to block the synthesis of β -alanine, so we could provide radiolabeled β -alanine and thus trace and quantify the different species of CoA in the cell (Jackowski & Rock, 1981). We measured no significant differences in the ratios or levels of CoA species in strains JE11864 and JE11865, with or without *acs*⁺ induction (Fig. 3, Fig. S5). The above results suggested that excess Ac-CoA generated by Acs was either consumed quickly by Ac-CoA utilization pathways, or hydrolyzed into acetate and CoA, effectively re-establishing a balance between free CoA and other CoA species. Regardless of the mechanisms, the data show that the cell maintained CoA homeostasis under conditions where Acs activity was elevated.

In a *pat* strain, elevated Acs levels cause a low-energy physiological state

Acetate metabolism and energy production during growth on 10 mM acetate rely on the synthesis of Ac-CoA by Acs. In the wild-type cell, the Acs enzyme consumes ATP, and releases AMP and pyrophosphate (reactions 1, 2). The synthesis of Ac-AMP (reaction 1) is unfavorable, but is driven by the hydrolysis of pyrophosphate into 2 moles of orthophosphate by pyrophosphate phosphohydrolase (reaction 3). AMP is converted to ADP via an ATP-consuming step (reaction 4). Thus the activation of acetate to Ac-CoA by Acs has a net cost of two ATP equivalents plus the lost energy released by the hydrolysis of pyrophosphate into orthophosphate.

We measured the levels of ATP, ADP and AMP over the course of 90 min after induction of *acs*⁺ in strains JE11864 (Δ *acs* / *acs*⁺) and JE11865 (Δ *acs pat* / *acs*⁺). Ninety minutes after the addition of inducer, the energy charge in strain JE11865 dropped to 0.17, in sharp contrast with strain JE11864, where the energy charge was 0.71 (Fig. 4, Fig. S3).

Although we detected AMP in vitro, and the procedures used to analyze the nucleotide content of the cell preserve labile nucleotides (Bochner & Ames, 1982), we decided to investigate whether AMP or Ac-AMP accumulated in the Pat-deficient strain overexpressing *acs*⁺. We addressed this question by analyzing the Acs reaction products in vitro, using

homogeneous Acs in a chemically defined reaction mixture; HPLC was used to resolve products from reagents. As shown in figure 5B, Ac-AMP was detectable only when CoA was absent and inorganic pyrophosphatase was present in the reaction mixture. In the presence of CoA, however, we did not detect Ac-AMP (Fig. 5D). Given that CoA and inorganic phosphatase are present in the cell, we conclude that AMP, not Ac-AMP must be what accumulates in a Pat-deficient strain overexpressing *acs*⁺.

Excessive ATP hydrolysis to AMP during growth on acetate leads to growth arrest

We took two approaches to determine whether the accumulation of AMP was the problem caused by excessive Acs activity. We contrived two ways to synthesize Ac-CoA from acetate using pathways that did not have Ac-AMP as an intermediate, nor yield AMP as a product. We reasoned that if growth arrest were avoided through the use of the alluded pathways, we could infer that Ac-CoA was not the cause of the phenotype. The first pathway we used was the acetate kinase/phosphotransacetylase (AckA-Pta) pathway (Brown et al., 1977). To do this, we placed *ackA*⁺ and allele *pta105* under the control of an L-(+)-arabinose-inducible promoter. Allele *pta105* encodes a Pta enzyme with increased catalytic efficiency that allows *acs* strains cells to grow on 10 mM acetate (Brinsmade & Escalante-Semerena, 2007). The second pathway we used was a codon-optimized *Giardia lamblia* *acs*⁺ gene (Fig. S7); *Gl* Acs is an ADP-forming Ac-CoA synthetase (Sánchez et al., 2000, Sánchez et al., 1999, Sánchez & Muller, 1996). Plasmids pACKA6 (*ackA*⁺ *pta105*) and plasmid pGLACS2 (*Gl acs*⁺) were brought individually into the Pat-deficient and Pat-proficient strains, yielding strains JE13780 (Δ *acs* / pACKA6), JE13786 (Δ *acs pat* / pACKA6), JE13920 (Δ *acs* / pGLACS2), and JE13921 (Δ *acs pat* / pGLACS2). As shown in figure 6, overexpression of these Ac-CoA synthesizing systems did not cause a growth defect in Pat-deficient strains, even when 1 mM L-(+)-arabinose was used to induce expression of the genes. Results of control experiments are shown in figure S6.

Increased CoA biosynthesis partially alleviates the negative effect caused by high Acs activity

We wanted to know whether increased synthesis of CoA would affect the growth arrest caused by increased levels of Acs activity. We increased the total CoA level in strain JE11865 (Δ *acs pat panD* / *pacs*⁺) by overproducing a feedback resistant variant of the pantothenate kinase (CoaA) enzyme (Rock et al., 2003). The resulting strain was named JE12386 (Δ *acs*⁺ *pat panD* / *pacs*⁺ *coaA2*); the expression of *acs*⁺ and *coaA2* was induced by L-(+)-arabinose. The feedback-resistant variant encoded by allele *coaA2* was CoaA^{R106A}, which is not under negative allosteric control by CoA (Rock et al., 2003). Increased levels of CoaA^{R106A} in the cell allowed strain JE12386 (Δ *acs pat panD* / *pacs*⁺ *coaA2*) to grow on acetate only in the presence of pantothenate when *acs*⁺ was overexpressed (Table 3, lines 6, 8). Concentrations 100 μ M L-(+)-arabinose resulted in growth arrest that the cell could not escape even when 1 mM pantothenate was added to the medium (data not shown).

Carbon sources, other than acetate, restore growth of a *pat* strain that overexpresses *acs*⁺

We studied the effect of carbon sources (*i.e.*, glycerol, glucose, pyruvate, citrate, and succinate) on the growth arrest that a *pat* strain that overexpresses *acs*⁺ undergoes in minimal medium + acetate. For this purpose, we inoculated strains JE11864 (Δ *acs pat panD* / *pacs*⁺) and JE11865 (Δ *acs pat panD* / *pacs*⁺) into medium containing 10 mM acetate + L-(+)-arabinose (500 μ M; to induce *acs*⁺ expression). After a 24-h incubation period, an alternative carbon source was added to the medium, and growth behavior was monitored as a function of time. As shown in figure 7 are the responses to glycerol and glucose, which restored growth of strain JE11865, but additional acetate did not. Pyruvate, succinate and citrate also relieved growth arrest (data not shown).

Isocitrate dehydrogenase (IcdA) activities are similar in the *pat*⁺ and *pat* strains that synthesize low or high levels of Acs

We investigated the possibility that the accumulation of AMP inhibited acetate metabolism. We focused on the branch point metabolite isocitrate, which can either proceed through isocitrate dehydrogenase (IcdA) and the TCA cycle to generate NADH and ATP, or through isocitrate lyase and the glyoxalate bypass to retain carbon for growth (Kornberg, 1966). This branch point is controlled by phosphorylation of IcdA, which is catalyzed by the IcdA kinase/phosphatase AceK (LaPorte, 1993).

E. coli and *S. enterica* growing solely on acetate, or other fatty acids that are degraded via β -oxidation to Ac-CoA, depend on the glyoxalate bypass to avoid the oxidation of Ac-CoA to CO₂. Notably, AMP is a positive allosteric effector of the phosphatase activity of AceK (Miller *et al.*, 2000, Nimmo & Nimmo, 1984, Zheng & Jia, 2010). Thus, the accumulation of AMP could result in active IcdA, which would divert isocitrate away from the glyoxalate bypass, with the concomitant limitation in carbon and reducing power needed for growth.

We tested this idea directly by measuring IcdA activity after induction of *acs*⁺ in *pat*⁺ and *pat* strains. We did not detect any significant difference in the specific activity of IcdA between the two strains. The specific activity of IcdA expressed in $\mu\text{mol}/\text{min}/\text{mg}$ of total cellular protein in strain JE9960 ($\Delta\text{acs pat} / \text{pacs}^+$) was 0.3 ± 0.01 when *acs*⁺ expression was induced, and 0.4 ± 0.01 when *acs*⁺ was not induced. In strain JE9912 ($\Delta\text{acs} / \text{pacs}^+$), the specific activity of IcdA was 0.3 ± 0.01 when *acs*⁺ expression induced, and 0.4 ± 0.01 when *acs*⁺ was not induced.

DISCUSSION

Evidence reported here supports the idea that the SDPADS plays a role in maintaining adenosine nucleotide homeostasis in *S. enterica*. Although our data were obtained under conditions that bypassed the contributions of the transcriptional control of *acs*⁺, we created a physiological condition where the posttranslational control of Acs activity was needed to avoid a low-energy state in the cell. Since in the wild-type cell *acs*⁺ expression is carefully controlled, it is plausible that the SDPADS is a backup system that controls Acs activity under conditions that de-regulate *acs*⁺ expression.

Recently published work performed with *S. enterica*, implicated the SDPADS in the control of many enzymes, ribosomal proteins, etc (Wang *et al.*, 2010). If this alleged broad involvement of the SDPADS in *S. enterica* cell function is correct, it is remarkable that strains lacking SDPADS activities are not dysfunctional. Clearly, our understanding of the role of the SDPADS in *S. enterica* physiology is limited, and needs to be investigated further. However, work reported here provide insights into a role of the SDPADS in energy homeostasis, which may be widespread in nature, given that Ac-CoA synthetases (Acs, AMP-forming) in other organisms are also under SDPADS control (Gardner *et al.*, 2006, Gardner & Escalante-Semerena, 2009, Crosby *et al.*, 2010, Hallows *et al.*, 2006, Schwer *et al.*, 2006). It is plausible that the SDPADS may also play a role in maintaining CoA homeostasis, however, data in support of this possibility are not available.

Why is Acs regulated at the posttranslational level?

It is clear that elevated, uncontrolled Acs activity has negative effects during growth on acetate, and that, whatever the problem is, it is avoided by the SDPADS. Data shown in figure 2 confirm that the problem is caused by elevated Acs activity, not by elevated Acs protein levels. The data indicate that Pat-dependent control of Acs is needed to prevent the depletion of ATP, a problem that is exacerbated by the accumulation of AMP (Fig. 4). The

combination of low ATP and high AMP dramatically decreases the energy charge of the cells, to a level (0.17), that is well below the threshold for viability (0.5) (Chapman et al., 1971). Since ATP is required for numerous metabolic reactions, it is not surprising that such a steep reduction in the ATP pool would arrest growth.

The effect of the lack of Pat on the cellular ATP pool was evident immediately after *acs*⁺ induction in a Pat-deficient strain, even though growth arrest was not observed until ~2 h post induction (Fig. 1). The efficiency of the posttranslational control of Acs was clear in a *pat*⁺ strain, in which Acs activity was held at a basal level during growth on 10 mM acetate, regardless of the level of *acs*⁺ induction (Fig. 2). In contrast, in the *pat* strain Acs activity was much higher (>30 fold) than the activity of the enzyme in a *pat*⁺ strain, a result that supports the conclusion that the SDPADS is important to control Acs activity.

Given the detrimental effects of uncontrolled Acs activity, it is remarkable that a less active form of Acs has not been selected for through evolution. Clearly these variants exist (e.g. Acs^{G266S}), and can support growth on acetate (Table 1). Most likely, however, the absence of less active Acs enzymes reflects a physiological need for high Acs activity, though the conditions under which such level of activity is required have not been identified. One possibility is that high Acs activity may be needed to respond to sudden environmental changes, when transcriptional regulation of *acs*⁺ expression would be too slow to produce sufficient Acs protein or other acetate activating enzymes to mount an appropriate response. Under such physiological conditions, the availability of the SDPADS would allow the cell to activate or inactivate Acs as needed.

A physiological condition where the concentration of ATP is low and the concentration of AMP is high, would make it difficult for the cell to catalyze reaction 4, *i.e.* it would not synthesize ADP, and if the ADP levels were too low for the ATP synthase to work efficiently, the ATP pool would be replenished at a slow rate or not at all. This appears to be the reason why uncontrolled Acs activity leads to growth arrest. In stark contrast, no growth defect was evident when ADP-forming Ac-CoA synthesizing pathways were overproduced in the Pat-deficient strain (Fig. 6, S6), suggesting that the problem created by an uncontrolled Acs enzyme was not caused by excessive synthesis of Ac-CoA, but to the physiological condition created by low ATP and high AMP levels.

The higher level of NAD⁺ observed in the Pat-deficient strain when *acs*⁺ was overexpressed (Fig. S2), suggests that, upon growth arrest, electron flow through the electron transport system increases, probably in an effort to augment the proton motive force needed to fuel ATP synthase.

Why would increased synthesis of CoA help the cell escape growth arrest?

The positive effect that increased synthesis of CoA has on growth arrest (Table 3) may be due to increased ATP synthase activity caused by a larger proton motive force generated by the higher flux of Ac-CoA through the glyoxylate shunt. This interesting observation needs to be further investigated.

Ways of escaping the low-energy state caused by too much Acs activity

The negative effect of high Acs activity in a Pat-deficient strain is not irreversible, and can be corrected by the addition of other carbon sources (except acetate) (Fig. 7). The immediate response to glucose and glycerol suggests that the net gain of ATP through substrate level phosphorylation reactions coupled to the lower branch of glycolysis is enough to jumpstart the cells. In the case of glucose, an additional mol of ATP would be generated when Ac-CoA is metabolized to acetate via the AckA-Pta pathway.

There was an approximately two-fold difference in the growth rate of the *pat* and *pat*⁺ strains that overexpressed *acs*⁺ in the presence of glucose and glycerol. The strain that could not control Acs (*pat*) grew faster than the one that could (*pat*⁺). To explain this observation we posit that the uncontrolled Acs activity may activate any free acetate generated during the catabolism of glucose or glycerol, maintaining higher Ac-CoA levels, whose metabolism via the glyoxylate shunt would generate a larger proton motive force that, in turn, would augment the pool of ATP in the cell. The fact that high levels of acetate (80 mM) did not restore growth of the *pat* strain overexpressing *acs*⁺ could be explained in terms of a failure to restore the energy charge. An important difference between the effect of glycerol or glucose and that of acetate, is that the catabolism of glycerol and glucose yields a net gain of ATP through the lower branch of glycolysis via substrate level phosphorylation (SLP) reactions. In contrast, acetate would not yield any ATP via SLP reactions, since ATP generation during growth on acetate relies on the activity of ATP synthase, whose activity would be compromised due to low levels of ADP.

How does the *pat* strain that overexpresses *acs*⁺ maintain CoA homeostasis?

This is a very intriguing question that was not addressed in this work. Plausible explanations would be increased levels of thioesterase activity or activities of Ac-CoA dependent biosynthetic enzymes. These possibilities need to be investigated.

EXPERIMENTAL PROCEDURES

Strain constructions

All strains used in this study were derivatives of *Salmonella enterica* sv Typhimurium strain LT2. Strain genotypes are described in Table 4. Chromosomal mutations were introduced by phage P22-mediated transduction (Chan *et al.*, 1972, Schmieger, 1971, Schmieger & Backhaus, 1973). Plasmids were introduced by electroporation (Inoue *et al.*, 1990).

Growth media and conditions

Cultures were grown at 37°C in no-carbon essential (NCE) minimal medium (Berkowitz *et al.*, 1968) supplemented with sodium acetate (10 mM), L-methionine (0.5 mM), MgSO₄ (1 mM), trace minerals (Atlas, 1995), and ampicillin (100 µg/ml). L-(+)-arabinose was added at indicated concentrations to induce expression of *acs*⁺ from the P_{araBAD} promoter. Strains harboring allele *panD623::Tn10d(tet*⁺*)* were supplemented with β-alanine (0.5 µM) during growth on minimal medium, unless otherwise stated.

Growth analysis

For experiments presented in table 1, table 3 and in figure 6, figure 7, strains were grown in triplicate in 96-well microtiter dishes; samples (5 µl) of overnight cultures grown in nutrient broth (NB) (Davis *et al.*, 1980) supplemented with ampicillin (100 µg/ml) was used to inoculate 195 µl of fresh minimal medium. Growth was analyzed using a computer-controlled BioTek ELx808-1 Ultra microplate reader (BioTek Instruments, Inc). Cell density measurements at 650 nm were acquired every 1800 s for 36 h; plates were shaken for 1745 s between readings. Data were analyzed using the GraphPad Prism v4 software package (GraphPad Software). L-(+)-Arabinose concentrations between 10 µM and 1.3 mM were used in this study. The growth defect of the Pat-deficient strain was induced by 50 µM arabinose, when growth behavior was analyzed using a microplate reader, and by 100 µM when cultures were grown in shake tubes and flasks. The need for higher concentration of inducer was probably due to the difference in aeration. Higher L-(+)-arabinose concentrations were used to ensure homogeneous expression of Acs (Morgan-Kiss *et al.*,

2002); higher L-(+)-arabinose concentrations did not have any effect on the growth behavior of Pat-proficient strains.

To test the effect of *acs*⁺ induction at different times (Fig. 1), strains JE9912 (Δacs / *pac*s⁺) and JE9960 (Δacs *pat* / *pac*s⁺) were grown overnight at 37°C in nutrient broth (NB) supplemented with ampicillin (100 µg/ml), then sub-cultured 1:100 into 5 ml of the minimal acetate medium described above. Cultures were grown in triplicate shaking at 37°C. L-(+)-Arabinose was added at designated times to a final concentration of 250 µM; growth was monitored using a Spectronic 20 spectrophotometer (Milton Roy).

Random mutagenesis of *acs*⁺

Plasmid pACS7 (*P_{araBAD}-acs⁺ bla⁺*) (Starai et al., 2005a) was transformed into the mutagenic *E. coli* strain XL1-Red (Stratagene), and the resulting strain was cultured as per manufacturer's instructions. Transformants were outgrown twice overnight in 5 ml lysogenic broth [LB, (Bertani, 1951, Bertani, 2004)] supplemented with ampicillin (100 µg/ml). Plasmid was isolated using a Wizard Plus SV Miniprep kit (Promega).

The pool of mutagenized pACS7 plasmid was electroporated into strain JE9894 (Δacs *pat*). Transformants were plated onto solid medium containing: sodium acetate (10 mM); L-methionine (0.5 mM); MgSO₄ (1 mM); trace minerals (Balch & Wolfe, 1976); ampicillin (100 µg/ml); and L-(+)-arabinose (250 µM). Isolates were re-streaked onto the same medium to confirm growth. The phenotype was confirmed in liquid cultures as described above. Plasmid pACS21 was obtained from an isolate showing robust growth under L-(+)-arabinose (250 µM) induction. The plasmid was sequenced at the DNA Sequence Laboratory of the University of Wisconsin-Madison Biotechnology Center, using BigDye™ protocols (ABI PRISM), and the sequence was compared to that of wild-type *acs* from *S. enterica* sv Typhimurium LT2 by BLAST (Altschul et al., 1990).

Site-directed mutagenesis of *acs*⁺

Plasmids pACS23 and pACS26—The QuikChange® II XL kit (Stratagene) was used, per manufacturer's protocols, to introduce the G266S mutation in plasmids pACS7 and pACS10. Mutagenic primers 5'-ATCCTTTATACCTCCAGCTCCACCGCAAGC-3' and 5'-GCTTGCCGGTGGAGCTGGAGGTATAAAGGAT-3' were designed using the QuikChange® Primer Design Program (www.stratagene.com/qcprimerdesign). DNA sequencing was performed as described above to confirm the mutation. The resulting plasmids were named pACS23 (encoding Acs^{G266S} in pBAD30) and pACS26 (encoding Acs^{G266S} in pTYB1), respectively. Plasmid pACS23 was used in complementation experiments, while plasmid pACS26 was used to purify Acs^{G266S} protein.

Plasmid pACS24—Site-directed mutagenesis was performed as described above using primers 5'-GCCAAAACCCGTTCCGGCGCAATTATGCGCCGCATTTTG-3' and 5'-CAAAATGCGGCGCATAATTGCGCCGGAACGGGTTTTTGGC-3' to introduce the K609A mutation into plasmid pACS7.

Plasmid pACS28—The *acs8* allele from plasmid pACS24 was amplified using primers 5'-GAGAACCAACCATATGAGCCAAACACAT-3' and 5'-AACCCCGCTCTTCCGCATGACGGCATCGCGATGGC-3' to add 5' *Nde*I and 3' *Sap*I restriction sites. The PCR product was digested with *Nde*I and *Sap*I and cloned into pTYB1 (New England Biolabs) digested with the same enzymes. The resulting plasmid pACS28 was used to overproduce Acs^{K609A} protein.

Construction of other plasmids

Plasmid pACS27—Plasmid pACS7 was digested with *KpnI* and *XbaI* to remove the *acs*⁺ gene, which was cloned into pBAD33 (Guzman et al., 1995) digested with the same enzymes.

Plasmid pCOAA8—The *coaA* gene from *S. enterica* TR6583, which encodes pantothenate kinase, was cloned into pBAD33's *KpnI* - *HindIII* sites using primers 5'-GTCAGGTACCGATGTAGCAATAGCAGCCAGA-3' and 5'-CACGAAGCTTTATTTCCGCAATCTTACCTGT-3' to produce plasmid pCOAA6. The feedback insensitive R106A *coaA2* allele (Rock et al., 2003) was generated using mutagenic primers 5'-GTAAAAGCACGACAGCAGCGGTTCTGCAAGCGTTGC-3' and 5'-GCAACGCTTGCGAGAACCGCTGCTGTCGTGCTTTTAC-3' designed similarly as plasmids pACS23 and pACS26 to produce plasmid pCOAA7. The *coaA2* allele was cloned into the *KpnI* - *HindIII* sites of pBAD18cm from pCOAA7 to produce pCOAA8.

Plasmid pACKA6—Plasmid pPTA11 encoding the *S. enterica pta*⁺ gene was subjected to quick-change mutagenesis with primers 5'-AACATTTCCGTGCAGACTCGTTGTTAGTGAC-3' and 5'-CGAGTCTGCACGGAAATGTTCCAGCAT-3' to construct the *pta105* allele in pPTA100. The *ackA*⁺ allele from *S. enterica* TR6583 was amplified with 5'-GCGAATTCGCCACGTATCATAAATAGGTACTTC-3' and 5'-GTCAGACCCGGGAGGCTGGCGGTGTGAAATCA-3' and cloned 5' of the *pta105* allele in pPTA100 yielding plasmid pACKA6.

Plasmid pGLACS2—Plasmid pGLACS1 encoding *Giardia lamblia* ATCC 50803 *acs*⁺ (locus tag GLP_457_48101_50281) was synthesized with *E. coli* codon optimization by GenScript. A ribosome binding site 5' of the gene and restriction sites *SacI* and *XbaI* were added to the synthetic gene to facilitate expression and cloning. *E. coli* codon-optimized *G. lamblia acs*⁺ was excised and cloned into the same site in plasmid pBAD33 to yield plasmid pGLACS2.

Chemical synthesis of Ac-AMP

The synthesis of Ac-AMP was performed as described without modifications (Berg, 1956).

Purification of Acs proteins

Wild-type and variant Acs proteins were overproduced and purified by chitin affinity chromatography as described (Starai et al., 2002). Protein concentrations were determined by the turbidity of the sample in 20% (w/v) trichloroacetic acid (Kunitz, 1952). Proteins were resolved by SDS-PAGE and stained with Coomassie Blue, and protein purity was determined by band densitometry using a Fotodyne imaging system with Foto/Analyst v. 5.00 software (Fotodyne Inc) for image acquisition and TotalLab v.2005 software for analysis (Nonlinear Dynamics).

Quantification of Ac-CoA synthetase activity

Purified enzymes—Acs activity was assayed by coupling AMP formation to NADH oxidation (Williamson & Corkey, 1969). In this assay, myokinase converts AMP and ATP into two molecules of ADP. Pyruvate kinase converts ADP and phosphoenolpyruvate into ATP and pyruvate. Pyruvate is reduced by the NADH-dependent lactate dehydrogenase; pyruvate reduction is monitored by the decrease in the absorbance at 340 nm as a function of time. Assay conditions were as described (Reger et al., 2007). ATP and CoA were kept at 2.5 mM and 1.5 mM, respectively. Enzyme activity was determined at acetate

concentrations of: 5 μ M–5 mM (for Acs^{WT}); 1–100 mM (for Acs^{G266S}); 100 μ M–25 mM (for Acs^{L641P}); and 100 μ M–100 mM (for Acs^{K609A}). Reactions (1 ml) were incubated 5 min at 37°C, then started by addition of 10 pmol Acs enzyme. NADH consumption was monitored at 340 nm for 15 min using a computer-controlled PerkinElmer LAMBDA 40 UV/VIS spectrophotometer (PerkinElmer, Inc.); data were analyzed using the UV KinLab software package (PerkinElmer, Inc.). Kinetic parameters were calculated using the Prism v. 4.0a software package (GraphPad Software, Inc.).

HPLC analysis of Ac-AMP—The Acs assay described above was incubated at 37°C for up to 1 h in the presence of 0.1U inorganic pyrophosphatase where indicated. Aliquots were taken at the specified time and stopped on ice with the addition of 1 mM HCl. Products were separated and measured by HPLC as described (Horswill & Escalante-Semerena, 2002) using solvent A [KH₂PO₄ (100mM) + tetrabutylammonium bromide (TBAB, 2mM)], and solvent B [KH₂PO₄ (100 mM) + TBAB (2mM) + acetonitrile (15% v/v)]. A standard curve was generated with authentic Ac-AMP ranging between 250 pmol and 10 nmol, with R² values of 0.99. Activity was reported in nmol of acetyl-AMP/min/mg purified Acs.

Cell-free extracts—Strains JE9912 (Δ acs / acs⁺) and JE9960 (Δ acs pat / acs⁺) were grown in minimal medium containing 10 mM acetate in flasks. When the density of the cultures reached OD₆₀₀ ~ 0.2, 100 ml of each culture were harvested and frozen. L-(+)-Arabinose was added to a final concentration of 1.3 mM to the remaining culture, which was incubated for an additional hour before 100 ml of each culture were harvested by centrifugation (Beckman/Coulter Avanti J25-I refrigerated centrifuge, equipped with a JLA-16.250 rotor; 15 min at 4°C; 6,000 $\times$ g), and frozen (–80°C) until used. The frozen pellets were suspended in HEPES buffer [(pH 7.5, 50 mM) + NaCl (100 mM) + lysozyme (1 mg/ml)], and disrupted by sonication (60 s, 28% duty, 2 s pulses, setting 4) with a 500 Sonic Dismembrator (Fisher Scientific). Extracts were dialyzed against the same buffer without lysozyme before Acs activity was assayed (as described above) in the absence or presence of acetate (0.2 mM). Activity determined in the absence of acetate from the reaction mixture served as control for background oxidation of NADH. Specific activity reflects the release of one AMP molecule per two molecules of NADH oxidized. Equal amounts and dilutions of total protein (determined by A₂₈₀) from each of the cell-free extracts were resolved by SDS-PAGE prior to the transfer of proteins onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore). Acs proteins were detected by Western blot analysis (Winston *et al.*, 1990) using primary antibody rabbit α -Acs (Harlan Laboratories, Madison, WI), and visualized with alkaline phosphatase conjugated goat anti-rabbit secondary antibody (Thermo Scientific).

Nucleotide labeling

Strains JE11864 (Δ acs panD / acs⁺) and JE11865 (Δ acs panD pat / acs⁺) were grown overnight in LB supplemented with ampicillin (100 μ g/ml), and sub-cultured into minimal MOPS medium (Neidhardt *et al.*, 1974) supplemented with K₂HPO₄ (0.2 mM), sodium acetate (10 mM), β -alanine (0.5 μ M), L-methionine (0.5 mM), ampicillin (100 μ g/ml) and [³²P]_i H₃PO₄ (30 mCi/ml, American Radiolabeled Chemicals). All experiments were done in triplicate. Cultures were inoculated using a 1:66 dilution of the inoculum.

Nucleotides were extracted and neutralized as described (Bochner & Ames, 1982). Samples were spotted on polyethyleneimine (PEI) Cellulose F thin layer chromatography (TLC) plates (EMD Chemicals Inc); TLC plates were developed as described (Bochner & Ames, 1982). Briefly, the first dimension used a mobile phase containing guanidine HCl (0.9 M, pH 6), and the second dimension used a mobile phase that contained (NH₄)₂SO₄ (1.2 M) + ethylenediamine tetraacetic acid (EDTA, 100 mM) adjusted to pH 3.5 with concentrated

(NH₄)HSO₄. All TLC plates were exposed to X-ray film (Kodak XAR-2) for 36 h and developed using standard procedures. Exposed autoradiograms were scanned into imaging software (Adobe Photoshop), and spots were identified by nucleotide standards separated with the same procedure and exposed to UV light. Spots corresponding to ATP, ADP and AMP were quantified by as described above. Percent adenine nucleotides were calculated based on the total digital light units (DLU) for each adenine nucleotide spot divided by the sum of the three species. Quantification of ATP, ADP and AMP were normalized to the number of maximum possible ³²P label incorporated into each molecule. Energy charge was calculated with the equation $[ATP] + \frac{1}{2}[ADP] / [ATP] + [ADP] + [AMP]$. See supplemental figures 2 and figure 3 for raw DLU/OD counts and TLC scans.

CoA labeling

Strains JE11864 ($\Delta acs\ panD / acs^+$) and JE11865 ($\Delta acs\ panD\ pat / acs^+$) were grown overnight in LB supplemented with ampicillin (100 µg/ml). All experiments were done in triplicate in minimal NCE medium supplemented with trace minerals, MgSO₄ (1 mM), NH₄Cl (30 mM), acetate (10 mM), β-alanine (0.5 µM), L-methionine (0.5 mM), β-[1-¹⁴C]-alanine (1 mM, 55 mCi/mmol American Radiolabeled Chemicals). Cultures were inoculated using a 1:66 dilution of the inoculum. Different CoA derivatives were extracted as described (Wadler & Cronan, 2007). Dried samples were suspended in a mixture of CoA-SH (50 mM), Ac-CoA, and malonyl-CoA (Ma-CoA) in NH₄AcO (50 mM, pH 5) + CH₃CN (1.2%, v/v) + *tris*(2-carboxyethyl)phosphine (TCEP, 5 mM). This mixture was separated with HPLC using a reverse phase C18 column (Altima HP C18-AQ 5m). The conditions used were a modification of described methodology (Iram & Cronan, 2006). The starting conditions were 98.8% buffer A (50 mM NH₄AcO, pH 5) and 1.2% buffer B (CH₃CN), which were applied for 4 min, followed by a 31-min linear gradient that increased buffer B to 10.4%. Peaks corresponding to Ma-CoA (retention time (RT) = 17–20 min), CoA-SH (RT = 21–24 min) and Ac-CoA (RT = 27–30 min) were collected in scintillation vials, the radioactivity content of which was determined using a Packard Tri-Carb 2100TR Liquid Scintillation Analyzer. See figure S4 for a representative HPLC chromatogram.

Isocitrate dehydrogenase activity assay

IcdA activity was measured one hour after *acs*⁺ induction in strains JE9912 ($\Delta acs / acs^+$), and JE9960 ($\Delta acs\ pat / acs^+$) according to LaPorte (LaPorte *et al.*, 1985).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- Altschul SF, Gish W, Miller W, Myers EW. Basic local alignment search tool. *J. Mol. Biol.* 1990; 215:403–410. [PubMed: 2231712]
- Atkinson DE. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry.* 1968; 7:4030–4034. [PubMed: 4972613]
- Atlas, R. *Handbook of Media for Environmental Microbiology.* Boca Raton: CRC Press; 1995. p. 6
- Balch WE, Wolfe RS. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl. Environ. Microbiol.* 1976; 32:781–791. [PubMed: 827241]

- Beatty CM, Browning DF, Busby SJ, Wolfe AJ. Cyclic AMP receptor protein-dependent activation of the *Escherichia coli* *acsP2* promoter by a synergistic class III mechanism. *J. Bacteriol.* 2003; 185:5148–5157. [PubMed: 12923087]
- Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD. Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat. Chem. Biol.* 2009; 5:593–599. [PubMed: 19561621]
- Berg P. Acyl adenylates: The synthesis and properties of adenylate acetate. *J. Biol. Chem.* 1956; 222:1015–1023. [PubMed: 13367068]
- Berkowitz D, Hushon JM, Whitfield HJ Jr, Roth J, Ames BN. Procedure for identifying nonsense mutations. *J. Bacteriol.* 1968; 96:215–220. [PubMed: 4874308]
- Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.* 1951; 62:293–300. [PubMed: 14888646]
- Bertani G. Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. *J. Bacteriol.* 2004; 186:595–600. [PubMed: 14729683]
- Blander G, Guarente L. The sir2 family of protein deacetylases. *Annu. Rev. Biochem.* 2004; 73:417–435. [PubMed: 15189148]
- Bochner BR, Ames BN. Complete analysis of cellular nucleotides by two-dimensional thin layer chromatography. *J. Biol. Chem.* 1982; 257:9759–9769. [PubMed: 6286632]
- Brinsmade SR, Escalante-Semerena JC. In vivo and in vitro analyses of single-amino acid variants of the *Salmonella enterica* phosphotransacetylase enzyme provide insights into the function of its N-terminal domain. *J. Biol. Chem.* 2007; 282:12629–12640. [PubMed: 17339319]
- Brown TD, Jones-Mortimer MC, Kornberg HL. The enzymic interconversion of acetate and acetyl-coenzyme A in *Escherichia coli*. *J. Gen. Microbiol.* 1977; 102:327–336. [PubMed: 21941]
- Browning DF, Beatty CM, Sanstad EA, Gunn KE, Busby SJ, Wolfe AJ. Modulation of CRP-dependent transcription at the *Escherichia coli* *acsP2* promoter by nucleoprotein complexes: anti-activation by the nucleoid proteins FIS and IHF. *Mol. Microbiol.* 2004; 51:241–254. [PubMed: 14651625]
- Browning DF, Beatty CM, Wolfe AJ, Cole JA, Busby SJ. Independent regulation of the divergent *Escherichia coli* *nrfA* and *acsP1* promoters by a nucleoprotein assembly at a shared regulatory region. *Mol. Microbiol.* 2002; 43:687–701. [PubMed: 11929525]
- Castilho BA, Olfson P, Casadaban MJ. Plasmid insertion mutagenesis and *lac* gene fusion with mini-mu bacteriophage transposons. *J. Bacteriol.* 1984; 158:488–495. [PubMed: 6327606]
- Chan RK, Botstein D, Watanabe T, Ogata Y. Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium*. II. Properties of a high-frequency-transducing lysate. *Virology.* 1972; 50:883–898. [PubMed: 4565618]
- Chapman AG, Fall L, Atkinson DE. Adenylate energy charge in *Escherichia coli* during growth and starvation. *J. Bacteriol.* 1971; 108:1072–1086. [PubMed: 4333317]
- Chohnan S, Furukawa H, Fujio T, Nishihara H, Takamura Y. Changes in the size and composition of intracellular pools of nonesterified coenzyme A and coenzyme A thioesters in aerobic and facultatively anaerobic bacteria. *Appl. Environ. Microbiol.* 1997; 63:553–560. [PubMed: 9023936]
- Chohnan S, Izawa H, Nishihara H, Takamura Y. Changes in size of intracellular pools of coenzyme A and its thioesters in *Escherichia coli* K-12 cells to various carbon sources and stresses. *Biosci. Biotechnol. Biochem.* 1998; 62:1122–1128. [PubMed: 9692193]
- Crosby HA, Heiniger EK, Harwood CS, Escalante-Semerena JC. Reversible N(epsilon)-lysine acetylation regulates the activity of acyl-CoA synthetases involved in anaerobic benzoate catabolism in *Rhodopseudomonas palustris*. *Mol. Microbiol.* 2010; 76:874–888. [PubMed: 20345662]
- Davis, RW.; Botstein, D.; Roth, JR. A manual for genetic engineering: advanced bacterial genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1980.
- Frey PA, Arabshahi A. Standard free energy change for the hydrolysis of the alpha, beta-phosphoanhydride bridge in ATP. *Biochemistry.* 1995; 34:11307–11310. [PubMed: 7547856]
- Gardner JG, Escalante-Semerena JC. *Bacillus subtilis*, the sirtuin protein deacetylase encoded by the *srtN* gene (formerly *yhdZ*), and functions encoded by the *acuABC* genes control the activity of acetyl-CoA synthetase. *J. Bacteriol.* 2009; 191:1749–1755. [PubMed: 19136592]

- Gardner JG, Grundy FJ, Henkin TM, Escalante-Semerena JC. Control of acetyl-coenzyme A synthetase (AcsA) activity by acetylation/deacetylation without NAD(+) involvement in *Bacillus subtilis*. *J. Bacteriol.* 2006; 188:5460–5468. [PubMed: 16855235]
- Garrity J, Gardner JG, Hawse W, Wolberger C, Escalante-Semerena JC. N-lysine propionylation controls the activity of propionyl-CoA synthetase. *J. Biol. Chem.* 2007; 282:30239–30245. [PubMed: 17684016]
- Guzman LM, Belin D, Carson MJ, Beckwith J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol.* 1995; 177:4121–4130. [PubMed: 7608087]
- Hallows WC, Lee S, Denu JM. Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. *Proc. Natl. Acad. Sci. U S A.* 2006; 103:10230–10235. [PubMed: 16790548]
- Horswill AR, Escalante-Semerena JC. Characterization of the propionyl-CoA synthetase (PrpE) enzyme of *Salmonella enterica*: Residue Lys592 is required for propionyl-AMP synthesis. *Biochemistry.* 2002; 41:2379–2387. [PubMed: 11841231]
- Ikeuchi Y, Kitahara K, Suzuki T. The RNA acetyltransferase driven by ATP hydrolysis synthesizes N4-acetylcytidine of tRNA anticodon. *Embo J.* 2008; 27:2194–2203. [PubMed: 18668122]
- Ingram-Smith C, Martin SR, Smith KS. Acetate kinase: not just a bacterial enzyme. *Trends Microbiol.* 2006; 14:249–253. [PubMed: 16678422]
- Inoue H, Nojima H, Okayama H. High efficiency transformation of *Escherichia coli* with plasmids. *Gene.* 1990; 96:23–28. [PubMed: 2265755]
- Iram SH, Cronan JE. The beta-oxidation systems of *Escherichia coli* and *Salmonella enterica* are not functionally equivalent. *J. Bacteriol.* 2006; 188:599–608. [PubMed: 16385050]
- Jackowski S, Rock CO. Regulation of coenzyme A biosynthesis. *J. Bacteriol.* 1981; 148:926–932. [PubMed: 6796563]
- Kornberg HL. The role and control of the glyoxylate cycle in *Escherichia coli*. *Biochem. J.* 1966; 99:1–11. [PubMed: 5337756]
- Kumari S, Beatty CM, Browning DF, Busby SJ, Simel EJ, Hovel-Miner G, Wolfe AJ. Regulation of acetyl coenzyme A synthetase in *Escherichia coli*. *J. Bacteriol.* 2000a; 182:4173–4179. [PubMed: 10894724]
- Kumari S, Simel EJ, Wolfe AJ. Sigma(70) is the principal sigma factor responsible for transcription of *acs*, which encodes acetyl coenzyme A synthetase in *Escherichia coli*. *J. Bacteriol.* 2000b; 182:551–554. [PubMed: 10629211]
- Kunitz M. Crystalline inorganic pyrophosphatase isolated from baker's yeast. *J. Gen. Physiol.* 1952; 35:423–450. [PubMed: 14898026]
- LaPorte DC. The isocitrate dehydrogenase phosphorylation cycle: regulation and enzymology. *J. Cell. Biochem.* 1993; 51:14–18. [PubMed: 8381789]
- LaPorte DC, Thorsness PE, Koshland DE Jr. Compensatory phosphorylation of isocitrate dehydrogenase. A mechanism for adaptation to the intracellular environment. *J. Biol. Chem.* 1985; 260:10563–10568. [PubMed: 3897222]
- Miller SP, Chen R, Karschnia EJ, Romfo C, Dean A, LaPorte DC. Locations of the regulatory sites for isocitrate dehydrogenase kinase/phosphatase. *J. Biol. Chem.* 2000; 275:833–839. [PubMed: 10625615]
- Morgan-Kiss RM, Wadler C, Cronan JE Jr. Long-term and homogeneous regulation of the *Escherichia coli araBAD* promoter by use of a lactose transporter of relaxed specificity. *Proc. Natl. Acad. Sci. U S A.* 2002; 99:7373–7377. [PubMed: 12032290]
- Neidhardt FC, Bloch PL, Smith DF. Culture medium for enterobacteria. *J. Bacteriol.* 1974; 119:736–747. [PubMed: 4604283]
- Nimmo GA, Nimmo HG. The regulatory properties of isocitrate dehydrogenase kinase and isocitrate dehydrogenase phosphatase from *Escherichia coli* ML308 and the roles of these activities in the control of isocitrate dehydrogenase. *Eur. J. Biochem.* 1984; 141:409–414. [PubMed: 6329757]
- North BJ, Verdin E. Sirtuins: Sir2-related NAD-dependent protein deacetylases. *Genome Biol.* 2004; 5:224. [PubMed: 15128440]

- Reger AS, Carney JM, Gulick AM. Biochemical and crystallographic analysis of substrate binding and conformational changes in acetyl-CoA synthetase. *Biochemistry*. 2007; 46:6536–6546. [PubMed: 17497934]
- Rock CO, Park HW, Jackowski S. Role of feedback regulation of pantothenate kinase (CoaA) in control of coenzyme A levels in *Escherichia coli*. *J. Bacteriol.* 2003; 185:3410–3415. [PubMed: 12754240]
- Sánchez LB, Galperin MY, Muller M. Acetyl-CoA synthetase from the amitochondriate eukaryote *Giardia lamblia* belongs to the newly recognized superfamily of acyl-CoA synthetases (Nucleoside diphosphate-forming). *J. Biol. Chem.* 2000; 275:5794–5803. [PubMed: 10681568]
- Sánchez LB, Morrison HG, Sogin ML, Muller M. Cloning and sequencing of an acetyl-CoA synthetase (ADP-forming) gene from the amitochondriate protist, *Giardia lamblia*. *Gene*. 1999; 233:225–231. [PubMed: 10375639]
- Sánchez LB, Muller M. Purification and characterization of the acetate forming enzyme, acetyl-CoA synthetase (ADP-forming) from the amitochondriate protist, *Giardia lamblia*. *FEBS Lett.* 1996; 378:240–244. [PubMed: 8557109]
- Sasse, J. Detection of proteins. In: *Current Protocols in Molecular Biology*. Ausubel, FA.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K., editors. New York: Wiley Interscience; 1991. 10.16.11–10.16.18
- Schmieger H. The fate of the bacterial chromosome in P22-infected cells of *Salmonella typhimurium*. *Mol. Gen. Genet.* 1971; 110:238–244. [PubMed: 4929818]
- Schmieger H, Backhaus H. The origin of DNA in transducing particles in P22-mutants with increased transduction-frequencies (HT-mutants). *Mol. Gen. Genet.* 1973; 120:181–190. [PubMed: 4568531]
- Schwer B, Bunkenborg J, Verdin RO, Andersen JS, Verdin E. Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2. *Proc. Natl. Acad. Sci. U S A.* 2006; 103:10224–10229. [PubMed: 16788062]
- Shin S, Song SG, Lee DS, Pan JG, Park C. Involvement of *iclR* and *rpoS* in the induction of *acs*, the gene for acetyl coenzyme A synthetase of *Escherichia coli* K-12. *FEMS Microbiol. Lett.* 1997; 146:103–108. [PubMed: 8997713]
- Starai VJ, Celic I, Cole RN, Boeke JD, Escalante-Semerena JC. Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science*. 2002; 298:2390–2392. [PubMed: 12493915]
- Starai VJ, Escalante-Semerena JC. Acetyl-coenzyme A synthetase (AMP forming). *Cell. Mol. Life Sci.* 2004a; 61:2020–2030. [PubMed: 15316652]
- Starai VJ, Escalante-Semerena JC. Identification of the protein acetyltransferase (Pat) enzyme that acetylates acetyl-CoA synthetase in *Salmonella enterica*. *J. Mol. Biol.* 2004b; 340:1005–1012. [PubMed: 15236963]
- Starai VJ, Gardner JG, Escalante-Semerena JC. Residue Leu-641 of acetyl-CoA synthetase is critical for the acetylation of residue Lys-609 by the Protein acetyltransferase enzyme of *Salmonella enterica*. *J. Biol. Chem.* 2005a; 280:26200–26205. [PubMed: 15899897]
- Starai VJ, Garrity J, Escalante-Semerena JC. Acetate excretion during growth of *Salmonella enterica* on ethanolamine requires phosphotransacetylase (EutD) activity, and acetate recapture requires acetyl-CoA synthetase (Acs) and phosphotransacetylase (Pta) activities. *Microbiology*. 2005b; 151:3793–3801. [PubMed: 16272400]
- Starai VJ, Takahashi H, Boeke JD, Escalante-Semerena JC. Short-chain fatty acid activation by acyl-coenzyme A synthetases requires SIR2 protein function in *Salmonella enterica* and *Saccharomyces cerevisiae*. *Genetics*. 2003; 163:545–555. [PubMed: 12618394]
- Starai VJ, Takahashi H, Boeke JD, Escalante-Semerena JC. A link between transcription and intermediary metabolism: a role for Sir2 in the control of acetyl-coenzyme A synthetase. *Curr. Opin. Microbiol.* 2004; 7:115–119. [PubMed: 15063846]
- Studer SV, Mandel MJ, Ruby EG. AinS quorum sensing regulates the *Vibrio fischeri* acetate switch. *J. Bacteriol.* 2008; 190:5915–5923. [PubMed: 18487321]
- Thao S, Chen S-S, Zhu H, Escalante-Semerena JC. Nε-Lysine acetylation of a bacterial transcription factor inhibits its DNA-binding activity. *PLoS One*. 2010 In Press.

- Thauer RK, Jungermann K, Decker K. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* 1977; 41:100–180. [PubMed: 860983]
- Vallari DS, Jackowski S, Rock CO. Regulation of pantothenate kinase by coenzyme A and its thioesters. *J. Biol. Chem.* 1987; 262:2468–2471. [PubMed: 3029083]
- Vetting MW, de Carvalho LP, Roderick SL, Blanchard JS. A novel dimeric structure of the RimL Nalpha-acetyltransferase from *Salmonella typhimurium*. *J. Biol. Chem.* 2005; 280:22108–22114. [PubMed: 15817456]
- Vetting MW, Magnet S, Nieves E, Roderick SL, Blanchard JS. A bacterial acetyltransferase capable of regioselective *N*-acetylation of antibiotics and histones. *Chem. Biol.* 2004; 11:565–573. [PubMed: 15123251]
- Wadler C, Cronan JE. Dephospho-CoA kinase provides a rapid and sensitive radiochemical assay for coenzyme A and its thioesters. *Anal. Biochem.* 2007; 368:17–23. [PubMed: 17603993]
- Wang Q, Zhang Y, Yang C, Xiong H, Lin Y, Yao J, Li H, Xie L, Zhao W, Yao Y, Ning ZB, Zeng R, Xiong Y, Guan KL, Zhao S, Zhao GP. Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. *Science*. 2010; 327:1004–1007. [PubMed: 20167787]
- Way JC, Davis MA, Morisato D, Roberts DE, Kleckner N. New *Tn10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. *Gene*. 1984; 32:369–379. [PubMed: 6099322]
- Williamson, JR.; Corkey, BE. Assays of intermediates of the citric acid cycle and related compounds by fluorometric enzyme methods. In: *Methods in Enzymology*. Lowenstein, JM., editor. New York: Academic Press; 1969. p. 494-497.
- Winston, SE.; Fuller, SA.; Hurrell, JGR. Western blotting. In: Ausubel, FA.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K., editors. *Current Protocols in Molecular Biology*. New York: Wiley Interscience; 1990. pp. 10.18.11–10.18.16
- Zhao K, Chai X, Marmorstein R. Structure and substrate binding properties of CobB, a Sir2 homolog protein deacetylase from *Escherichia coli*. *J. Mol. Biol.* 2004; 337:731–741. [PubMed: 15019790]
- Zheng J, Jia Z. Structure of the bifunctional isocitrate dehydrogenase kinase/phosphatase. *Nature*. 2010; 465:961–965. [PubMed: 20505668]

Figure 1. Overexpression of acs^+ arrests growth in the absence of Pat

A. Strain JE9912 ($\Delta acs / pacs^+$); B. Strain JE9960 ($\Delta acs pat / pacs^+$). Both strains were grown in medium containing 10 mM acetate. Expression of acs was induced by addition of L-(+)-arabinose (250 μ M) during lag (0 h incubation, filled squares), early exponential (8 h, triangles), mid-exponential (10.5 h, inverted triangles), late exponential (13 h, diamonds), or early stationary (15.75 h, circles) growth phase. Controls (open squares) were not induced. Symbols with arrows on the top of the graph point to the time of induction indicated on the x-axis. Graphed points represent the mean of three independent measurements; average standard error = 0.067; $pacs^+$ refers to plasmid pACS7.

Figure 2. Acs activity is elevated in the Pat deficient strain when acs^+ is overexpressed
 Strains JE9912 ($\Delta acs / pacs^+$) and JE9960 ($\Delta acs pat / pacs^+$) were grown in medium containing acetate (10 mM). Expression of acs^+ was induced by addition of L-(+)-arabinose (1.3 mM) when growth was in early log phase ($\text{OD}_{600} \sim 0.2$). Acs activity was measured from crude extract and compared before and after induction of both strains. Equal amounts of total protein were loaded on two identical SDS-PAGE gels and diluted 10-fold twice. One gel was stained with Coomassie Blue (Sasse, 1991), and the other one was used to transfer proteins onto a PVDF membrane for Western blot analysis. Triplicate assays were performed on two independent cultures for each assay time point. $pacs^+$ refers to plasmid pACS7 (Table 4).

Figure 3. Elevated Acs activity does not appear to affect CoA homeostasis in a Pat-deficient strain

Ac-CoA (circles), CoASH (triangles), and Ma-CoA (squares) were measured at 15, 40, and 90 min after the addition of water (uninduced expression, solid lines, filled shapes) or L-(+)-arabinose (1.3 mM; induced expression, dashed lines, open shapes) to cultures of Pat-proficient (JE11864, $\Delta acs panD / pacs^+$) and Pat-deficient (JE11865, $\Delta acs pat panD / pacs^+$) strains growing on low acetate (10 mM). Each point represents the average of triplicate measurements from independent cultures. The experiment was performed at least four times; $pacs^+$ refers to plasmid pACS7.

Figure 4. Elevated Acs activity in the Pat-deficient strain decreases the energy charge

The levels of ATP (squares), ADP (circles) and AMP (triangles) were measured 0, 20, 45, and 90 min after the addition of water (uninduced expression; solid lines, filled shapes), or L-(+)-arabinose (1.3 mM, induced expression; dashed lines, open shapes) to cultures of Pat-proficient (JE11864, $\Delta acs panD / pacs^+$) and Pat-deficient (JE11865, $\Delta acs panD pat / pacs^+$) strains growing on acetate (10 mM). Details of the procedures used to resolve and quantify adenine nucleotides are described under Experimental Procedures. Each point represents triplicate measurements from independent cultures. The experiment was performed at least twice; $pacs^+$ refers to plasmid pACS7 (Table 4).

Figure 5. Ac-AMP formation by Acs in vitro

Representative HPLC analysis of the products of the Acs reaction. In a reaction mixture that contained ATP, acetate, and inorganic pyrophosphatase (concentrations described under Experimental Procedures) the specific activity of Acs, measured as nmol of Ac-AMP / min / mg of Acs = 0.89 ± 0.14 (panel B). The rate of Ac-AMP formation was linear between 15–60 min. Ac-AMP was not detected in the absence of pyrophosphatase (panel C) or in the presence of CoA in the 60 min. time period (panel D).

Figure 6. Overexpression of ADP-forming acetate activation pathways does not lead to growth arrest

Strains JE13780 ($\Delta acs / packA^+ -pta105$), JE13786 ($\Delta acs pat / packA^+ -pta105$), JE13920 ($\Delta acs / pGlacs^+$) JE13921 ($\Delta acs pat / pGlacs^+$) were grown in medium containing 10 mM acetate in the presence of 0 μM (circles), 500 μM (squares) and 1 mM (triangles) L-(+)-arabinose. Growth curves were performed twice in triplicate; average standard error = 0.004. Plasmid $packA^+ -pta105$ refers to pACKA6 and $pGlacs$ refers to pGLACS2.

Figure 7. Addition of alternate carbon sources relieves growth arrest caused by overexpression of *acs*⁺
 Strain JE11864 ($\Delta acs\ panD / pacs^+$; squares) and JE11865 ($\Delta acs\ panD\ pat / pacs^+$; triangles) were grown in media containing 10 mM acetate in the presence of 0 μ M (solid lines, filled shapes) or 500 μ M (dashed lines, open shapes) L-(+)-arabinose; β -alanine was present in the medium at 0.5 μ M. This concentration of β -alanine did not affect the growth phenotype. Glucose (13.5 mM), glycerol (10 mM), or acetate (80 mM) was added to each culture when it had reached stationary phase for at least 10 h; the arrow indicates the point of addition. With the exception of acetate, the addition of a carbon source restored growth of

the Pat-deficient strain synthesizing high levels of Acs; *pacs*⁺ refers to plasmid pACS7. Growth curves were performed at least twice in triplicate; average standard error = 0.02.

Table 1

High Acs activity causes growth arrest in the absence of Pat

Acs variant	Line #	Strain	Relevant Genotype	L-(+)-arabinose (μM, inducer)					
				0		25		250	
				Max OD ₆₅₀	Doubling Time (h)	Max OD ₆₅₀	Doubling Time (h)	Max OD ₆₅₀	Doubling Time (h)
WT	1	JE9912	Δacs / pacs ⁺	0.20	3	0.2	3	0.2	4
	2	JE9960	Δacs pat / pacs ⁺	0.20	3	0.1	9	0.01	CNBD
L641P	3	JE10494	Δacs / pacs ⁴	0.10	24	0.2	3	0.05	CNBD
	4	JE10495	Δacs pat / pacs ⁴	0.05	CNBD	0.2	4	0.05	CNBD
G266S	5	JE10119	Δacs / pacs ⁷	0.10	45	0.1	17	0.1	20
	6	JE10275	Δacs pat / pacs ⁷	0.10	43	0.2	10	0.2	15
K609A	7	JE10125	Δacs / pacs ⁸	0.05	CNBD	0.05	ND	0.05	CNBD
	8	JE10724	Δacs pat / pacs ⁸	0.05	CNBD	0.05	ND	0.05	CNBD

Strains encoding Acs variants were grown in NCE medium + acetate (10 mM). Expression of *acs* alleles was induced by addition of the indicated concentration of L-(+)-arabinose. Growth curves were performed in triplicate; average standard error = 0.003. pacs⁺ (encodes Acs^{WT}), pacs⁴ (encodes Acs^{L641P}), pacs⁷ (encodes Acs^{G266S}), pacs⁸ (encodes Acs^{K609A}), and refer to plasmids pACS7, pACS8, pACS23, and pACS24, respectively. WT, wild type; CNBD, could not be determined.

Table 2

Kinetic parameters of variant Acs proteins.

Line #	Protein	K_m acetate (mM)	k_{cat} acetate (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)
1	Acs ^{WT}	41 ± 12 (4)	9,400 ± 1,500 (200)	229 ± 40
2	Acs ^{L641P}	410 ± 190 (140)	1,500 ± 600 (100)	3.6 ± 2.3
3	Acs ^{G266S}	11,000 ± 2,000 (3000)	3,200 ± 400 (200)	0.29 ± 0.04

K_m and k_{cat} values were calculated for Acs variants based on activity of Acs (10 nM) incubated with ATP (2.5 mM), CoA (1.5 mM), and varying concentrations of acetate. Acs proteins were purified from a *pat cobB* strain, and were therefore unacetylated. Kinetic parameters were not determined for Acs^{K609A} because this variant lacked activity above a no-enzyme control. WT, wild type. Reported kinetic parameters reflect the mean and range of three independent determinations. Average standard error is reported in parentheses.

Table 3

Increased levels of CoA alleviate the negative effect of high Acs levels

Strain	Genotype	Line #	Pantothenate (mM)	L-(+)-Arabinose (μM)			
				None		100	
				ΔOD ₆₅₀	Doubling Time (h)	ΔOD ₆₅₀	Doubling Time (h)
JE12383	Δ <i>acs</i> / <i>pacs</i> ⁺ pBAD18 <i>cat</i> ⁺	1	0	0.2	5.1	0.2	4.8
		2	10	0.2	4.6	0.2	4.5
JE12384	Δ <i>acs</i> / <i>pacs</i> ⁺ <i>pcoaA2</i>	3	0	0.2	4.8	0.2	4.8
		4	10	0.2	3.7	0.2	3.5
JE12385	Δ <i>acs pat</i> / <i>pacs</i> ⁺ pBAD18 <i>cat</i> ⁺	5	0	0.2	4.6	NG	ND
		6	10	0.2	4.1	NG	ND
JE12386	Δ <i>acs pat</i> / <i>pacs</i> ⁺ <i>pcoaA2</i>	7	0	0.2	4.4	NG	ND
		8	10	0.2	3.8	0.1	12.5

All strains are TR6583 (*metE205 ara-9*) derivatives with the *pantD626::Tn10Δ(cat⁺)* allele, thus they were β-alanine auxotrophs; β-alanine was present in the medium at 0.5 μM. This concentration of β-alanine did not affect the growth phenotype. Strains JE12383, JE12384, JE12385 and JE12386 were grown on minimal medium + acetate (10 mM) + the indicated concentration of L-(+)-arabinose ± pantothenate (10 mM). Expression of allele *coaA2* was co-induced with *acs*⁺ with L-(+)-arabinose, with plasmid pBAD18 *cat*⁺ serving as the empty-vector control. Growth curves were performed in triplicate; average standard error = 0.006. Plasmids *pcoaA2* (encodes CoaA R106A) and *pacs*⁺ (encodes Acs WT) refer to plasmid pCOAA8 and pACS7, respectively. ΔOD₆₅₀ = final - initial OD₆₅₀; in all cases the initial OD₆₅₀ = 0.1; ND, not determined; NG, no growth.

Table 4

Strains and plasmids used in this work.

Strain or plasmid	Relevant genotype	Source or reference
<i>S. enterica</i> strains		
LT2	Wild type	Laboratory collection
LT2 derivatives:		
DM290	<i>panD623::Tn10d(ter⁺)</i>	D.M. Downs
TR6583	<i>metE205 ara-9</i>	K. Sanderson via J. Roth
TR6583 derivatives:		
JE7449	<i>pat10::MudJ(lacZ⁺ kan⁺)^d</i>	Laboratory collection
JE7758	Δ <i>acs2</i>	(Starai <i>et al.</i> , 2005b)
JE9912	Δ <i>acs2</i> / pACS7	
JE9944	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i>	
JE9960	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i> / pACS7	
JE10560	Δ <i>acs2</i> / pACS27	
JE10561	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i> / pACS27	
JE10119	Δ <i>acs2</i> / pACS23	
JE10125	Δ <i>acs2</i> / pACS24	
JE10275	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i> / pACS23	
JE10494	Δ <i>acs2</i> / pACS8	
JE10495	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i> / pACS8	
JE10724	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i> / pACS24	
JE11552	Δ <i>acs2 panD623::Tn10d(ter⁺)</i>	
JE11553	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i> <i>panD623::Tn10d(ter⁺)</i>	
JE11864	Δ <i>acs2 panD623::Tn10d(ter⁺)</i> / pACS7	
JE11865	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i> <i>panD623::Tn10d(ter⁺)</i> / pACS7	
JE12383	Δ <i>acs2 panD623::Tn10d(ter⁺)</i> / pACS7 pBAD18 <i>cat⁺</i>	
JE12384	Δ <i>acs2 panD623::Tn10d(ter⁺)</i> / pACS7 pCOAA8	
JE12385	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i> <i>panD623::Tn10d(ter⁺)</i> pACS7 / pBAD18 <i>cat⁺</i>	
JE12386	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i> <i>panD623::Tn10d(ter⁺)</i> pACS7 / pCOAA8	
JE13780	Δ <i>acs2</i> / pACKA6	
JE13786	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i> / pACKA6	
JE13920	Δ <i>acs2</i> / pGLACS2	
JE13921	Δ <i>acs2 pat10::MudJ(lacZ⁺ kan⁺)</i> / pGLACS2	
<i>E. coli</i> strains		
XL1-Red	<i>endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> <i>mutD5 mutS mutT Tn10 (ter⁺)</i>	Stratagene
Plasmids		

Strain or plasmid	Relevant genotype	Source or reference
Cloning vectors		
pGEM-T Easy	Cloning vector, <i>bla</i> ⁺	Promega
pBAD18cm	Complementation vector, <i>P_{araBAD} cat</i> ⁺	(Guzman et al., 1995)
pBAD30	Complementation vector, <i>P_{araBAD} bla</i> ⁺	(Guzman et al., 1995)
pBAD33	Complementation vector, <i>P_{araBAD} cat</i> ⁺	(Guzman et al., 1995)
pTYB1	Overexpression vector, <i>bla</i> ⁺	New England Biolabs
Acs complementation plasmids		
pACS7	<i>acs</i> ⁺ cloned into pBAD30	(Starai et al., 2005a)
pACS8	<i>acs4</i> (<i>Acs</i> ^{L641P}) cloned into pBAD30	(Starai et al., 2005a)
pACS21	<i>acs7</i> (<i>Acs</i> ^{G266S}) cloned into pBAD30, original mutant isolate	
pACS23	<i>acs7</i> (<i>Acs</i> ^{G266S}) cloned into pBAD30	
pACS24	<i>acs8</i> (<i>Acs</i> ^{K609A}) cloned into pBAD30	
pACKA6	<i>ackA</i> ⁺ <i>pta105</i> cloned into pBAD30	
pACS27	<i>acs</i> ⁺ cloned into pBAD33	
pGLACS2	<i>G. lamblia acs</i> ⁺ cloned into pBAD33	
Acs overexpression plasmids		
pACS10	<i>acs</i> ⁺ cloned into pTYB1	(Starai et al., 2002)
pACS12	<i>acs4</i> (<i>Acs</i> ^{L641P}) cloned into pTYB1	(Starai et al., 2005a)
pACS26	<i>acs7</i> (<i>Acs</i> ^{G266S}) cloned into pTYB1	
pACS28	<i>acs8</i> (<i>Acs</i> ^{K609A}) cloned into pTYB1	
Other plasmids		
pCOAA6	<i>coaA</i> ⁺ cloned into pBAD33	
pCOAA7	<i>coaA2</i> (<i>CoaA</i> ^{R106A}) cloned into pBAD33	
pCOAA8	<i>coaA2</i> (<i>CoaA</i> ^{R106A}) cloned into pBAD18cm	

Unless otherwise noted, all the strains and plasmids were constructed during the course of this work.

^a MudJ is an abbreviation of MudI1734(*lacZ*⁺ *kan*⁺) (Castilho *et al.*, 1984)

^b Tn10d(*ter*⁺) is an abbreviation of Tn10ΔD16ΔD17 (Way *et al.*, 1984)