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Acetate Consumption Activity Directly Determines the Level of Acetate Accumulation During *Escherichia coli* W3110 Growth

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***Escherichia coli* excretes acetate during aerobic growth on glycolytic carbon sources, which has been explained as an overflow metabolism when the carbon flux into the cell exceeds the capacity of central metabolic pathways. Non-acetogenic growth of *E. coli* on gluconeogenic carbon sources like succinate or in carbon-limited slow growth conditions is believed an evidence for the explanation. However, we found that a strain defected in the *acs* (acetyl Co-A synthetase) gene, the product of which is involved in scavenging acetate, accumulated acetate even in succinate medium and in carbon-limited low growth rate condition, where as its isogenic parental strain did not. The *acs* promoter was inducible in noncatabolite repression condition, whereas the expression of the *ackA–pta* operon encoding acetate kinase and phosphotransacetylase for acetate synthesis was constitutive. Results in this study suggest that *E. coli* excretes and scavenges acetate simultaneously in the carbon-limited low growth condition and in non-acetogenic carbon source, and the activity of the acetate consumption pathway directly affects the accumulation level of acetate in the culture broth.**

Keywords: Acetate metabolism, acetyl CoA synthetase, phosphotransacetylase, acetate kinase, glyoxylate shunt

Escherichia coli excretes acetate as a major product of its aerobic metabolism as well as of mixed-acid fermentation [1, 7, 13]. Acetate synthesis mainly depends on the Pta–AckA (Pta, phosphotransacetylase; AckA, acetate kinase) pathway converting acetyl CoA *via* acetyl phosphate to acetate [35]. The genes encoding Pta and AckA form a single operon in the order of *ackA* to *pta* and are transcribed by the same promoter. During the growth transition to stationary phase, pyruvate oxidase (PoxB), an alternative acetate synthesis pathway, converts pyruvate to acetyl phosphate that is in turn converted to acetate spontaneously [4]. *E. coli* reuses excreted acetate after the favorable carbon sources are exhausted [3, 35]. Acetate is activated to acetyl-CoA by the reverse reaction of the Pta–AckA pathway through acetyl phosphate or by acetyl CoA synthetase (*acs* gene product, ACS) [3, 17]. ACS and the Pta–AckA pathway operate at low and high concentrations of acetate, respectively, owing to the different K_m values for acetate [17].

Aerobic acetogenesis of *E. coli* has been explained as the "overflow" metabolism [1, 9, 10]. In this explanation, acetate excretion is the result of unbalance between the fast carbon influx into the central metabolism and the limited capacity of the tricarboxylic acid (TCA) cycle or respiration. Indeed, the acetate excretion is not observed when the specific growth rate of *E. coli* is lower than a certain threshold growth rate (μ_c), owing to the carbon limitation in continuous cultivations or the quality of carbon sources in batch cultures [1, 6, 9, 10]. In this context, aerobic acetogenesis of *E. coli* has been proposed as a means of generating extra ATP to support faster growth [12].

The activities of Pta and AckA are independent of carbon sources [3, 10]. In anaerobic or glucose starvation conditions, the levels of acetate kinase and phosphotransacetylase were higher as compared with those in aerobic or glucose-rich conditions [13, 25]. Expression of the *acs* gene is repressed by glucose and induced by acetate [31]. Kumari *et al.* [15] showed that the expression of *acs* depends on cyclic AMP receptor protein (CRP) and the oxygen regulator FNR, as well the glyoxylate shunt repressor IclR, its activator FadR, and many enzymes involved in acetate metabolism. Tight control of *acs* expression depends on culture condition, and the relatively consistent activity of Pta and AckA shows the possible control of the acetate

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accumulation profile of *E. coli* by differential *acs* expression. Lin *et al.* [18] reported that the overexpression of ACS in *E. coli* resulted in a significant reduction in acetate accumulation during glucose fermentation and enhanced acetate assimilation when acetate was used as a carbon source. In this study, we report that *E. coli* W3110 constitutively synthesized acetate even when it grew on succinate, which is known as an acetate-nonproducing carbon source, and the activity of the acetate consumption pathway affected the accumulation level of acetate in the culture broth.

Materials and Methods

Strains and Plasmids

All the strains used for physiological experiments were derivatives of *E. coli* W3110. An *acs* mutant constructed by using a part of the *acs* coding region (1.5-kb EcoRV–EcoRI fragment) from the Kohara library 638 clone [14]. The kanamycin gene block from plasmid pUC4K (GE Healthcare Bio-Sciences, Uppsala, Sweden) was inserted into the BclI site of the *acs* coding region, and the resulting *acs*::Km^r segment was integrated into the chromosome of the *polA*^{ts} strain CP367 [26] by homologous recombination promoted in high temperature under the pressure of kanamycin-resistant selection. The *acs*::Km^r mutation was confirmed by genetic mapping and the PCR- amplified *acs* region, and then P1-transduced to the W3110 strain.

Strains containing single-copy transcriptional fusions of *lacZ* to the *ackA–pta* promoter and *acs* promoter in W3110 Δ(*lacZ*) were constructed by using pRS45 and bacteriophage lambda RS415 [32]. From the 405 clone of the Kohara library [14], a 2,079-bp PvuII–PvuII fragment DNA containing the upstream region of the *ackA–pta* operon and 84 amino acids of AckA was used as the *ackA–pta* promoter [11]. A 1,397 bp Klenow-filled XhoI–ClaI fragment from pSK122 [31] was used as a *acs* promoter.

For the construction of an *acs* derepressed W3110 derivative, the *acs* allele of CV101 isolated by Rosenzweig *et al.* [29] was amplified using primers 5'-ATATGAATTCTGAGAACACAAATGGGC-3' (the underlined region binds to the 1040 to 1056 bases of IS30) and 5'-ACGCGTCGACTTACGATGGCATCGCG-3' (the underlined region binds to the C terminal of the *acs* gene), and Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN, U.S.A.). The EcoRI–SalI-digested 2,873-bp fragment containing IS30 R-end and the *acs* coding region was subcloned into pBRINT-Cm [2] and integrated into the *lacZ* site of the chromosome by using JC7623 (*sbcBC recBC* strain, from CGSC). The *cat* gene-tagged *acs* derepressed allele (manifested as *acs*^{derep}-Cm) was transduced to W3110 by phage P1vir, yielding W3110 *acs*^{derep}-Cm.

Culture Conditions

LB media supplemented with appropriate antibiotics were used for the strain construction and maintenance. M9 media supplemented with glucose or succinate 0.2%, respectively, were used for the analysis of growth and metabolic products accumulation profiles. For batch cultures, the bacterial cells were grown in a shaking incubator maintained at 37°C. The chemostat cultivations were carried out at 37°C in a 2.5-l fermentor (Korea Fermentor Co., Inchon, Korea), which contained 1 l of M9 minimal medium with continuous feeding of the media and extracting the same volume of the culture broth. The air flow rate was fixed at 1 l/min, and dissolved oxygen was maintained above 20% of its saturation level by manually adjusting the agitation speeds in the range of 500–900 rpm. pH was kept constant at 7.0 by the addition of 2 M NaOH.

Analytical Methods

Optical density (OD) was measured at 600 nm (Ultrospec 2000 UV/visible spectrophotometer; GE Healthcare Bio-Sciences, Uppsala, Sweden). The glucose concentration in the medium was measured with a glucose analyzer (Model 2300; YSI Co., Yellow Springs, OH, U.S.A.). The concentrations of acetate, D-lactate, and pyruvate in the sample were assayed with enzymatic test kits (Roche Applied Science, Indianapolis, IN, U.S.A.). β-Galactosidase activity was measured by a modified Miller assay as described previously [37].

Results

An *acs* Mutant Produced Acetate in Succinate Medium

In order to examine the effect of ACS expression on the acetate accumulation profile, we constructed an *acs* mutant of *E. coli* W3110 and analyzed the acetate accumulation in the culture broth. In a glucose minimal medium, the *acs* mutant and its parental strain showed similar acetate production profiles during the exponential growth phase (Fig. 1A). The *acs* mutation was not expected to affect acetate accumulation during the exponential growth phase on glucose because *acs* is repressed in the presence of glucose [15, 31]. During the stationary phase, however, the *acs* mutant did not use up all the excreted acetate and remained at 0.1 g/l (1.7 mM) of acetate in the culture broth whereas the parental strain consumed all the produced acetate (Fig. 2A). The result shows that ACS is responsible for the lower level scavenging of acetate below 0.1 g/l. Acetate above this level was consumed by the reverse direction of the Pta–AckA pathway as expected [17].

The *acs* mutant excreted and accumulated acetate in the culture broth when grew on succinate, which has been known as a non-acetogenic substrate for *E. coli* (Fig. 1B). Acetate had accumulated concurrently with cell growth and reached 0.06 g/l at the stationary phase after using up 2 g/l of succinate. The mutant could not reuse acetate during the stationary phase. As reported, the parental strain did not accumulate acetate in the culture broth when it grew on succinate. The wild type and the *acs* mutant showed similar cell yields on succinate. These results show that *E. coli* excretes acetate even when it grows on succinate, whereas derepressed acetyl CoA synthetase scavenges the excreted acetate.

Expression of *ack–pta* and *acs* Promoters in Batch Cultures

To investigate the effect of expression of acetate metabolic genes compared with the acetate accumulation profile of

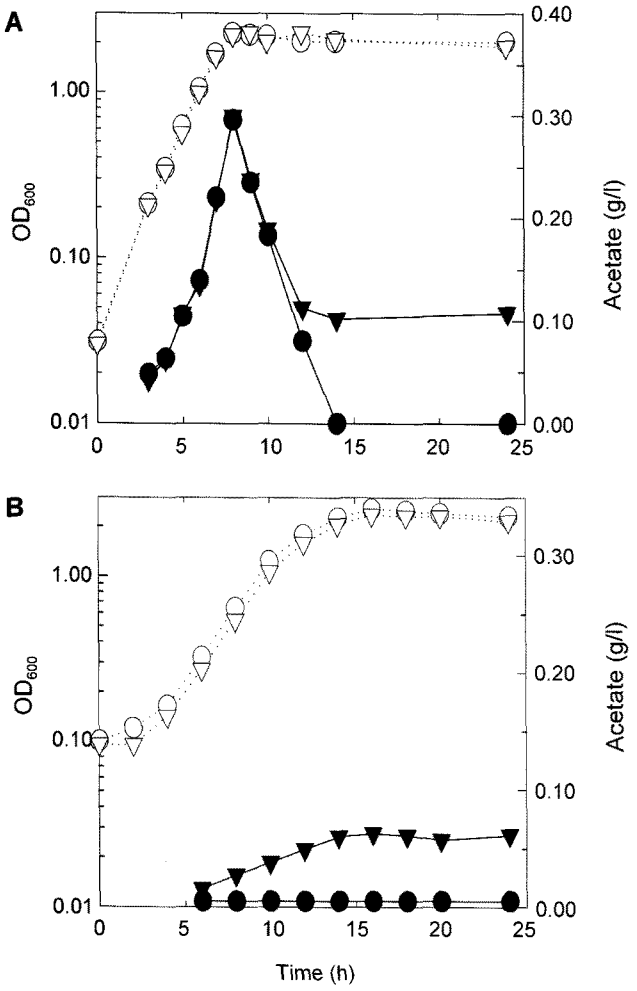

Fig. 1. Effect of *acs* mutation on the acetate profile in M9–glucose (**A**) and M9–succinate (**B**) media.
W3110 (circle) and W3110 *acs* mutant (inverted triangle) were grown in 0.2% glucose and 0.6% succinate and sampled at various time points. The concentration of acetate in the culture broth was determined with the Enzymatic BioAnalysis kit from Boehringer Mannheim Co. Open and filled symbols represent cell growth measured at OD_{600} and acetate concentration, respectively.

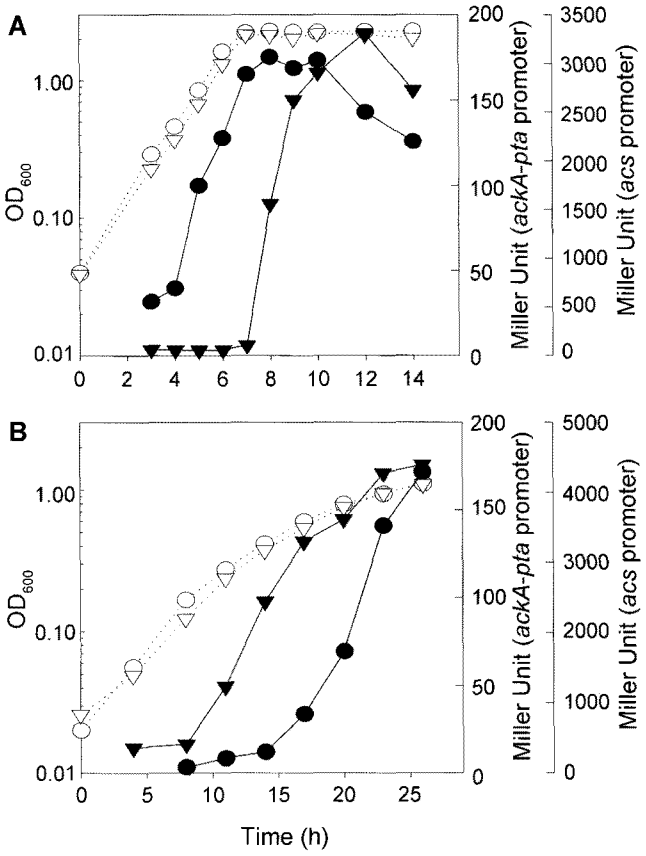

Fig. 2. Expression profiles of the *ackA–pta* and *acs* promoters in M9–glucose (**A**) and M9–succinate (**B**) medium.
W3110 Δ(*lacZ*) derivatives carrying the transcriptional *lacZ* fusion to the *ackA–pta* (circle) and *acs* (inverted triangle) promoters were grown in 0.2% of carbon sources and sampled at various time points. Open and filled symbols represent cell growth measured at OD_{600} and Miller units, respectively.

E. coli on glucose and succinate, we constructed single-copy transcriptional fusions of *lacZ* to the *ackA–pta* and *acs* promoters, respectively, in W3110 Δ(*lacZ*) and measured the promoter activities.

Because the Pta–AckA pathway is known as the major acetate utilization pathway, we examined whether acetate induces the expressions of these promoters. Exponentially growing cells harboring each promoter fusion in glucose M9 medium were washed with M9 buffer and then resuspended in M9–acetate (0.6%). Expression of the *ackA–pta* promoter did not increase in this condition, indicating that this promoter is not induced by acetate (data not shown). The expression pattern and level of the *ackA–pta* promoter in glycerol minimal medium and casamino acid were very similar to that in glucose, meaning that this promoter is not under the control of catabolite repression either (data not shown). The increase of promoter activity in the stationary phase, shown in Fig. 2A, is consistent with the increase of the amount of AckA and Pta in glucose-starvation condition, demonstrated by two-dimensional protein gel [25].

Fig. 2B showed the *ackA–pta* promoter expressed even in succinate, which is coincident with previous reports for enzymatic activities of Pta and AckA [3, 10]. In our condition, phosphotransacetylase activity of 16 h culture in 0.2% succinate medium was 1.511 U (specific activity is expressed as mmol of NADH formed per minute per milligram of protein), compared with 1.561 U of 6 h culture in 0.2% glucose medium.

The transcriptional response of *acs* to carbon sources was examined previously [16]. The cells grown on glucose quickly induced *acs* transcription when glucose was used up and shifted to the acetate consumption phase (Fig. 2A). The expression patterns of the single-copied *acs–lacZ* fusion were very similar with that analyzed using plasmid-

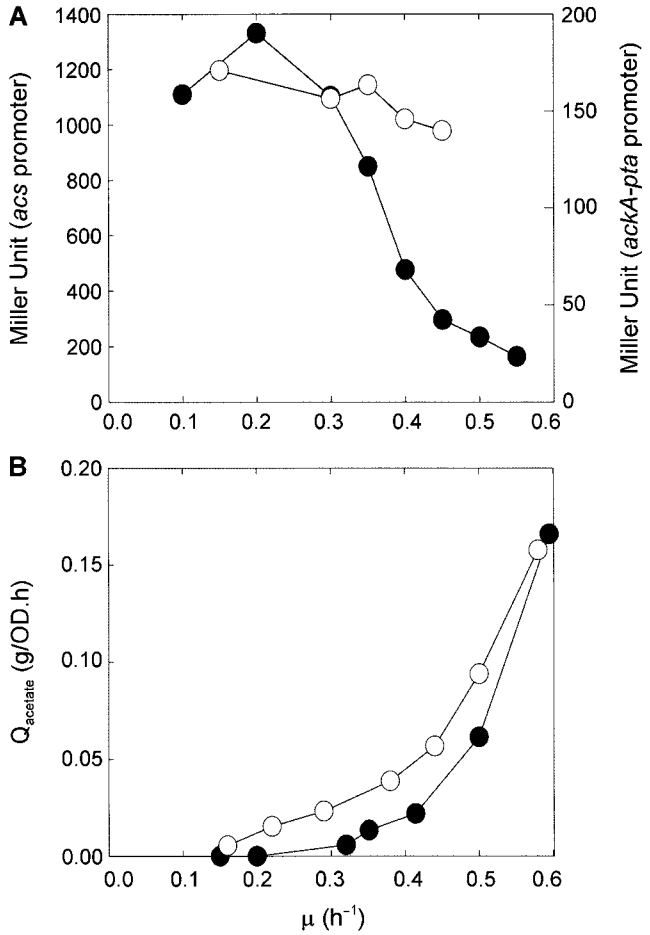

Fig. 3. A. Expression of *ackA–pta* and *acs* promoters in the glucose-limited continuous culture.
W3110 Δ(*lacZ*) derivatives carrying the transcriptional *lacZ* fusion to the *ackA–pta* (open circle) and *acs* (filled circle) promoters grown in 0.2% M9–glucose were shifted to continuous culture with a changed dilution rate and sampled at various μ values. **B.** Profiles of acetate production rate ($Q_{acetate}$) of W3110 (filled circle) and *acs* mutant (open circle) with various growth rates (μ). The detailed method is described in the text.

encoded *acs–cat* fusion [31]. As with other noncatabolite repressible carbon sources, the *acs* promoter was derepressed for the exponential phase in the succinate medium (Fig. 2B).

Expression of *ackA–pta* and *acs* Promoters in Chemostats

In the glucose-limited continuous culture, *E. coli* is known to not excrete acetate under certain threshold growth rates (μ_c) [6]. In order to examine the possible effect of expression of acetate metabolic genes on the acetate accumulation pattern, we first assayed the expression from the *ackA–pta* and *acs* promoters in glucose-limited continuous culture using the transcriptional fusion strains. The *acs* promoter activated when the dilution rate (*i.e.*, specific growth rate) was lower than 0.35 h^{-1}. In contrast, the *ackA–pta* promoter expressed constitutively with whole growth rate range whereas it decreased slightly with the increase of the growth

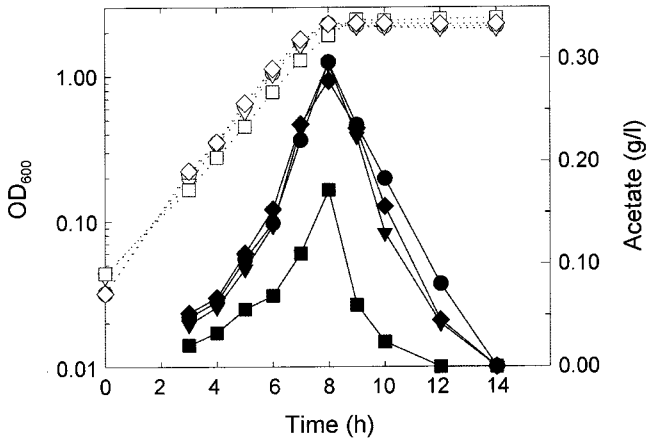

Fig. 4. Effect of derepression of *acs* and *aceBAK* on the acetate profiles of glucose batch culture.
W3110 (circle), W3110 acs^{derep}-Cm (inverted triangle), W3110 *iclR* and *fadR* double mutant (diamond), and W3110 *iclR fadR* acs^{derep}-Cm (square) were grown in 0.2% M9–glucose medium and sampled at various time points. Open and filled symbols represent cell growth measured at OD_{600} and acetate concentration, respectively.

rate (Fig. 3A). Notley and Ferenci [24] demonstrated three representative patterns of promoter expression in response to the growth rate in glucose-limited chemostats in *E. coli*; RpoS (*e.g.*, *osmY::lacZ*), cAMP (*e.g.*, *malT::lacZ*), and endoinducer (*e.g.*, *lamB::lacZ*) dependent patterns. The pattern of *acs* expression, shown in Fig. 4A, did not exactly match with any of the three patterns. Previously, it was reported that the *acs* gene is RpoS dependent, glucose repressible, and acetate inducible [15, 31]. The *acs* expression in glucose chemostats may be a combination of cAMP, endoinducer (acetate), and RpoS-dependent patterns.

Acetate Accumulation of the *acs* Mutant at Lower Growth Rate in a Glucose-Limited Culture

We used α-methyl glucose, a glucose analog, to make the glucose-limited culture condition as described by Andersen and von [1] and analyzed the specific acetate production rate ($Q_{acetate}$) at various growth rates (Fig. 3B). In 50 ml of M9 minimal medium with 0.2% of glucose and various amounts of α-methyl glucose, W3110 and *acs* mutant were cultivated and the OD and acetate concentration measured during the exponential growth phase. The $Q_{acetate}$ value was determined by multiplying the slope of acetate accumulation to OD by the growth rate of each culture ($Q_{acetate}=g_{acetate}$ OD^{-1} h^{-1}). The growth rate of wild type showing measurable $Q_{acetate}$ was 0.35 h^{-1} whereas that of *acs* mutant was 0.22 h^{-1}. Because *acs* mutation did not affect the growth pattern in the glucose medium (Fig. 1A), the difference in the $Q_{acetate}$ profiles should be the effect of the lack of ACS activity in the *acs* mutant. The polymorphism of *E. coli* alleles in an evolutionary study in the glucose-limited, long-term chemostat experiments showed coincidence with our result [29]. The majority of the polymorphism in the study was elucidated

Table 1. Strains, plasmids, and phages used in this study.

Strains/plasmids/phages	Characteristics	Source
Strains		
W3110	Wild-type *E. coli* K-12	Lab stock
W3110 Δ(*lacZ*)	*lacZ* deletion	Lab stock
W3110 *acs*::Km	*acs* inactivation Km cassette from pUC4K	This work
W31110 acs^{derep}-Cm	Derepressed *acs* integrated in *lacZ* site using pBRINT-Cm	This work
W3110 Δ(*lacZ*) P*acs*::*lacZ*	Single-copy *acs* promoter *lacZ* fusion using RS415 lysogenization	This work
W3110 Δ(*lacZ*) P*ack*::*lacZ*	Single-copy *ackA* promoter *lacZ* fusion using RS415 lysogenization	This work
W3110 *iclR fadR*	Wild type with *iclR-7 fadR613*::Tn*10*	This work
W3110 acs^{derep}-Cm *iclR fadR*	Derepressed *acs* clone with *iclR-7 fadR613*::Tn*10*	This work
CP367	$polA^{ts}$	[26]
CV101	Derepressed *acs* (acs^{derep})	[29]
JC7623	*sbcBC recBC*	CGSC
JRG861a	iclR-7	[31]
RS3032	*fadR613*::Tn*10*	[31]
Plasmids		
pUC4K	Km cassette harboring vector	GE Healthcare
pRS45	Promoterless *lacZ* fusion vector	[32]
pSK122	*acs* clone	[31]
pBRINT-Cm	*lacZ* integration vector	[2]
Phages		
405 clone of Kohara library	Kohara clone including *acs* gene	[14]
RS415	Lambda harboring *lac* operon fragment	[32]
P1	Generalized transducing phage	Lab stock

as a null mutation of *acs* and accumulated acetate at a growth rate of 0.2 h^{-1}.

The accumulation of acetate at the lower growth rate by *acs* mutant could be the effect of its inability to scavenge the excreted acetate, implying wild-type *E. coli* might excrete acetate even if it grows below the certain threshold growth rate (μ_c) and reuses them using derepressed ACS. On the basis of the O_2 consumption rate (QO_2) profile during chemostat, the acetate accumulation only above the certain threshold growth rate (μ_c) was explained by that the respiratory metabolic capacity of *E. coli* was saturated at μ_c and then the excess carbon flux from glycolysis flowed into the acetate production pathway [1, 9]. However, our results show that *E. coli* may excrete acetate even before the QO_2 limitation started and the expression of *acs* veils that.

Derepression of *acs* Decreased Acetate Accumulation

The scavenging of acetate by derepressed ACS activity at lower growth rate or in succinate media suggests that constitutive expression of ACS could be an effective approach to reduce the accumulation of acetate in the batch culture. To test this hypothesis, we constructed W3110 acs^{derep}-Cm harboring chromosomal incorporation of the derepressed *acs* allele from CV101, which was isolated by Rosenzweig *et al.* [29] and expressed ACS constitutively even in the presence of glucose. The avoidance of glucose repression in CV101 was due to an insertion of the IS30 element at the -37 site of the *acs* promoter (personal communication). The IS30 end offered a new -35 site of bacterial promoter.

In the glucose minimal medium, W3110 acs^{derep}-Cm showed a 38% increase of ACS activity (5.5–7.7 U) at the exponential phase (OD_{600}=0.8) and a 116% increase (11.7–24.5 U) at the stationary phase, respectively, comparing with those of wild type (the specific activity of ACS is expressed as mmole NADH formed per minute milligram of protein). However the acetate accumulation profile of this strain in the exponential phase was not significantly different from that of wild type (Fig. 4). We also found that the *acs* clone, originated from pGEM7Zf (+) (Promega), did not significantly reduce the acetate production in the exponential phase in the glucose medium (data not shown).

The apparent no-effect of constitutive expression of ACS could be the result of repression of the glyoxylate shunt, the next step of acetate catabolism [19, 20]. It was reported that the derepression of the glyoxylate shunt by introducing mutations in the *iclR* and *fadR* genes increased the rate of utilization of exogenous acetate [21]. IclR and FadR are known as repressors of the *aceBAK* operon encoding the genes involved in the glyoxylate shunt [19, 20, 36] and the *iclR-7* mutant is known to partially release the expression of *aceBAK*, even in glucose medium [34]. We transduced *iclR-7* (from JRG861a) and *fadR613*::Tn*10* (RS3032) to W3110 and W3110 acs^{derep}-Cm, as described in the previous report [31], and observed the acetate

accumulation phenotype. The W3110 acs^{derep}-Cm strain containing *iclR* and *fadR* showed drastically decreased acetate production during growth on glucose, compared with wild type, *iclR* and *fadR* double mutant, and acs^{derep}-Cm strain (Fig. 4).

Discussions

In this report, we showed that an W3110 *acs* mutant, which could not scavenge the acetate in the culture broth completely, accumulated acetate in the succinate batch culture and at very low specific growth rate in glucose-limited condition. These results suggest that *E. coli* constitutively secretes acetate even while it grows on gluconeogenic carbon sources and under a carbon limitation with very low growth rate. In theses conditions, the *acs* gene was derepressed (Fig. 2B) or induced (Fig. 3A) in the wild-type *E. coli*. However, the derepression of *acs* alone was not sufficient to consume all of the excreted acetate in the culture broth. As shown in Fig. 4, the acs^{derep} allele alone did not significantly affect the acetate profile in glucose medium unless the glyoxylate shunt enzymes were also derepressed. Lin *et al.* [18] also showed that the overexpression of ACS resulted in a significant reduction in acetate accumulation in glucose fermentation, but they did not clarity if the reduction was also observed during the exponential phase. This means that the derepression of both the acetate consumption pathway from acetate to acetyl-CoA and the assimilation of acetyl-CoA through the glyoxylate shunt are required to decrease the acetate accumulation in the glucose medium.

The *aceBAK* operon encoding the glyoxylate shunt enzymes is repressed by glucose, induced by short fatty acids including acetate, and derepressed in succinate medium [19, 20, 34]. Therefore, we supposed that *aceBAK* is expressed coordinately with the *acs* gene in succinate and at the slowly growing conditions. As shown in Fig. 4, the derepression of both the *acs* gene and *aceBAK* operon decreased acetate accumulation, even in the glucose batch culture. Based on these results, we suggest that the non-acetate accumulation phenotype of *E. coli* in succinate medium or in carbon-limited conditions is not because it does not produce acetate but because it reuses acetate using derepressed ACS and glyoxylate shunt. Sophisticated control of the expression of the acetate consumption pathway consisting of the ACS and glyoxylate shunt would be an approach of significance to avoid acetate accumulation in *E. coli* fermentation.

When *E. coli* produced and re-assimilated acetate simultaneously, as shown in Figs. 2B and 4B, the net result was waste of biological energy; where 1 mole of acetyl CoA converted to acetate *via* the Pta–AckA pathway accompanies 1 mole of ATP production, whereas activation of 1 mole of acetate to acetyl CoA consumes 2 mole of ATP. Besides, in succinate medium or in the carbon-limited conditions with lower growth rate below μ_c, the aerobic metabolism capacity of *E. coli* is not supposed to be saturated. The hypothesis that the aerobic acetogenesis of an overflow metabolism is for additional ATP generation cannot explain the constitutive production and re-assimilation of acetate in those conditions [1, 9, 10].

Acetate synthesis may provide *E. coli* a competitive advantage. In the natural habitat, the organism that consumes the common-interest nutrient faster can out-compete other species. In terms of energy efficiency, acetate synthesis is less effective than the complete oxidation of nutrient through the TCA cycle and respiration. Therefore, acetate synthesis induces a lower intracellular energy status, or at least maintains the energy status at a certain level so that it can maintain or increase the maximum uptake rate of nutrient. *E. coli* grown in conditions that reduce the energy status of the cell, like anaerobic culture, showed a larger glucose uptake rate than in aerobic condition [5]. In addition, the majority of clones of a polymorphic population of *E. coli* in a glucose-limited, long-term continuous culture had accumulated genetic changes that increase the glucose uptake kinetics [29]. Therefore, it seems that gaining a predominance of nutrient uptake through the less economical but faster acetate synthesis may be beneficial for the competition. In addition to the induction of a less economical catabolism, the excretion of acetate may be directly a part of adaptation that enhances glucose uptake through increase of the total pool size of phosphoryl donor for enzyme I of PTS by the action of acetate kinase [8, 29].

The other potential role of acetate synthesis, even in the condition known as non-acetogenic, is providing acetyl phosphate that is proposed to be involved in various cellular regulations. As a phosphate donor of two-component regulatory signal transduction in bacteria, acetyl phosphate is known to be a general signal of cellular metabolism [22, 27, 28, 30]. Acetyl phosphate is also known to be involved in gene expression, starvation survival [25], virulence of *Streptococcus pneumoniae* [33], and to function as a signal of carbon/nitrogen balance affecting PHB metabolism in *Cyanobacter* [23].

Acknowledgments

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