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Acetyl-CoA synthetase overexpression in *Escherichia coli* demonstrates more efficient acetate assimilation and lower acetate accumulation: a potential tool in metabolic engineering

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Abstract The overexpression of acetyl-CoA (CoA) synthetase (ACS) in *Escherichia coli* showed significant reduction in acetate during glucose fermentation. It also greatly enhanced acetate assimilation when acetate was used as a carbon source. These features are ideal for applications in metabolic engineering. ACS overexpression can be strategically applied to reduce acetate byproduct, recover wasted carbon, and redirect carbon flux toward more favorable pathways. The native *acs* gene was cloned and overexpressed in *E. coli*. Studies showed significant effects on acetate production and assimilation in cultures grown in minimal and complex media with glucose or acetate as the carbon source.

Introduction

Escherichia coli is known to produce significant amounts of acetate during excess glucose fermentation in high cell density cultures. Under aerobic conditions, acetate is the major byproduct produced. High acetate accumulation is harmful to cell growth as it decouples transmembrane pH gradients. This negatively affects internal osmotic pressure, intracellular pH and amino acid synthesis. An excellent review on the acetate switch is provided by Wolfe (2005). Many strategies have been successfully utilized to reduce acetate production during glucose metabolism in order to increase product formation. Examples include overexpression of phosphoenolpyruvate carboxylase (Farmer and Liao 1997), overexpression of the non-indigenous pathway acetolactate synthase (Aristidou et al. 1995), and inactivation of acetate kinase-phosphotransacetylase (ACKA-PTA) and pyruvate oxidase (Dittrich et al. 2005; Lin et al. 2005b; Sanchez et al. 2005; Tomar et al. 2003; Vadali et al. 2004c).

In this study, an alternative concept addressing the acetate conundrum in *E. coli* is presented. Acetate can be utilized as a carbon source from secreted acetate or exogenous acetate. Enhancing the capability of *E. coli* to assimilate acetate would reduce harmful effects of acetate, recycle wasted carbon, and increase carbon flux toward desired pathways. This would provide a potentially useful tool in metabolic engineering. For this strategy, acetyl-Coenzyme A (CoA) synthetase (ACS) is overexpressed in *E. coli* to enhance the assimilation of acetate and the activation to acetyl-CoA. ACS carries an irreversible reaction that converts acetate to acetyl-CoA via two enzymatic steps. The first step is the reaction of acetate with adenosine triphosphate (ATP) to form acetyl-adenosine monophosphate (AMP). Acetyl-AMP then reacts with CoA to form acetyl-CoA-releasing AMP (Kumari et al. 1995). *E. coli acs* has been cloned and characterized, and its regulation has been studied (Kumari et al. 1995, 2000). ACS is regulated at the transcriptional level. It functions anabolically as it is active during stationary phase to enable cells to utilize secreted acetate. ACS has high affinity for acetate (Km of 200 μM), which allows it to function at low acetate concentrations. On the other hand, the reversible ACKA-PTA can assimilate acetate only at high acetate concentrations (Km of 7 to 10 mM) (Wolfe 2005). The overexpression of ACS in *E. coli* showed significant reduction in acetate during glucose metabolism. It also greatly enhanced the assimilation of acetate when used as the sole carbon source. These characteristics warrant ACS

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overexpression as a positive approach to coping with acetate in *E. coli* fermentations.

Materials and methods

Strains and plasmids

The wild-type *E. coli* MG1655 was used for ACS expression experiments. The expression vector used to overexpress ACS is pUC19. The native *E. coli acs* was cloned by polymerase chain reaction (PCR) from purified *E. coli* genomic DNA. The forward primer used is 5' GCTCTA GAGCATATGAGCCAAATTCACAAACACACC 3' and the reverse primer used is 5' GGAATTCCTTACGATGG CATCGCGATAGC 3'. The forward primer is flanked by *Xba*I and the reverse primer is flanked by *Eco*RI both at the 5' ends. The MasterTaq kit from Eppendorf was used for PCR. The restriction enzymes were purchased from Promega and New England Biolabs. The ligation kit used was from Sigma Aldrich. The *acs* PCR product (2.0 kb) was thoroughly digested with *Xba*I and *Eco*RI, and then subsequently ligated into pUC19 (2.7 kb) at the *Xba*I and *Eco*RI sites. This new plasmid was designated pNC5 (4.6 kb). Then pNC5 was transformed into MG1655 to form the experimental strain MG1655(pNC5), and pUC19 was transformed into MG1655 to form the control strain MG1655(pUC19). Strains MG1655(pNC5) and MG1655 (pUC19) confer ampicillin resistance.

Medium and experimental conditions

The minimal medium used in experiments was M9. M9 consists of the autoclaved salt solution (7 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl and 1 g/l NH_4Cl) added with filter-sterilized thiamine, $MgSO_4$ and $CaCl_2$ at final concentrations of 0.6 μg/l, 0.1 and 0.1 mM, respectively. The complex medium used was Luria-Bertani (LB) (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl). All the media were added with isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM for induction. Ampicillin was added to a final concentration of 100 mg/l for selection pressure. Glucose and acetate were added to the media as carbon sources at different concentrations depending on the experiment. All the media used were adjusted to pH 7.5.

All experiments were performed aerobically. Flask cultures were grown at 37°C and 250 rpm. The fermentation time varied depending on the experiment. Each flask contained 50 ml of medium. The inoculum used per flask was 100 μl from an overnight-grown LB culture double-washed before use. All flask experiments were performed in triplicates.

Batch reactor experiments were carried out in a 1.4-l New Brunswick Scientific Bioflo 110 fermenters with a working volume of 600 ml. A 1% (v/v) inoculum was used from an overnight culture grown from a single colony for 12 h. The pH was measured using a glass electrode and controlled at pH 7.0 using 1.5N HNO_3 and 2N Na_2CO_3. The temperature was maintained at 37°C, and the agitation speed was constant at 500 rpm. The inlet airflow used was 1.0 vvm. The dissolved oxygen was monitored using a polarographic oxygen electrode (New Brunswick Scientific) and maintained above 50% saturation throughout the experiment.

Analytical techniques

Optical density (OD) was measured at 600 nm with a spectrophotometer (Bausch and Lomb Spectronic 1001); the culture was diluted to the linear range with 0.15 M NaCl and the dilution factor was accounted in the calculation of OD. For analyzing acetate and glucose, 1 ml of culture was centrifuged and the supernatant was then

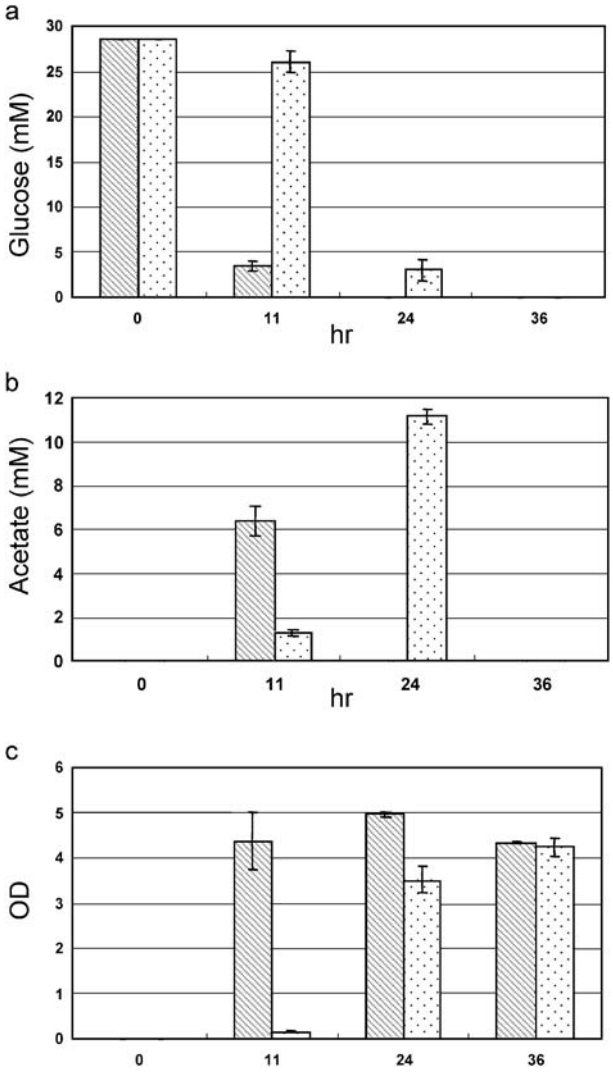

Fig. 1 Analysis of cultures grown in M9 medium with glucose. **a** Remaining glucose concentration after 11, 24, and 36 h of growth. **b** Acetate concentration after 11, 24, and 36 h of growth. **c** OD after 11, 24, and 36 h of growth. *Hatched bar* is strain MG1655(pNC5) overexpressing ACS, and *dotted bar* is the control strain MG1655 (pUC19)

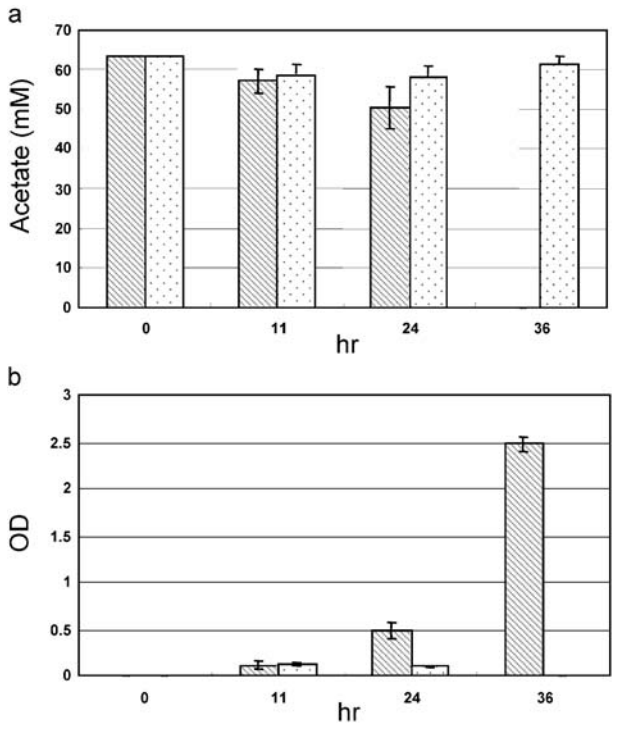

Fig. 2 Analysis of cultures grown in M9 medium with acetate. **a** Remaining acetate concentration after 11, 24, and 36 h of growth. **b** OD after 11, 24, and 36 h of growth. *Hatched bar* is strain MG1655 (pNC5) overexpressing ACS, and *dotted bar* is the control strain MG1655(pUC19)

filtered through a 0.45-μm syringe filter for high-performance liquid chromatography (HPLC) analysis. The HPLC system (Shimadzu-10A Systems, Shimadzu, Columbia, MD) used was equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), an ultraviolet detector (Shimadzu SPD-10A) and a differential refractive index detector (Waters 2410, Waters, Milford, MA). A 0.6-ml/min mobile phase using 2.5 mM H_2SO_4 solution was applied to the column. The column was operated at 55°C.

Enzyme assay

Crude extracts for the ACS assay were prepared by taking 20 OD units of culture [OD_{660nm}×vol (ml)=20] and centrifuging the appropriate volume at 5,000×*g* and 4°C for 20 min. The cell pellet was then washed once in 15 ml of ice-cold 100 mM Tris-HCl buffer (pH 8.0). The pellet was then centrifuged again and resuspended in 10 ml of the same buffer. The cells were then subjected to sonication for 10 min in an ice bath. The sonicated cells were centrifuged at 5,000×*g* and 4°C for 60 min to remove the cell debris. The supernatant was then used for the enzyme assay.

The ACS activity was measured by a modified method of Jones and Lipmann (1955) and Brown et al. (1977). ACS activates acetyl-CoA and AMP from the catalysis of acetate, ATP and CoA. Hydroxylamine is added in the assay to react with the acetyl-CoA formed in the reaction. This liberates the CoA and acetohydroxamate, which can be measured at 520 nm. The enzyme assay was performed in triplicates and the specific enzyme activities were expressed in units of U/mg, which is micromole of acetate converted to acetyl-CoA per minute time and milligram protein. Total protein concentration of the crude extract was measured by Lowry's method (Sigma Lowry Reagent, Modified) using bovine serum albumin as standard.

Results

The effect of overexpression of ACS on acetate accumulation and assimilation in *E. coli* was examined. The native *E. coli acs* was cloned from the genomic DNA and inserted into the expression vector pUC19. This plasmid designated pNC5 was used to overexpress ACS in the wild-type strain MG1655. Based on the enzyme assay, strain MG1655 (pNC5) exhibited specific ACS activity ninefold higher than the control strain MG1655(pUC19) (data not shown). Aerobic experiments were performed to compare strain MG1655(pNC5) with the control strain MG1655(pUC19). Cultures of MG1655(pNC5) grown in minimal (M9) medium with glucose exhibited faster glucose consumption than the control strain MG1655(pUC19). After 11 h of fermentation, strain MG1655(pNC5) consumed 88% of the initial glucose, whereas the control strain MG1655 (pUC19) only consumed 9% (Fig. 1a). This result also

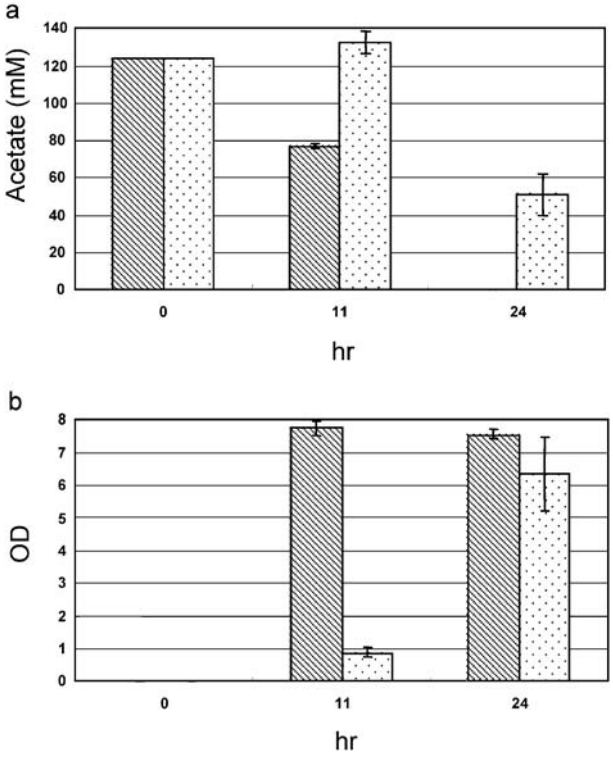

Fig. 3 Analysis of cultures grown in LB medium with acetate. **a** Remaining acetate concentration after 11 and 24 h of growth. **b** OD after 11 and 24 h of growth. *Hatched bar* is strain MG1655(pNC5) overexpressing ACS, and *dotted bar* is the control strain MG1655 (pUC19)

correlated with the faster growth rate of MG1655(pNC5) with ACS overexpressed. After 11 h of fermentation, strain MG1655(pNC5) reached 4.4 OD_{600}, whereas the control strain MG1655(pUC19) took 36 h to reach 4.2 OD_{600} (Fig. 1c). During the fermentation, the acetate accumulation was observed to be substantially lower in cultures of MG1655(pNC5) than in cultures of MG1655(pUC19) (Fig. 1b). Secreted acetate in the culture was reassimilated faster by strain MG1655(pNC5) than strain MG1655 (pUC19) (Fig. 1b). The lower acetate accumulation and faster acetate assimilation observed in the cultures of strain MG1655(pNC5) were apparently due to the overexpression of ACS. Increased ACS levels presumably enhanced the activation of acetate to acetyl-CoA. This metabolic utilization of acetate can contribute to lipid biosynthesis or oxidation via the tricarboxylic acid (TCA) cycle (Brown et al. 1977). This could explain why overexpression of ACS in strain MG1655(pNC5) increased its glucose consumption and growth rates.

Aerobic experiments were performed in M9 medium supplemented with acetate to explore how overexpression of ACS improves the consumption of acetate as the sole carbon source. Results showed that MG1655(pNC5) was able to consume the supplemented 64 mM of acetate in less than 36 h (Fig. 2a). The control strain MG1655(pUC19) was not able to consume any of the acetate in 36 h; therefore, it did not grow. Strain MG1655(pNC5) reached 2.5 OD_{600} in less than 36 h (Fig. 2b). The results demonstrate the significant impact of ACS overexpression in acetate-grown cultures. A similar experiment was carried out with a LB medium instead of minimal medium to show that acetate consumption is equally affected by ACS overexpression in a complex medium. The LB medium was supplemented with 124 mM of acetate. After 11 h, strain MG1655(pNC5) consumed 38% of the acetate, whereas the control strain MG1655(pUC19) did not consume any (Fig. 3a). All 124 mM of acetate was consumed in less than 24 h by strain MG1655(pNC5). Strain MG1655(pUC19) consumed only 59% of the supplemented acetate after 24 h. Cultures of strain MG1655(pNC5) were at 7.8 OD_{600} at the 11th hour of fermentation and remained constant for the rest of the 24 h (Fig. 3b). The cultures of the control strain MG1655 (pUC19) did not reach 6.3 OD_{600} until after 24 h of fermentation. These results clearly demonstrated the effectiveness of ACS overexpression on improving acetate utilization. Secreted acetate and supplemented acetate can both be efficiently assimilated to enhance carbon flux toward product formation when ACS is overexpressed in *E. coli*.

Experiments in batch reactor were also carried out to examine the effect of ACS overexpression on acetate accumulation during growth. The experiments were performed under aerobic conditions with the LB medium supplemented with glucose. The cultures of strain MG1655 (pNC5) in the bioreactor showed the same characteristics as those in the flasks. Acetate accumulation was lower in the cultures of strain MG1655(pNC5) than in the cultures of the control strain MG1655(pUC19) (data not shown). Strain MG1655(pNC5) also assimilated the secreted acetate faster than strain MG1655(pUC19). Overall, the biomass yield (Yx/s) and specific growth rate were higher for strain MG1655(pNC5).

Discussion

Many favorable product formation pathways branch from the acetyl-CoA node in the central metabolic network of *E. coli*. Products not indigenous to *E. coli* such as isoamyl acetate and poly(3-hydroxybutyrate) require acetyl-CoA as a substrate for formation. The application of ACS overexpression in increasing the flux toward acetyl-CoA from acetate assimilation can be potentially useful for enhancing the production of these products. Isoamyl acetate production was increased when pantothenate kinase was overexpressed to increase the intracellular acetyl-CoA pool in isoamyl acetate-producing strains (Vadali et al. 2004a,b). The production of native metabolites such as the specialty chemical succinate can also be increased by empowering the strain with efficient acetate assimilation via ACS overexpression. One example of such an application is in genetically engineered *E. coli* strains that can solely produce succinate under aerobic conditions (Lin et al. 2005a–c). This strain requires acetyl-CoA and oxaloacetate for succinate production through the glyoxylate cycle and TCA cycle. A mixed carbon source feed of acetate and glucose coupled with overexpression of ACS can enhance the carbon flux toward acetyl-CoA and oxaloacetate to increase succinate production. Overall, ACS overexpression in *E. coli* offers the advantages of reduced acetate accumulation and enhanced acetate assimilation, both of which can improve cell growth and product formation.

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References

Aristidou AA, San K-Y, Bennett GN (1995) Metabolic engineering of *Escherichia coli* to enhance recombinant protein production through acetate reduction. Biotechnol Prog 11:475–478

Brown TDK, Jones-Mortimer MC, Kornberg HL (1977) The enzymic interconversion of acetate and acetyl-coenzyme A in *Escherichia coli*. J Gen Microbiol 102:327–336

Dittrich CR, Vadali RV, Bennett GN, San K-Y (2005) Redistribution of metabolic fluxes in the central aerobic metabolic pathway of *E. coli* mutant strains with deletion of the *ackA-pta* and *poxB* pathways for the synthesis of isoamyl acetate. Biotechnol Prog 21:627–631

Farmer WR, Liao JC (1997) Reduction of aerobic acetate production by *Escherichia coli*. Appl Environ Microbiol 63:3205–3210

Jones ME, Lipmann F (1955) Aceto-CoA-kinase. Methods Enzymol 1:585–595

Kumari S, Tishel R, Eisenbach M, Wolfe AJ (1995) Cloning, characterization, and functional expression of *acs*, the gene which encodes acetyl coenzyme A synthetase in *Escherichia coli*. J Bacteriol 177:2878–2886

Kumari S, Beatty CM, Browning DF, Busby SJM, Simel EJ, Hovel-Miner G, Wolfe AJ (2000) Regulation of acetyl coenzyme A synthetase in *Escherichia coli*. J Bacteriol 182:4173–4179

Lin H, Bennett GN, San K-Y (2005a) Fed batch culture of a metabolically engineered *Escherichia coli* strain designed for high level succinate production and yield under aerobic conditions. Biotechnol Bioeng 90:775–779

Lin H, Bennett GN, San K-Y (2005b) Genetic reconstruction of the aerobic central metabolism in *Escherichia coli* for the absolute aerobic production of succinate. Biotechnol Bioeng 89:148–156

Lin H, Bennett GN, San K-Y (2005c) Metabolic engineering of aerobic succinate production systems in *Escherichia coli* to improve process productivity and achieve the maximum theoretical succinate yield. Metab Eng 7:116–127

Sanchez AM, Bennett GN, San K-Y (2005) Novel pathway engineering design of the anaerobic central metabolic pathway in *Escherichia coli* to increase succinate yield and productivity. Metab Eng 7:229–239

Tomar A, Eiteman MA, Altman E (2003) The effect of acetate pathway mutations on the production of pyruvate in *Escherichia coli*. Appl Microbiol Biotechnol 62:76–82

Vadali RV, Bennett GN, San K-Y (2004a) Applicability of CoA/acetyl-CoA manipulation system to enhance isoamyl acetate production in *Escherichia coli*. Metab Eng 6:294–299

Vadali RV, Bennett GN, San K-Y (2004b) Enhanced isoamyl acetate production upon manipulation of the acetyl-CoA node in *Escherichiacoli*. Biotechnol Prog 20:692–697

Vadali RV, Bennett GN, San K-Y (2004c) Production of isoamyl acetate in *ackA-pta* and/or *ldh* mutants of *Escherichia coli* with overexpression of yeast ATF2. Appl Microbiol Biotechnol 63:698–704

Wolfe AJ (2005) The acetate switch. Microbiol Mol Biol Rev 69:12–50