Inhibition of *ackA* and *pta* genes using two specific antisense RNAs reduced acetate accumulation in batch fermentation of *E. coli* BL21 (DE3)

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Abstract
Expression of foreign proteins in *E. coli* is normally inhibited by exogenous production of acetate. To overcome this problem, various strategies have been proposed and tested to reduce the extent of acetate accumulation. Although these strategies can improve the outcome, the implementation of their proposed techniques is not practical. Because to achieve optimal results, it requires extremely tight control conditions and the actual cost is very high. Furthermore, a simple knockout mutation of the target metabolic pathway would not be appropriate because the acetate pathway plays an important physiological role in *E. coli*. In this study, we employed an antisense RNA strategy as an elaborated metabolic engineering tool to partially block biosynthesis of two major acetate pathway enzymes, acetate kinase (ACK) and phosphotransacetylase (PTA). The fragments of antisense cassette were cloned sequentially in pBluescriptsk+ and completed cassette subcloned in pLT10T3. The function of this cassette was evaluated with RT-PCR and ACK and PTA assay. The effect of cassette on cell physiology was monitored by determination of optical density, glucose consumption and acetate production. We found that the antisense method partially reduced mRNA levels of the target genes, lowered the concentration of acetate in culture media and increased growth rate and final cell density in antisense-regulated strain. This strategy could provide us with a useful, inexpensive and practical tool to achieve a large-scale protein production system.

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INTRODUCTION

It has been observed that *E. coli* converts 10 to 30% of carbon flux to acetate in a glucose-containing media even when the culture is fully aerated. Acetate production is a main problem in batch fermentations that leads to a reduced recombinant protein yields (Babaeipour *et al*., 2008; Shojaosadati *et al*., 2008; Reiling *et al*., 1985; Mori *et al*., 1979; Landwall and Holme, 1977). Several approaches have been employed to avoid the accumulation of acetate in the medium. Acetate is produced mainly through the actions of phosphotransacetylase (encoded by *pta gene*) and acetate kinase (encoded by *ackA*) through the following reactions respectivly: 1) acetyl-CoA + Pi↔acetyl-P + CoA; 2) acetyl-P + ADP↔acetate + ATP. Recently genetic methods have been applied to obtain *pta* mutants, which shows decreased acetate excretion (Bauer *et al*., 1990). However, in some cases, the *pta* knockout mutation leads to high pyruvate production, which is also undesirable (Diaz-Ricci *et al*., 1991). Construction of mutants with modified glucose uptake rates (Chou *et al*., 1994) and channeling of carbon flux away from acetate production towards other end metabolites have been also reported (Aristidou *et al*., 1994; Dedhia *et al*., 1994; Diaz-Ricci *et al*., 1991; Bauer *et al*., 1990). In recent years, the use of antisense RNA is considered for partial repression

of target gene. Antisense RNAs are very effective in the inhibition of target gene expression in a sequence-specific manner. This technology can provide us with an inexpensive tool to down-regulate target genes. In this study we have applied antisense RNA strategy, to obtain an *E. coli* strain with a limited production of acetate. This would help to maintain a longer life culture, and a better production of recombinant protein. E. *coli* BL21 (DE3), is a widely used host strain for over-expression of recombinant proteins. We have synthesized antisense RNAs against *pta* and *ackA* genes to lower acetate production and improve our recombinant protein expression system.

MATERIALS AND METHODS

Strains, media, and culture conditions: XL1-Blue (endA1 gyrA96 (nal^R) thi-1 recA1 relA1 lac glnV44 F'[: Tn10 proAB$^+$ lacI^q Δ (lacZ) M15] hsdR17 (r_K^- m_K^+), Tetracyline resistant) (Stratagene) for plasmid construction.BL21 (DE3) (F$^-$ ompT gal dcm lon hsdS_B (r_B^- m_B^-) λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) (Novagen) for observation of effect of antisense cassette.

All strains were grown at 37ºC on Luria-Bertani (LB) (Applichem) complex medium in process stages and stored on glycerol-stock at -80ºC. Ampicillin (50 µg/ml) was added as a selection pressure for strains which have plasmid. LB medium was used for cells growth and strain construction, and M9 medium was used for preparation of seed culture. For physiological characterizations, all strains were cultured in M9 modified medium consisted of 55.5 mM glucose, 86.1 mM K_2HPO_4, 55.1 mM KH_2PO_4 ,10.4 mM Citric acid, 18.9 mM $(NH_4)_2SO_4$, 8.1 mM $MgSO_4.7H_2O$, and 1 ml trace element solution per liter. The trace element solution contained 10.1 mM $FeSO_4.7H_2O$, 10.1 mM $MnCl_2.4H_2O$, 10 mM $CoSO_4.7H_2O$, 10.2 mM $CaCl_2.2H_2O$, 1.2 mM $CuCl_2.2H_2O$, and 1 mM $ZnSO_4.7H_2O$ g per liter in 1 M HCl. Batch cultivation were carried out in a 2 l bench-top bioreactor (Inforse AG Ch-4103, Switzerland) with the working volume of 1 l, including two six-blade Rushton impellers with a speed range of 50-1200 rpm. 300 µl of freezer stock were grown overnight (16-18 h) at 37ºC in 50 ml of M9 medium in a 250-ml flask. All cultures were grown at 37ºC, and ampicillin (120 µg /ml) was added as a selection pressure for plasmid-bearing strains.

Batch cultivation: A batch culture was initially established by the addition of 100 ml of an overnight-incubated seed culture (CDW = 0.4-0.6 g/l) to the bioreactor containing 900 ml of M9 modified medium. The pH was maintained at 7 by the addition of 25% (w/v) NH_4OH or 3 M H_3PO_4 solutions. Dissolved oxygen was controlled at 30-40% (v/v) of air saturation by controlling of both the inlet air (which was enriched with pure oxygen) and agitation rate. Foam was controlled by the addition of silicon-antifoaming reagent. The growth continued until depletion of initial glucose in the medium, as indicated by a rapid increase in the dissolved oxygen concentration.

Construction of plasmids with antisense cassette: The plasmid, derived from pLT10T3 (BCCM), was constructed to produce the antisense RNAs (asRNA) targeted against two enzymes, acetate kinase (ACK) and phosphotransacetylase (PTA); this plasmid was named PL6 (Fig. 1). Antisense cassette included the promoter of *ackA*, antisense to both *ackA* and *pta*, and the termination sequence of *pta*. Each was obtained from the *E. coli* K12 genome (NCBI databank) (Kakuda *et al*., 1994) using polymerase chain reaction (PCR) amplification with a DNA thermal cycler (AutoQ server thermal cycler, Quanta biotech Ltd). As same as done previously (Kim and Cha, 2003) the promoter region, extending from -79 to +21, was selected to include an open complex region for transcription initiation, -10 (TATA box), -35, a spacer region between these two regions, and the UP element located from -40 to -60 of the *ackA* gene (Aiyar *et al*., 1998; Mooney *et al*., 1998). The lengths of antisense genes against *ackA* and *pta* were determined 155 bp for *ackA* and 141 bp for *pta*, including a ribosome binding site (RBS). The ρ-independent terminator region of the original *pta* gene was selected as a termination sequence following each antisense gene (Mooney *et al*., 1998). All primers used in PCR for the construction of the antisense cassette are listed in Table 1.

Analytical procedures: Cell growth was monitored by measuring the optical density at 600 nm in a spectrophotometer (DU-640; Cecil). Samples for metabolite measurements were taken every 1hour and spun down in a centrifuge with 9000 g for 5 min (Biofuge Pico; Heraeus). The supernatant was retained and stored at -80ºC until assayed. The glucose concentration in the medium was measured using the GOD-PAP kit (Biolab, France). Acetate concentrations were determined with enzymatic test kits (Boehringer-Mannheim, Darmstadt, Germany). Relative activities

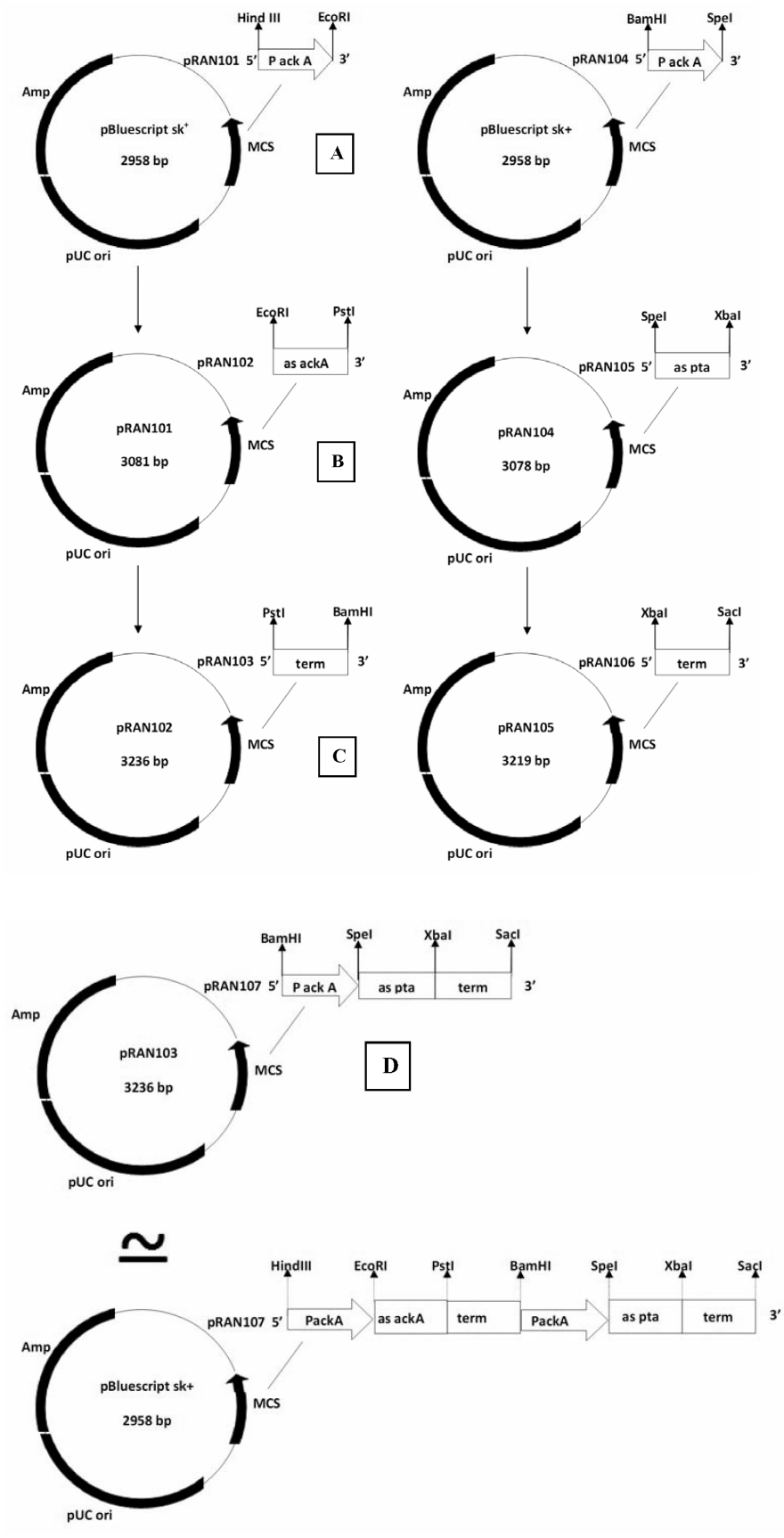

Figure 1. Schematic diagram of stages of recombinant plasmid construction.

Table 1. Primers used for PCR amplification of promoter of *ackA*, antisense *ackA*, antisense *pta*, and termination sequence of *pta*.

Target gene	Name of primer	Sequence of primers (5' → 3')
Promoter of *ackA*	Forward (for *asackA*)	5' GA.AGC.TTG.GCA.TAG.ACT.CAA.GAT.ATT.TC 3'
	Reverse (for *asackA*)	5' AAG.AAT.TCG.TCA.GGG.AGC.CAT.AGA.G 3'
	Forward (for *aspta*)	5' CCG.GAT.TCT.AGA.CTC.AAG.ATA.TTT.CTT.CC 3'
	Reverse (for *aspta*)	5' GGA.ACT.AGT.GTC.AGG.GAG.CCA.TAG.AG 3'
Antisense *ackA*	Forward (for *asackA*)	5' CTG.CAG.TAC.GCT.CTA.TGG.CTC.CC 3'
	Reverse (for *asackA*)	5' CGG.AAT.TCC.TCT.TCA.CCA.TTT.ACT.GC 3'
Antisense *pta*	Forward (for *aspta*)	5' TTC.TAG.AGC.TGT.TTT.GTA.ACC.CGC.C 3'
	Reverse (for *aspta*)	5' CAC.TAG.TAT.TGC.ACG.GAT.CAC.GCC 3'
terminator region of *pta*	Forward (for *asackA*)	5' CTG.CAG.TCT.CTC.GTC.ATC.ATC.CGC 3'
	Reverse (for *asackA*)	5' AAG.GAT.CCA.TGC.AGC.GCA.GTT.AAG.C 3'
	Forward (for *aspta*)	5' CCT.CTA.GAT.CTC.GTC.ATC.ATC.GCA 3'
	Reverse (for *aspta*)	5' GAG.CTC.ATG.CAG.CGC.AGT.TAA.G 3'

of ACK and PTA were measured by applying the coupled reaction of Brown *et al*. (1977). Cell extracts were prepared in this way: 50 ml of culture broth at mid-log phase (~1.0 of OD_{600}) from flask culture as described above was harvested at 5000 rpm for 10 min at 4ºC and rinsed two times with 30 ml of washing buffer (10 mM $MgCl_2$, 10 mM sodium phosphate (pH 7.5), 1mM EDTA). The cells were suspended in 1 ml of washing buffer, sonicated for 5 min on ice, and centrifuged at 13000 rpm for 30 min at 4ºC. Protein concentration in the supernatant, as crude extract, was measured by the Bradford method using the Bio-Rad dye reagent with bovine serum albumin as a protein standard. ACK assay was done in the mixture with this composition, 15 mM malic acid, 3.75 mM CoA, 22.5 mM NAD^+, 20 µg/ml malate dehydrogenase, 20 µg/ml citrate synthase, 60 mM ATP, 1 U/ml PTA, 225 mM Tris-HCl (pH 7.6), 4.5 mM $MgCl_2$, and 12.5 mM sodium acetate. PTA assay was achieved in the reaction mixture of 15 mM malic acid, 3.75 mM CoA, 22.5 mM NAD^+ ,20 µg/ml malate dehydrogenase, 20 µg/ml citrate synthase, 10 mM AcP, 225 mM Tris-HCl (pH 7.6), 4.5 mM $MgCl_2$. The reaction was initiated by adding of crude extract with equal protein concentration. Activities of ACK and PTA were measured by monitoring of absorbance at 340 nm (1 U= 1 µmol/min, ε_{340} = 6.22 mM/cm).

Semi-quantitative analysis of mRNA transcription by RT-PCR: Total RNA extraction was achieved in accord with the manner of Karunakaran and Kuramitsu (1996). Each culture (5 ml) was harvested at mid-log phase (~1.0 of OD_{600}). Samples were treated by an alkali lysis method and rinsed with ethanol. After drying, the RNA pellet was dissolved in DEPC treated double-distilled water and then treated with DNase I (Fermentas) to eliminate genomic DNA. Any remaining DNase was heat-inactivated at 65ºC for 10 min.

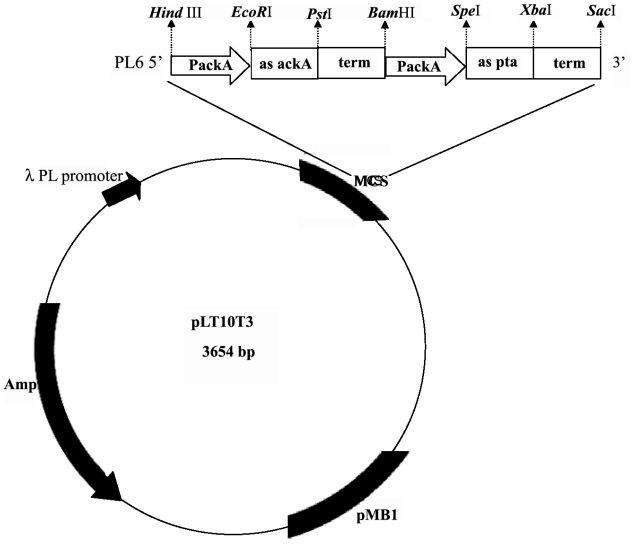

Figure 2. Schematic diagram of recombinant plasmid. PL6 is derived from pLT10T3 and contains the antisense genes against both *ackA* and *pta*. Each antisense gene was transcribed under the promoter of *ackA* and terminated by the termination sequence of *pta*.

Table 2. Primers used in RT-PCR for analysis of mRNA transcription for *ackA* and *pta*.

Target gene	Name of primer	Sequence of primers (5'→3')
ackA	Forward	5'CGATGCAGTAAATGGTGAAGAG3'
	Reverse	5' ATCAGCGCAGTGTAGGCAC 3'
	Forward	5' AGGAAGCGGCTTTAGGTG 3'
	Reverse	5' ATCAGCGCAGTGTAGGCAC 3'
pta	Forward	5' CCGTATTATTATGCTGATCC3'
	Reverse	5' GCTGTACCGCTTTGTAGG3'
	Forward	5'GTGCTGATGGAAGAGATCG3'
	Reverse	5' GCTGTACCGCTTTGTAGG3'

The RNA concentration of each sample was found out by absorbance at 260 nm using a UV-visible spectrophotometer (1 absorbance unit as 40 µg of RNA) (Roche applied science lab faqs, 2008). Quantitative analyses for mRNA transcription levels of *ackA* and *pta* genes were performed using reverse transcription (RT) PCR. Quantity of total RNA as a template for RT-PCR was chosen 5 µg in each reaction for first strand cDNA synthesis by reverse transcription. To avoid the interference of quick degradation of bacterial mRNA from the 5′ end, primers for each mRNA were designed to amplify a section of the gene in the later part of full mRNA (Fig. 2). Reaction compositions were total RNA (5 µg), 3′-end primer (1 µM), 5 U/µl AMV reverse transcriptase (1 µl), 10x RT buffer (2 µl), 10 mM dNTP mix (1 µl), 40 U/µl RNase inhibitor (0.5 µl), and DEPC-treated distilled water to 20 µl. The reaction was done in a thermal cycler at 48ºC for 50 min, then remaining reverse transcriptase was inactivated at 94ºC for 2 min; 2 µl of RT product was utilized as a template for PCR used for quantitative analysis. The PCR reaction was achieve in the following reaction mixture: 10x buffer (5 µl), RT product (2 µl), 10 mM dNTP mix (1 µl), forward and reverse primer (1 µM), Taq polymerase (2 U), and distilled water to 50 µl. Denaturation was done at 96ºC for 5 s, annealing at 60ºC for 5 s, and extension at 68ºC for the 28 s for *ackA* and 60 s for *pta*. The number of repeated cycles for amplification was selected 30 cycles for both *ackA* and *pta*. Reverse transcription was also done directly from total RNA without RT for inspection of genomic DNA contamination. The image of the gels was analyzed on a gel documentation system (Opticom500, Isogen life science). Intensities of bands were measured by total lab image analysis software (Isogen life science). All primers used in RT-PCR for analysis of mRNA transcription are listed in Table 2.

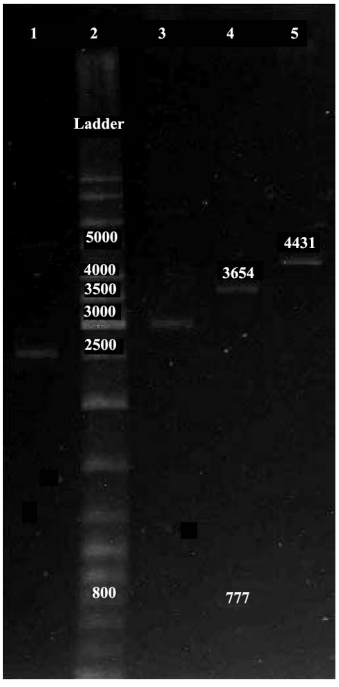

Figure 3. Electrophoresis diagram of recombinant plasmid. Lane 1 pLT10T3; Lane 2 ladder; Lane 3 PL6; Lane 4 PL6 digested with *Hind*III and SacI was released antisense cassette with 777bp; Lane 5 PL6 digested with *Bam*H1.

RESULTS

Plasmid with antisense cassette: All fragments of antisense cassette were amplified by PCR with the chromosomal DNA of *E. coli* K12 as the template.

Initially, the PCR products were cloned in pBluescript SK+ (Stratagene) respectively, according to Figure 1 and the complete cassette was then subcloned in pLT10T3. Plasmid containing antisense cassette, PL6, is shown in Figure 2 and Figure 3.

Antisense down-regulation of target transcripts and enzyme activities: Transcription levels of each target gene (*ackA* and *pta*) were investigated by semi-quantitative RT-PCR that was observed in Figure 4. Reverse transcriptions were done 3 times from each

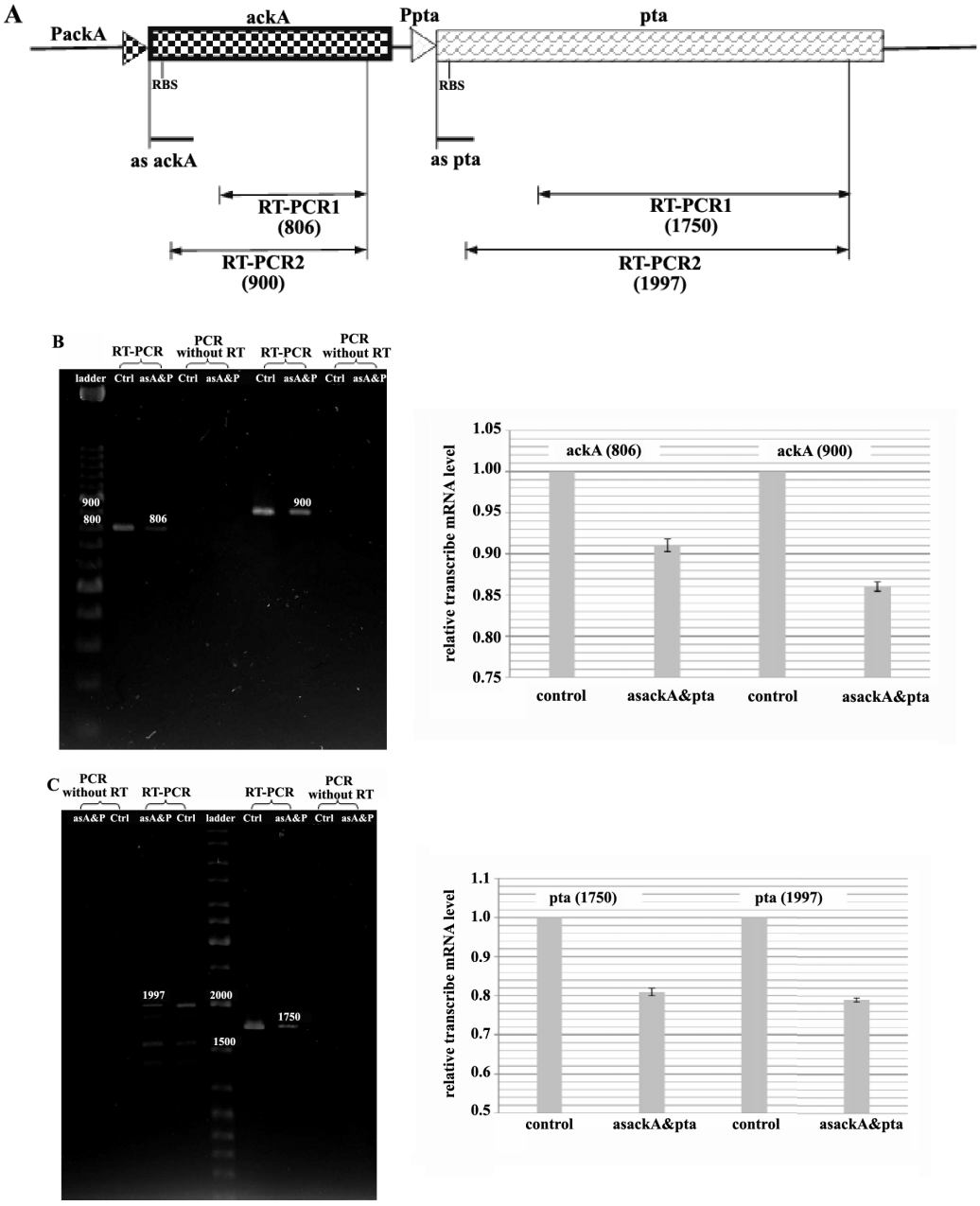

Figure 4. Semi-quantitative RT-PCR analysis for antisense cassette function. A: Schematic map of *ackApta* operon with antisense regulation and RT-PCR. B: RT-PCR on the region after the hybridization zone of asRNA and region involving the hybridization zone for *ackA*. C: RT-PCR on the region after the hybridization zone of asRNA and region involving the hybridization zone for *pta*. Reverse transcriptions were carried out 3 times from each total RNA sample prepared from separated series of cell cultures. Two PCRs were performed for a template got from each reverse transcription. DNA band intensity was analyzed, and the values were averaged to calculate relative transcribed mRNA levels of *ackA* and *pta* from the antisense-regulated strain compared to the control. Ctrl, control (pLT10T3); as A&P, as*ackA* and *pta* (pL6)] are PCR products after reverse transcription; the other lanes are control PCR samples without reverse transcription to check for any contamination of genomic DNA fragments in the PCR products. The error bars represent the standard errors from repeated RT-PCR reactions for each gene.

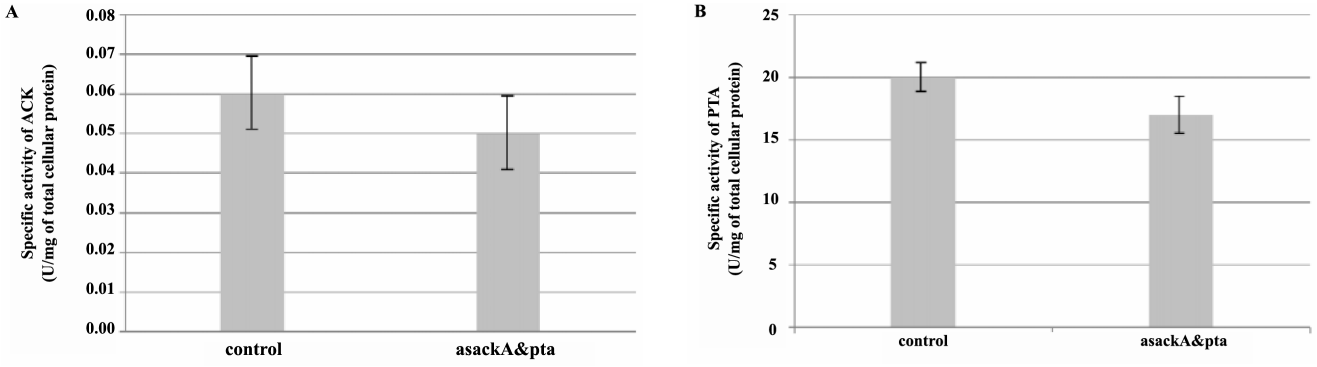

Figure 5. Specific activities of A: acetate kinase (ACK) and B: phosphotransacetylase (PTA) in *E. coli* BL21 strains harboring pLT10T3, and pL6 (*asackA* and *pta*). The error bars represent the standard errors from repeated assays of two separated culture series.

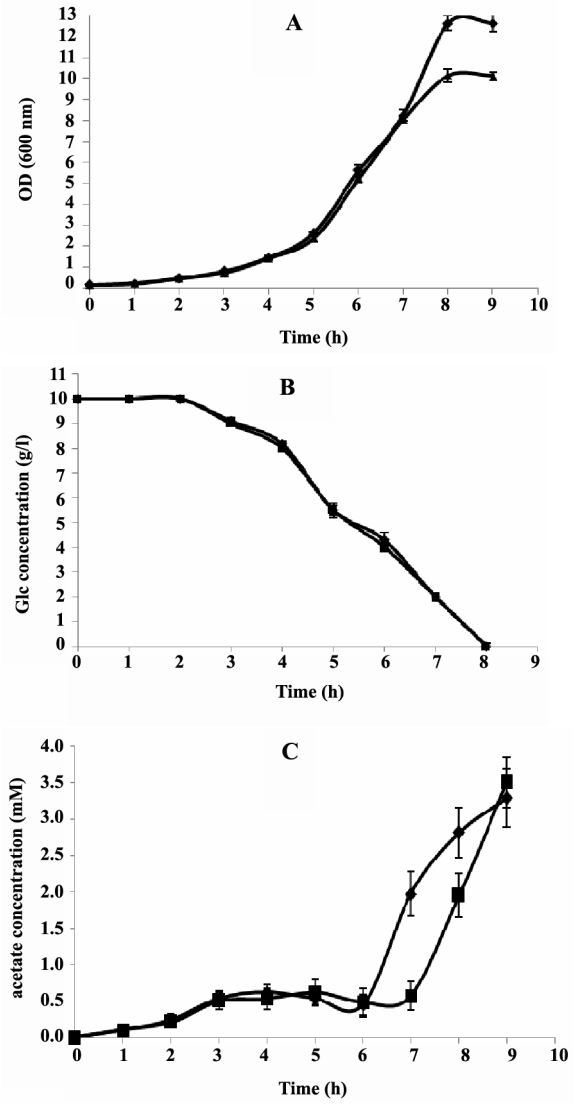

Figure 6. Time profiles of A: cell growth, B: residual glucose, and C: acetate concentration in the batch fermentation of *E. coli* BL21(DE3) Strains harboring (■) pLT10T3, and (♦) PL6 (*asackA* and *aspta*). Cells were grown in M9 with 10 g/l glucose media at 37°C. Values are average of triplicate experiments. The error bars represent the standard errors from 3 repeated experiments.

total RNA sample prepared from separated series of cell cultures. Two kinds of PCR fragments were amplified, 1) a fragment spanning after the antisense-hybridized zone until the end of mRNA coding region; 2) a longer fragment from an overlapping region with the antisense hybridized zone until nearly the end of mRNA coding region (Fig. 4A). PCRs were performed two times for each reverse transcription. DNA band intensity was analyzed and the values were averaged to calculate relative transcribed mRNAs of *ackA* and *pta* from the antisense-regulated strain compared to the control. The antisense regulated strains showed relatively lower levels (10-20% lower) of *ackA* and *pta* mRNA than the control strains (Fig. 4B, C). We also inspected the enzymatic activity of PTA and ACK to further confirm down-regulation action of antisense cassette. Even though error ranges were not small, we observed relatively lower activities (15-25% less) for each enzyme (PTA or ACK) in antisense regulated strains, compared to the control strains containing the parent plasmid pLT10T3 (Fig. 5).

Effect of antisense expression on cell physiology: The growth and the utilization rates of a sole carbon source (glucose) were measured in the culture of *E. coli* BL21 (DE3) transformed with the recombinant plasmid, PL6 (*asackA* and *aspta*), Compared with the control strain harboring the origin plasmid pLT10T3. According to Figure 6A, cells with antisense cassette showed similar growth rate compared to the control bacteria. But the final density of *E. coli* Bl21 (DE3) with antisense cassette is clearly higher than the control bacteria. In addition, glucose consumption rates were the same in both strains with and without antisense agents (Fig. 6B). However, the acetate production level was relatively lower for the antisense-regulated strain compare to the control bacteria (Fig. 6C).

DISCUSSION

Previous studies show that, acetate excretion occurs anaerobically during mixed-acid fermentation (Bock and Sawers 1996). It also happens aerobically when growth occurs on excess glucose (or other highly assimilable carbon sources) that inhibits respiration (Holms 1986), a behavior called the bacterial Crabtree effect (Crabtree 1929). Since high acetate concentration can inhibit growth and recombinant protein production (Luli and Strohl 1990; Majewski and Domach 1990; Shiloach *et al*., 1996; Han *et al*., 1992; Konstantinov 1990), therefore various operational strategies have been proposed and tested in the past to reduce the extent of acetate accumulation (Akesson *et al*., 2001; Delgado and Liao 1997; Farmer and Liao 1997; Aristidou *et al*., 1995; Aristidou *et al*., 1994; Chou *et al*., 1994; Dedhia *et al*., 1994; Hahm *et al*., 1994; Diaz-Ricci *et al*., 1991; Bauer *et al*., 1990; Majewski and Domach 1990; Robbins and Taylor 1989).

In this study we applied recently used antisense RNA approach for down-regulation of *ackA-pta* pathway. For the first time in this study, this protocol was applied in the *E. coli* Bl21 (DE3), as a widely used host strain for recombinant protein over-expression.

We performed semi-quantitative RT-PCRs and enzyme assays to ensure that antisense down-regulation was indeed occurring at the genetic and protein levels. Similar to the previously reported data (Kim and Cha 2003) transcribed mRNA levels of both *acKA* and *pta* genes were significantly lower (10-20% lower) in antisense down-regulated strains compare to the controls (Fig. 4B, C). We also obtained relatively reduced activities (15-25% lower) of both PTA and ACK enzymes under antisense-regulated conditions (Fig. 5A, B). Therefore, the reduced mRNA levels and their corresponding enzyme activities indicate that the partial down-regulation of target genes has been accomplished by using our constructed antisense expression system. As shown in Figure 4, the activity of ACK is much lower than PTA. These observations could be explained as following: Acetate is produced in two stages catalyzed by PTA and ACK enzymes; 1) acetyl-CoA + Pi↔acetyl-P + CoA [Keq = 9.26×10^{-3}], and 2) acetyl-P + ADP↔acetate + ATP [Keq = 161], respectively. This means that the second reaction that catalyzed by ACK is more spontaneous than the first one, catalyzed by PTA. Therefore, it is expected that PTA to be more active than ACK. We also observe an increase in the growth rate of the bacteria in presence of the antisense RNA, which might be an indication of a better growth condition that can prevent excessive cell death (Fig. 6A). As we expected, acetate concentration was less in bacteria regulated by antisense cassette compare to the control strains without antisenses (Fig. 6C). However, the concentration of acetate at the end of the growth curve was higher in bacteria with antisense RNA than control. This is quite logical, because control bacteria entered the stationary phase while the other one with cassette had continued to grow at a higher density. This observation can be concluded that decreasing of acetate by antisense RNA agents may lead to delay of death phase of *E. coli*. In particular, comparing the measured concentrations of glucose in the medium of both bacteria showed that equal amounts of glucose has been consumed, while the acetate discharge was lower in bacteria containing antisense RNA cassette. Therefore, it seems that the partial negative regulation of acetate pathway by antisense RNA technology could provide a useful, inexpensive and effective tool to overcome the limitation of accumulation of acetate in batch fermentation of *E. coli*. It could be also very useful in mass production of recombinant protein. Moreover, by selection of stronger promoters for antisense cassette, we can conduct more research to improve and optimize this technology

Acknowledgements

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