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Acs is essential for propionate utilization in *Escherichia coli*

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ABSTRACT

Bacteria like *Escherichia coli* can use propionate as sole carbon and energy source. All pathways for degradation of propionate start with propionyl-CoA. However, pathways of propionyl-CoA synthesis from propionate and their regulation mechanisms have not been carefully examined in *E. coli*. In this study, roles of the acetyl-CoA synthetase encoding gene *acs* and the NAD$^+$-dependent protein deacetylase encoding gene *cobB* on propionate utilization in *E. coli* were investigated. Results from biochemical analysis showed that, reversible acetylation also modulates the propionyl-CoA synthetase activity of Acs. Subsequent genetic analysis revealed that, deletion of *acs* in *E. coli* results in blockage of propionate utilization, suggesting that *acs* is essential for propionate utilization in *E. coli*. Besides, deletion of *cobB* in *E. coli* also results in growth defect, but only under lower concentrations of propionate (5 mM and 10 mM propionate), suggesting the existence of other propionyl-CoA synthesis pathways. In combination with previous observations, our data implies that, for propionate utilization in *E. coli*, a primary amount of propionyl-CoA seems to be required, which is synthesized by Acs.

1. Introduction

It is well known that short chain fatty acids, such as acetate, propionate and butyrate, can be utilized as sole carbon and energy source for bacteria. Among all these fatty acids mentioned above, oxidation of propionate is the most complicated and several pathways are involved in different bacteria [1]. However, all established and putative pathways for degradation of propionate start with propionyl-CoA.

Generally, in bacteria, propionyl-CoA can be synthesized from propionate through three different routs The first route is the propionyl-CoA synthetase (PrpE), which was firstly characterized in *Salmonella typhimurium* by Horswill and Escalante-Semerena in 1999 [2]. Subsequently, PrpE was also identified in other bacterial species, suggesting that it is a common route for propionyl-CoA synthesis in bacteria [3–5]. However, from the first beginning, Horswill and Escalante-Semerena found that, though PrpE was characterized to be a propionyl-CoA synthetase, *prpE* deletion mutant could still use propionate as carbon and energy source, since the acetyl-CoA synthetase (Acs) could compensate the lack of PrpE [2]. In fact, early studies already showed that, Acs could also use propionate as substrate [6–8], so it is not surprising to find out that *acs* serves as the second route for propionyl-CoA synthesis. The third route, a rather unusual route for propionyl-CoA synthesis, is consisted of two enzymes, the propionate kinase (PduW) and the phosphotransacetylase (Pta) [9]. So far, the third route has only been identified in *S. typhimurium*, and it is induced by 1,2-propanediol [10,11].

Regulation of propionyl-CoA synthesis has also been characterized. In 1996, Tsang and Escalante-Semerena found that, *cobB* mutant of *S. typhimurium* could not use propionate as carbon and energy source [11]. In 2002, CobB was characterized to be a NAD$^+$-dependent protein deacetylase responsible for deacetylation of Acs, which activates the acetyl-CoA synthetase activity of the protein [12]. Later on, PrpE was found to undergo propionylation *in vivo* which inactivate its activity, and CobB was found to be responsible for its de-propionylation [13]. Except for CobB, another known regulator for propionyl-CoA synthesis has been identified to be PrpR, a transcriptional activator for *prpBCDE* operon [14–16]. Very interestingly, in *S. typhimurium*, the function of PrpR needs 2-methylcitrate as co-activator [17,18].

Though progresses have been made on bacterial propionyl-CoA synthesis pathways, little is known about propionyl-CoA synthesis pathways and their regulations in *Escherichia coli*. To better understand how reversible acetylation regulates the propionyl-CoA synthetase activity of Acs, the protein was *in vitro* overexpressed and the effect of reversible acetylation on its propionyl-CoA

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synthetase activity was tested. To elucidate the role of Acs on propionyl-CoA synthesis in *E. coli*, the *acs* gene was deleted and the effect of *acs* deletion on bacterial growth on different concentrations of propionate was carefully examined. Furthermore, the effect of *cobB* deletion on propionate utilization of *E. coli* was also tested. The results are reported herein.

2. Material and methods

2.1. Bacterial strains, growth conditions, and plasmids

E. coli strain w3110 used in this work were derived from standard *E. coli* K-12 strain. Bacterial strains and plasmids used were listed in Table 1. Luria–Bertani broth (LB) was the rich medium used to grow *E. coli* strains. Ampicillin, kanamycin and chloramphenicol were added in medium if needed at 100, 50, and 25 μg/ml. For growth curve experiments, strains were grown to mid-log phase in LB medium, then collected by centrifugation at 8000*g* for 6 min and washed 3 times with E minimal medium (0.2 g $MgSO_4{\cdot}7H_2O$, 13.1 g $K_2HPO_4{\cdot}3H_2O$, 3.5 g $NaNH_4HPO_4{\cdot}4H_2O$, 2 g citric acid$\cdot H_2O$) [19], resuspended in E minimal medium and adjusted to OD_{600} = 0.8, 100 μl suspension culture were inoculated to 10 ml E minimal medium supplemented with varied concentrations of sodium propionate. Growth of bacteria at 37 °C was measured by taking OD_{600} every 2 days by using SyergyH1 Hybrid reader (Bio-Tek, USA). Experiments were performed in triplicates.

2.2. Unmarking of acs and cobB deletion mutants

The antibiotic resistance gene of the *acs* and *cobB* deletion mutants were eliminated with plasmid pCP20 as previously described [20]. Elimination of resistance gene was verified by antibiotic-resistance. Unmarked mutants were verified by PCR with primers designed up/downstream of target genes. Following primers were used: cobB-s, 5′-atctcttacctgtagctcgtgttccg-3′ (sense), cobB-a, 5′-aaaagtgggcgtgtattattccg-3′ (antisense); acs-s, 5′-cccctat gtgtaacaaataacca-3′ (sense), acs-a, 5′-tatcaggcctacaaaccgttac-3′ (antisense).

2.3. Overexpression and purification of proteins

Bacteria strains were first grown overnight in 5 ml LB medium, and then cultures were transferred into 500 ml fresh LB medium at 37 °C in shaking flasks. IPTG was added to a final concentration of 0.2 mM when OD_{600} reached to 0.6–0.8. Then cells were grown for an additional 3 h at 37 °C for *E. coli* BL21 (DE3)/pET32a-*cobB* and 12 h at 25 °C for *E. coli* DH5α/pTrcHis2C-*acs*, harvested by centrifugation and washed once with ice-cold PBS buffer, resuspended in 30 ml Binding Buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl and 10 mM imidazole), sonicated on ice at the intensity of 3 s burst at 200 W with a 5 s cooling period between each burst with an Ultrasonic Cell Disruptor (VCX750, SONICS, US) until cell suspension becomes clear. The lysate was centrifuged at 10,000*g* for 30 min at 4 °C and the supernatant was harvested. Proteins were purified as previously described [21]. Protein concentrations were measured by the Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, China) with BSA as standard, according to manufacturer's instructions.

2.4. In vitro enzymatic assay

Enzymatic activity of Acs was determined as previously described [2,22,23] with modifications. Briefly, 1 ml reaction mixtures contains 50 mM Tris–HCl (pH 7.5), 500 mM hydroxylamine (2.0 M solution of hydroxylamine was prepared before use by mixing equal volume of 4.0 M $NH_2OH{\cdot}HCl$ and 4.0 M KOH), 10 mM $MgCl_2$ and 2 mM DTT, 5 mM acyl substrate, 5 mM CoA and 5 mM ATP. The reaction mixture was preincubated at 37 °C for 5 min before the addition of Acs (5 μM). After addition of Acs, the reaction mixture was incubated at 37 °C for 10 min and terminated with the addition of equal volume of stop solution [2% (w/v) $FeCl_3$ in 2 M HCl, 5% (w/v) trichloroacetic acid (TCA)]. Reaction tubes were centrifuged at 10,000*g* for one minute to remove turbidity, and the color generated was measured at 540 nm. Samples without CoA were used as blank. The reaction product of acetylhydroxamate by acetyl-phosphate (sigma) served as the standard. The kinetic parameters and their standard errors were determined by non-linear regression to fit the data to the Michaelis–Menten equation.

2.5. Acetylation and CobB mediated deacetylation of Acs

Purified Acs was incubated with acetyl-phosphate (freshly prepared) in the buffer consisted of 300 mM NaCl and 50 mM Tris–HCl (pH 8.0) to obtain acetylated Acs (Ac-Acs). Ac-Acs was separated from the reaction mixture by dialysis, then deacetylated by CobB as previously described [24]. The acetylation level of Acs was analyzed by Western blot and enzyme activities of Acs, Ac-Acs and de-Acs (deacetylated by CobB) were determined as mentioned above.

2.6. Western blot analysis

Proteins were separated by 10% SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore). The PVDF membrane was blocked at 37 °C for 2 h in TBST [25 m M Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% Tween-20] containing 5% non-fat dry milk (NFDM). Primary rabbit anti-acetyl lysine polyclonal antibody (Cell Signaling Technology) was diluted (1:1000) in TBST/1% NFDM and incubated at 37 °C for 2 h. After washing

Table 1
Strains and plasmids used in this study.

Strain and plasmid	Relevant properties	Reference or source
E. coli strains		
W3110	Wild type strain, F-λ-IN(rrnD-rrnE)1 rph-1	Laboratory stock
RL001	W3110 Δ*cobB*::Km^r	Laboratory stock
RL002	W3110 Δ*acs*::Km^r	Laboratory stock
W3110 Δ*cobB*	W3110 derivative *cobB* unmark deleted	This study
W3110 Δ*acs*	W3110 derivative *acs* unmark deleted	This study
DH5α	Host for plasmid propagation	Laboratory stock
BL21(DE3)	Host for CobB protein expression	Laboratory stock
Plasmids		
pCP20	FLP helper plasmid, Apr, Cm^r	Yale CGSC
pTrcHis2C-*acs*	Acs-His expression plasmids, Ap^r	Laboratory stock
pET32a-*cobB*	CobB expression plasmids, Ap^r	Laboratory stock

with TBST triply, the membrane was incubated with HRP-conjugated goat anti-rabbit antibody (Cell Signaling Technology) for 1 h at 37 °C, and then detected according to the manufacturer's instructions.

3. Results

3.1. Expression, auto-acetylation and de-acetylation of Acs in vitro

The *acs* gene was expressed as a His-tagged protein under the control of a *trc* promoter in the *E. coli* expression vector pTrcHis2C as described previously [21], we induced and purified the protein by Ni^{2+} chromatography. A protein of 72 kDa was induced and purified as shown by SDS–PAGE (Fig. 1). Recently, it was reported that acetyl-phosphate can acetylate proteins nonenzymatically in *E. coli*[25]. So we tested the efficacy of acetyl-phosphate for Acs auto-acetylation under different conditions, including different concentrations of acetyl-phosphate and also different times of treatment. As shown in Fig. 2A and B, very obviously, auto-acetylation of Acs by acetyl-phosphate is both time and dose dependent, which is consistent with previous report [25]. In the presence of 10 mM acetyl-phosphate, four hours of treatment resulted in a remarkable increase of acetylation level of Acs. So this reaction condition was chosen for obtaining acetylated Acs (Ac-Acs) which was further used for deacetylation by CobB. From Fig. 2C, we could see that, in the presence of NAD^+, CobB could deacetylate Ac-Acs very effectively, which could be hindered by the known Sir2 inhibitor nicotinamide.

3.2. Effect of acetylation on Acs activities

Previous studies showed that reversible acetylation regulates the acetyl-CoA synthetase acitivity of Acs [12]. However, the effect of reversible acetylation on its propionyl-CoA synthetase activity has not been tested. We speculated that the propionyl-CoA synthetase activity of Acs could also be affected by reversible acetylation. To test this hypothesis, relative activities of Acs with different levels of acetylation were measured. From Fig. 2D, we could see that both acetyl-CoA synthetase activity and propionyl-CoA synthetase activity of Acs were reduced when Acs were acetylated by acetyl-phosphate, and both of the enzymatic activities could be restored after deacetylation by CobB. From Table 2, we could see

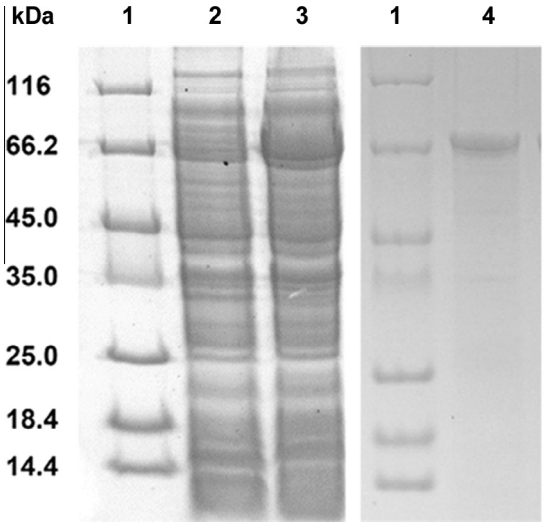

Fig. 1. SDS–PAGE analysis of Acs expression and purification. Lane 1, protein molecular marker. Lane 2 and 3, whole proteins of *E. coli* DH5α/pTrcHis2C-*acs* without/with IPTG induction. Lane 4, purified Acs protein.

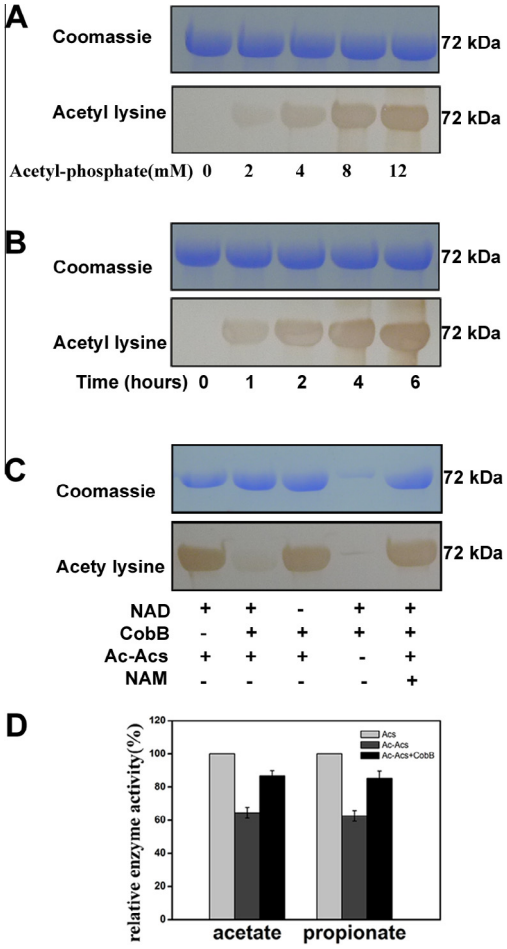

Fig. 2. Western blotting analysis of acs acetylation and regulation of Acs activity by acetylation and CobB-mediated deacetylation. (A) Acetylation of Acs with varying concentrations of acetyl-phosphate at 37 °C for 3 h. (B) Acetylation of Acs with 10 mM acetyl-phosphate as acetyl donor at 37 °C for different time as indicated. (C) Deacetylation of Acs by CobB. Acs was acetylated with 10 mM acetyl-phosphate at 37 °C for 4 h (Ac-Acs), dialyzed and then incubated with CobB in the presence of NAD^+ (1 mM) for 3 h at 37 °C. Nicotinamide (NAM, 10 mM) was added to the reaction as Sir2 inhibitor. (D) Effect of acetylation by acetyl-phosphate and deacetylation by CobB on Acs activity. The acetyl-CoA synthetase activity (acetate as substrate) and propionyl-CoA synthetase activity (propionate as substrate) of Acs was defined as a percentage of the activity measured for Acs before acetylation. Acs, before acetylation; Ac-Acs, acetylation; Ac-Acs + CobB, Acs was acetylation and then deacetylation by CobB; Error bars indicate SD of three measurements.

that acetylation of Acs resulted in a slightly increase of the K_m value for both acetate and propionate, suggesting a slight decrease of the affinity for both substrates. Meanwhile, acetylation of Acs also resulted in a significant decrease of the k_{cat} value for both acetate and propionate. Taken together, acetylation of Acs resulted in a significant decrease of k_{cat}/K_m value for both acetate and propionate, suggesting a significant decrease of catalytic efficiencies

Table 2
Steady-state kinetic parameters of Acs and Ac-Acs.

Substrate	Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Acetate	Acs	0.24 ± 0.028	1.62 ± 0.025	6.68 ± 0.821
Acetate	Ac-Acs	0.35 ± 0.014	0.99 ± 0.027	2.85 ± 0.067
Propionate	Acs	11.15 ± 0.527	0.56 ± 0.048	0.05 ± 0.002
Propionate	Ac-Acs	13.24 ± 0.463	0.28 ± 0.023	0.02 ± 0.002

Kinetic parameters of Acs and Ac-Acs at pH 7.5 and 37 °C. Enzyme activities were measured at varying concentrations of acetate or propionate in 50 mM Tris–HCl buffer, pH 7.5. The data are presented as the mean ± SD of triplicate tests.

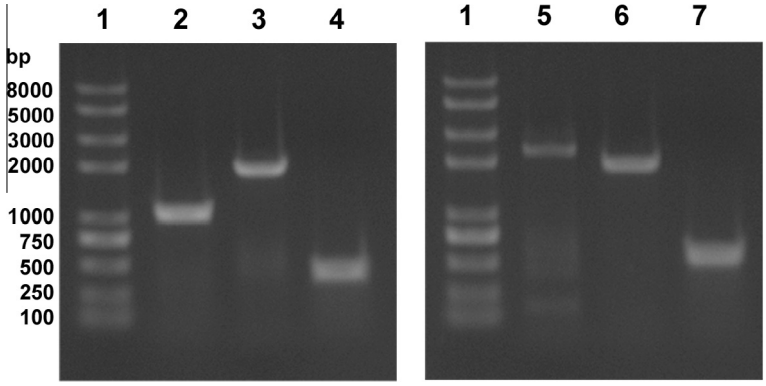

Fig. 3. PCR identification of the *cobB*, *acs* gene knockout strain. Lane 1, DNA marker; lane 2, 3, 4, PCR verification of *E. coli* W3110, W3110 Δ*cobB*::Km, W3110 Δ*cobB* with primers cobB-s and cobB-a; lane 5, 6, 7, PCR verification of *E. coli* W3110, W3110 Δ*acs*::Km, W3110 Δ*acs* with primers acs-s and acs-a.

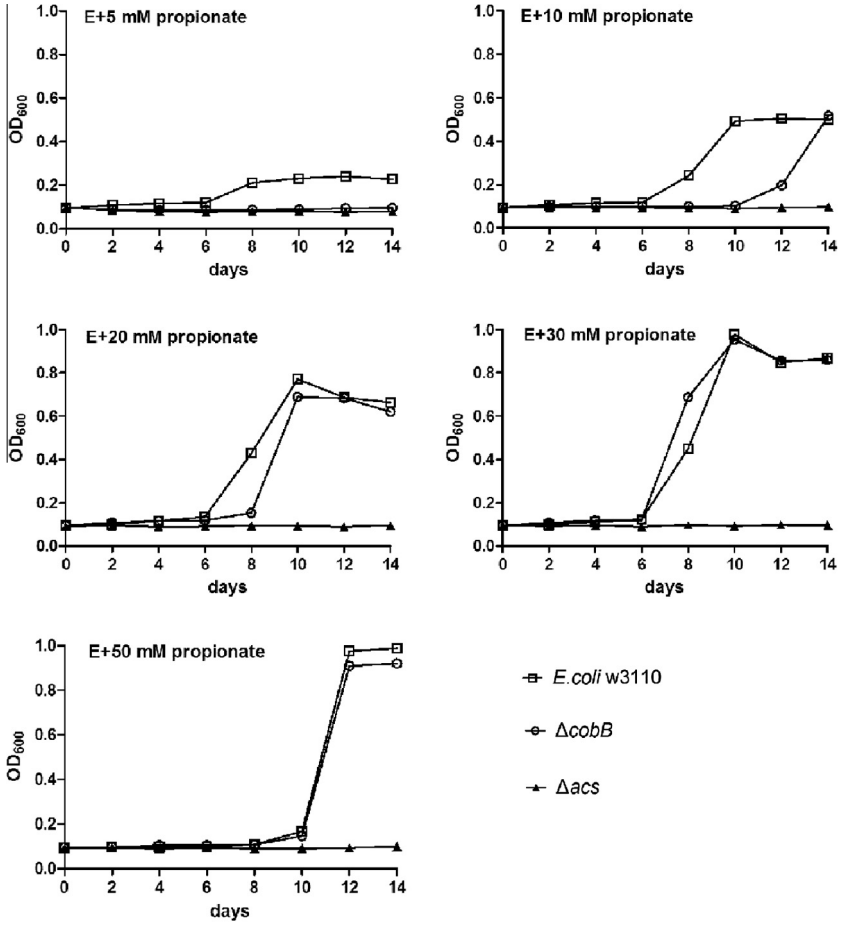

Fig. 4. Growth curves of *E. coli* W3110, Δ*cobB* and Δ*acs* mutant on E minimal medium with propionate as sole carbon and energy source. Concentration of propionate was as indicated.

for both substrates. Our results proved that, reversible acetylation could also regulate the propionyl-CoA synthetase activity of Acs (see Fig. 3).

3.3. Growth of *E. coli* Δ*acs* on propionate

Previous studies have shown that, in *S. typhimurium*, *prpE* and *acs* are jointed essential for growth on propionate [9]. However, the role of *acs* on propionate utilization in *E. coli* has never been tested. So the *acs* deletion mutant was constructed and unmarked, then grown in minimum medium with different concentrations of propionate as sole carbon source. As shown Fig. 4, the *acs* mutant failed to grow under all concentrations of propionate tested.

3.4. Growth of *E. coli* Δ*cobB* on propionate

Previously, Tsang and Escalante-Semerena reported that, in *S. typhimurium*, deletion of *cobB* resulted in blockage of propionate utilization of the bacteria [11]. To test if this is also the case in *E. coli*, the *cobB* mutant was grown in E minimum medium with

different concentrations of propionate as sole carbon source. As shown in Fig. 4, the *cobB* mutant did not grow under 5 mM propionate, and showed a severe growth defect under 10 mM of propionate. However, the *cobB* mutant only showed a slightly longer lag phase for growth on 20 mM propionate, and did not show any difference of growth in comparison with the wild type strain under higher concentrations of propionate (30 and 50 mM).

4. Discussion

In this study, we found that, in *E. coli*, reversible acetylation also modulates the propionyl-CoA synthetase activity of Acs, *acs* is essential for propionate utilization, and *cobB* plays a key role on propionate utilization under lower concentrations of propionate.

Previous study showed that, in *S. typhimurium*, deletion of *cobB* resulted in blockage of propionate utilization of the bacteria [11]. Later, CobB was shown to be a NAD^+-dependent protein deacetylase responsible for deacylation of Acs, and also responsible for depropionylation of PrpE [12,13]. Now, it is known that reversible acetylation regulates the acetyl-CoA synthetase activity of Acs, and reversible propionylation regulates the propionyl-CoA synthetase activity of PrpE [12,13]. Since *acs* and *prpE* were shown to be jointly essential for propionate utilization in *S. typhimurium*[9], the phenotype that *cobB* mutant cannot use propionate as carbon and energy source cannot be simply explained by the fact that CobB regulates the activity of PrpE through depropionylation. The effect of CobB on propionyl-CoA synthetase activity of Acs also needs to be examined. Our results showed that, reversible acetylation also regulates the propionyl-CoA synthetase activity of Acs. Thus, deletion of *cobB* not only leads to the inactivation of the propionyl-CoA synthetase activity of PrpE, but also that of Acs.

Though it has been reported that in *S. typhimurium*, *acs* and *prpE* are jointly essential for propionate utilization [9], previous study implied that these two genes may not be equally important for propionate utilization in *E. coli*. In 2002, Brock et al. found that, when *E. coli* was grown on minimum medium with propionate as sole carbon source, both *prpE* and *acs* mRNA levels were significantly increased, but PrpE could not be detected by following 2-D gel electrophoresis [26]. Our results showed that, in *E. coli*, deletion of *acs* led to blockage of propionate utilization in the presence of *prpE*, which is surprising. In fact, previous studies already showed that, in many bacteria the expression of the *prpBCDE* operon is under regulation of the transcriptional activator PrpR, which also exists in *E. coli*[14–16]. Very interestingly, PrpR was shown to need 2-methycitrate as co-activator [17,18]. And this means that, sufficient amount of propionyl-CoA is required for synthesis of 2-methylcitrate which is necessary for activating the expression of the *prpBCDE* operon. So, from our data, it seems that, in *E. coli*, Acs is responsible for synthesizing the primary amount of propionyl-CoA required for 2-methylcitrate synthesis. This may explain its essential role on propionate utilization.

In a attempt to test the role of *cobB* on propionate utilization in *E. coli*, we found that, unlike the phenotype reported in *S. typhimurium*, the *E. coli cobB* mutant only showed growth defect on lower concentrations of propionate (5, 10, 20 mM), and no growth defect could be observed above 30 mM of propionate. And these results may be explained by two reasons. The first reason is that, though Acs is under the regulation of CobB, the expression level of *acs* gene can be significantly induced by propionate [26]. Thus, the effect of hyper-acetylation of Acs may be partially alleviated by significant increase of gene expression induced by higher concentrations of propionate. And the second reason is that, other pathways of propionyl-CoA synthesis may exist in *E. coli* except for PrpE and Acs, which is not under the regulation of CobB. Since Acs is essential for propionate utilization and regulated by CobB, in any case, deletion of *cobB* will result in at least partial inactivation of Acs, and hence slower growth of the *cobB* mutant on propionate. The fact that the *cobB* mutant does not show any difference in growth under higher concentrations of propionate suggested that, other pathways of propionyl-CoA synthesis may exist in *E. coli* except for PrpE and Acs. Though these unknown pathways seem to be not under the control of CobB, they are not able to work in the absence of Acs.

Taken together, our data suggested that, in combination with previous observations, imply that, no matter how many pathways of propionyl-CoA synthesis from propionate there may exist in *E. coli*, a primary amount of propionyl \ l-CoA is required for subsequent assimilation of propionate, which is synthesized by Acs.

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