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ORIGINAL ARTICLE

Enhanced cytidine production by a recombinant *Escherichia coli* strain using genetic manipulation strategies

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Abstract Cytidine is a nucleoside molecule that is widely used as a precursor for antiviral drugs. In this study, a cytidine-producing strain Cyt18 was developed from *Escherichia coli* K-12 through 3-step genetic manipulation strategies. Cytidine deaminase gene (*cdd*) was firstly deleted from the *E. coli* K-12 strain to develop Cyt10. Furthermore, homoserine dehydrogenase gene (*thrA*) was inactivated from the Cyt10 strain to develop Cyt12, in which the intracellular aspartate concentration was expected to be increased. The recombinant plasmid pMG1105 containing an *pyrB-pyrA* operon from *Bacillus amyloliquefaciens* CYTI was constructed and was introduced into Cyt12 to obtain the Cyt18 strain. Compared to the Cyt12 strain, the cytidine production by the recombinant strain Cyt18 was increased by ~3-fold (722.9 mg/l vs. 249.3 mg/l).

Keywords Cytidine · *Escherichia coli* · Fermentation · Gene knockout · *pyrB-pyrA* operon

Introduction

Pyrimidine bases are the central precursors for RNA and DNA synthesis, of which intracellular pools are determined by de novo, salvage, and catabolic pathways (Andersen et al. 2008). As one of pyrimidine bases,cytosine can be attached to a ribose or deoxyribose ring resulting in cytidine, which is also required for DNA and RNA synthesis. However, the intracellular cytidine levels are very low and tightly controlled (Turnbough and Switzer 2008). To produce precursors for antiviral drugs, cytidine is either biologically acquired in a low yield or chemically synthesized through a very costly process. Therefore, developing an efficient strain for large-scale cytidine production is currently required.

Escherichia coli K-12 is one of the best-characterized organisms in molecular biology (Baba et al. 2006). Cytidine deaminase (CDA; EC 3.5.4.5) coded by cytidine deaminase gene (*cdd*) is a key enzyme involved in the pyrimidine salvage pathway (Sánchez-Quitian et al. 2011). CDA catalyzes the irreversible hydrolytic deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively (Yang et al. 1992). Therefore, deletion of the *cdd* gene may prevent cytidine and deoxycytidine consumption.

Aspartate is known as the precursor for both cytosine and homoserine synthesis (Asahi et al. 1996). In the homoserine synthesis pathway, aspartate is converted to homoserine catalyzed by a series of enzymes, in which homoserine dehydrogenase coded by *thrA* gene is a key one. In the homoserine dehydrogenase-deficient mutant, the intracellular aspartate concentration was expected to be increased (Asahi et al. 1996; Morbach et al. 1996). Thus, more aspartate can be used to synthesis cytidine. Aspartate carbamoyltransferase (ATCase; EC 2.1.3.2) (Chen and Slocum 2008) and carbamyl phosphate synthase (CPS; EC 6.3.5.5) are the first two key enzymes in the pyrimidine synthesis pathway, which are coded by *pyrB* and *pyrA* genes, respectively. In most Gram-positive bacteria (such as *Bacillus amyloliquefaciens* and *Bacillus subtilis*), bacteriophages, and

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all eukaryotes, CPS and ATCase play a similar role in pyrimidine nucleotide biosynthesis (Chen et al. 2007; Turnbough and Switzer 2008). Therefore, overexpression of CPS and ATCase enzymes could potentially be used to increase the cytidine production.

In this study, a *cdd*- and *thrA*-knockout recombinant *Escherichia coli* strain harboring the plasmid containing *pyrB-pyrA* operon was constructed and cytidine production by the recombinant strain was increased to 722.9 mg/l.

Materials and methods

Bacterial strains, plasmids and medium

Escherichia coli K-12 wild-type (WT) was used as the original strain for genetic manipulation (Blattner et al. 1997). *Bacillus amyloliquefaciens* CYTI was used to provide genomic DNA, pKD46, pKD3, pCP20, pMU3021, and pMG1105 plasmids. Luria-Bertani (LB) medium (Tryptone, 10 g/l; Yeast extract, 5 g/l; NaCl, 5 g/l; pH 7.0~7.2); Super Optimal Broth (SOB) medium (Yeast extract, 5 g/l; Bacto-tryptone, 20 g/l; NaCl, 0.5 g/l; KCl, 0.19 g/l; $MgCl_2$, 0.95 g/l; pH 7.0); Fermentation medium (Glucose, 10 g/l; $(NH_4)_2SO_4$, 1.8 g/l; Yeast extract, 1 g/l; Corn syrup, 2 ml/l; $FeSO_4{\cdot}7H_2O$, 80 mg/l; K_2HPO_4,7 g/l; KH_2PO_4, 1.5 g/l; $MgSO_4{\cdot}7H_2O$, 2 g/l; $MnSO_4$ H_2O, 1.5 μg/l; $ZnSO_4{\cdot}7H_2O$, 1.8 μg/l; Aspartate, 10 mg/l; 0.1 % Phenol red, 20 ml/l; pH 7.2).

PCR-mediated gene deletion

cdd and *thrA* genes were successively deleted from *Escherichia coli* K-12 by the Red recombination system as previously reported (Murphy et al. 2000; Datsenko and Wanner 2000; Baba et al. 2006; Lee et al. 2009), resulting in Cyt10 and Cty12, respectively, as follows.

PCR fragments composed of a selectable marker (chloromycetin-resistance gene, *cat*) flanked by 56-nt homology extensions and 23-nt priming sequences from pKD3, were generated. Oligonucleotides used for the generation of gene deletion fragments are shown in Table 1. Transformants carrying a λ Red helper plasmid (pKD46) were grown in 5 ml of SOB medium with 50 mg/l of ampicillin and 120 g/l of L-arabinose at 30 °C to an optical density at 600 nm (OD_{600}) of approximately 0.5 and then electro-competent cells were made by concentrating 100-fold and washing three times with ice-cold 10 % glycerol. 50 μl of electro-competent cells were electroporated (1.8-kV, 200-Ω, 25-μF electric pulse in a prechilled 0.2-cm-diameter cuvette) in the presence of 10–100 ng of PCR product and then transferred to 1 ml SOC medium (SOB medium containing 20 mM glucose), in which the cells were incubated for 1 h at 37 °C. Then one-half was spread onto LB agar plates

Table 1 Oligonucleotides for gene deletion and gene amplification

Gene	Direction	Sequence (5′→3′)
cdd	P1[a]	CCAACTTGCGGATAACTTGCAATCTGCA CTGGAACCTATTCTGGCAGACAAGTA CTTTGAGCGATTGTGTAGGCTGGAG
	P2 [a]	TGTGACAGCCGAGAGCTTTCAGCGTTGC GGAGGTGGCATCCCACTGAATCAACG GCTAACGGCTGACATGGGAATTAGC
	N1	ATCCACGTTTTCAAACCGCTTTTGC
	N2	TAAGCGAGAAGCACTCGGTCGATAC
thrA	P3 [a]	CGAGTGTTGAAGTTCGGCGGTACATCAG TGGCAAATGCAGAACGTTTTCTGCGT GTTTGAGCGATTGTGTAGGCTGGAG
	P4 [a]	CATCAAACCCGACGCTCATATTGGCACT GGAAGCCGGGGCATAAACTTTAACCA TGTAACGGCTGACATGGGAATTAGC
	N3	GACGCGTACAGGAAACACAG
	N4	GCCTCAACCGTGACTACATCT
pyrB-pyrA operon gene	S[b]	GCTACGCCATGGATGAATCATTTAACGG CGATGTCTGAACTGAGCG
	A[b]	TCTACTGCGGCCGCTCATACCGTAACGG CCGCCTTTGGATTGGTTTT

[a] Underline sequences are priming site sequences for template pKD3

[b] Underline sequences are priming site sequences for restriction sites

with 5 μg ml^{-1} chloramphenicol to select chloramphenicol-resistant transformants. After primary selection, mutants were maintained on medium without antibiotics. To remove the helper plasmid, individual colonies were purified nonselectively at 37 °C. Then, chloramphenicol-resistant mutants selected on plates were transformed with pCP20, and ampicillin-resistant transformants were selected at 30 °C, after which a few colonies were nonselectively purified at 42 °C and then investigated for loss of all antibiotic resistance. The majority lost the FRT-flanked resistance gene and the FLP helper plasmid simultaneously. Gene deletion and marker elimination were confirmed by using the appropriate antibiotic markers and the PCR method analysis (Figure 1) with the primers shown in Table 1. *thrA gene* was knocked out sequentially and knockout of the gene was confirmed by the same method.

Plasmid construction and co-expression of *pyrB-pyrA*

Standard procedures were used for plasmid preparation, restriction enzyme digestion, ligations, transformation, and agarose gel electrophoresis (Choi et al. 2010). The fragment *pyrB-pyrA* operon was PCR-amplified from genomic DNA of *Bacillus amyloliquefaciens* CYTI using primers shown in Table 1. PCR products were digested by *Not*I and *Xho*I and subcloned to pMU3021 to generate pMG1105, which was subsequently transformed into Cyt12 to produce Cyt18.

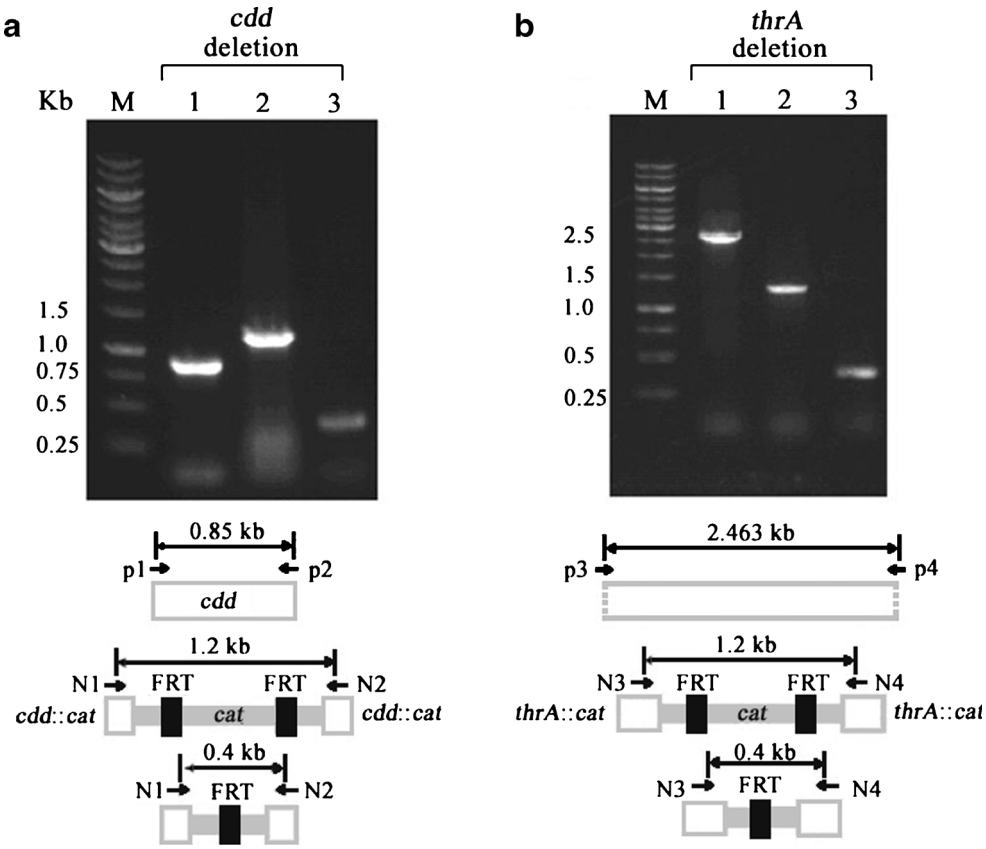

Fig. 1 PCR analysis of two gene deletion mutants. *P1* to *P4* and *N3* to *N4* refer to priming sites. PCR amplification for identifying the deletion of each target gene was performed using each *Escherichia coli* K-12 mutant genomic DNA as a template. **a** Deletion of *cdd* gene. *M* size marker; *lane 1 cdd* (0.85 kb); *lane 2 cdd*::*cat* (1.2 kb); *lane 3 cdd-cdd* (0.4 kb). **b** Deletion of *thrA* gene. *M* size marker; *lane 1 thrA* (2.463 kb); *lane 2 thrA*::*cat* (1.2 kb); *lane 3 thrA-thrA* (0.4 kb). The FRT (*black bar*)-flanked chloramphenicol-resistant cassette was amplified by PCR. The linear deletion PCR fragment (*white bar*) was transformed into a strain harboring the λ-Red recombinase, and then chloramphenicol-resistant transformants were selected. The selective marker was eliminated by FLP recombinase system

Escherichia coli Cyt18 strain was inoculated into a 250-ml flask containing 30 ml of LB medium with 50 μg/ml of tetracycline and incubated at 37 °C, 200 rpm. Bacteria were induced with 15 μl of 1 M IPTG, when growing to OD_{600} about 0.2–0.3. Expressed ATCase and CPS proteins were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assays for CPS and ATCase activities

ATCase and CPS activities were defined as the amount of enzyme that catalyzes the formation of 1 pmole of carbamyl aspartate and carbamyl phosphate, respectively, in 1 h at 37 °C (Delannay et al. 1999).

Fermentation

Fermentations were carried out in 250-ml flasks. The *Escherichia coli* seed culture was grown in 250-ml flasks containing 30 ml of LB medium to an OD_{600} about 12.0–16.0 within 8 h at 37 °C, 200 rpm. A 2-ml aliquot of seed culture and 50 μl of 1 M IPTG was transferred to a 500-ml baffled flask containing 50 ml of fermentation medium, and was then incubated at 37 °C, 220 rpm for 40 h. The pH was controlled at a minimum of 7.0 with 3 M NH_4OH.

Analytical procedures

Cytidine concentration in the fermentation broth was determined from the supernatant of cultivation samples (Zhang et al. 2011). pH values were measured with a pH meter (Mettler Toledo, China). Bacterial growth was expressed as the OD_{600} and measured by nucleic acid protein analyzer after dilution with distilled water. SDS-PAGE of proteins was carried out according to the method described by Laemmli (1970). The glucose concentration was measured using Biosensor Analyzer (Biology Institute of Shandong Academy of Sciences, China). The quantification of bases and pyrimidine compounds were measured by high-performance liquid chromatography (HPLC) under the following conditions: column, Agilent 5C18-250A (Agilent Technologies, USA); column size, 4.6 mm×250 mm; mobile phase, water:methylcyanide=96:4; flow rate, 1.0 ml/min; column temperature, 30 °C; detection, absorbance at 270 nm.

Statistical analysis

All experiments were conducted in triplicate and data were averaged and presented as mean±standard deviation (SD). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test were used to determine significant differences. Statistical significance was defined as $p<0.05$.

Results and discussion

Effects of deletion of *cdd* and *thrA* gene on cytidine production

Red recombination system is a simple and high efficient method to inactivate chromosomal genes in *Escherichia coli* (Datsenko and Wanner 2000; Murphy et al. 2000; Baba et al. 2006; Doublet et al. 2008). In this study, PCR products containing *cat* cassette flanked by 56-bp homology to the genes of *cdd* or *thrA* were used, and consequently these two genes were successfully deleted (Fig. 1).

CDA is considered as the only enzyme that can deaminate cytidine to uridine (Matsubara et al. 2006; Andersen et al. 2008). Results showed that cytidine production by *cdd*-knockout strain (Cty10) was obviously increased (129.69 mg/l vs. 1.48 mg/l; Table 2), suggesting that transformation of cytidine to uridine was efficiently prevented. However, the concentration of uracil, cytosine and uridine were also higher than the wild-type stains, presumably due to the degradation of cytidine to cytosine and conversion of cytosine to uracil, as well as synthesis of uridine from uracil (Matsubara et al. 2006). Some previous observations have revealed that the incorporation rate of exogenously supplied uridine through the salvage pathway during DNA synthesis is very poor in wild-type cells and that the CTP concentration is maintained at a normal level and increases only during DNA replication and repair (Tsen 1994; Matsubara et al. 2006; Tang et al. 2010). In the pyrimidine metabolism pathway, the disruption of the *cdd* gene was effective in blocking the flow of flux from the cytidine to uridine, and cytidine may accumulate. Therefore, it was also necessary to increase the concentration of cytidine by blocking the degradation pathway of cytidine. Moreover, enhanced cytidine synthesis was expected to occur in the Cyt10 strain, due to the deletion of the *cdd* gene. *cdd* was deleted to prevent the loss of CTP which is consumed in frequent DNA synthesis and repair.

Aspartate is one of important precursors for pyrimidine synthesis, and it is also consumed to synthesize homoserines by homoserine dehydrogenase (James and Viola 2002). Knockout of *thrA* gene coding homoserine dehydrogenase in Cty10 leads to enhanced cytidine production (249.28 vs. 129.69 mg/l; Table 2), which suggested that deletion of the *thrA* gene was helpful in increasing aspartate pool and consequently improving cytidine production, and cytidine production in Cyt12 was increased by 1.2-fold compared with that in Cyt10 (Table 2). In *thrA*-defective cells, high-aspartate pools may promote UMP accumulation to increase cytidine production through the degradation of excess UMP, which in turn stimulates the uridine phosphorylase reaction in the direction of cytidine synthesis (Morbach et al. 1996; Turnbough and Switzer 2008). In the homoserine dehydrogenase-deficient mutant, the intracellular concentration of aspartate was expected to be increased. The excess aspartate was used by the densitized UMP biosynthesis enzymes, and, therefore, the activity of UMP biosynthesis was improved (James and Viola 2002; Andersen et al. 2008). The relationship between the increase of the aspartate pool and homoserine dehydrogenase deficiency is not clear (Asahi et al. 1996), however, the accumulation amount of cytidine has indeed increased in our study.

Co-expression of *pyrB* and *pyrA* genes in Cty12

CPS and ATCase are the first two key enzymes involved in pyrimidine synthesis. *pyrB-pyrA* operon was cloned from *Bacillus amyloliquefaciens* CYTI and was overexpressed in Cyt12. Expression of *pyrB* and *pyrA* gene was identified by SDS-PAGE. CPS is composed of 118- and 50-kDa subunits and the molecular weight of ATCase is 34 kDa. As shown in Fig. 2, three extra bands (118, 50, and 34 kDa) appeared in the lysate of induced Cty18, which indicated that these two genes were well expressed. To further investigate whether overexpression of CPS and ATCase resulted in increased enzyme activities, the activities of two enzymes were detected. As

Table 2 Production of pyrimidine in four different strains after 40 h of culture[a]

Strain[a]	OD_{600}[b]	Concentration (mg/l)				Yield (%)[c]
		Uracil	Cytosine	Uridine	Cytidine	
WT	2.95±0.10	4.70±0.37	4.14±0.22	2.46±0.15	1.48±0.18	0.021
Cyt10	2.82±0.20	53.52±2.22	55.1±3.27	51.79±2.06	129.69±4.13	1.81
Cyt12	2.61±0.20	54.37±2.70	56.35±2.21	70.59±4.35	249.28±4.55	3.12
Cyt18	2.28±0.17	79.42±3.80	99.34±3.75	83.49±3.67	722.9±10.46	8.03

[a] Cultures were grown at 37 °C in fermentation medium supplemented with 100 g/l glucose. Data are the averages and standard deviations of three independent experiments

[b] OD_{600}, optical density at 600 nm

[c] Yield = 100 × cytidine produced/glucose consumed (g/g)

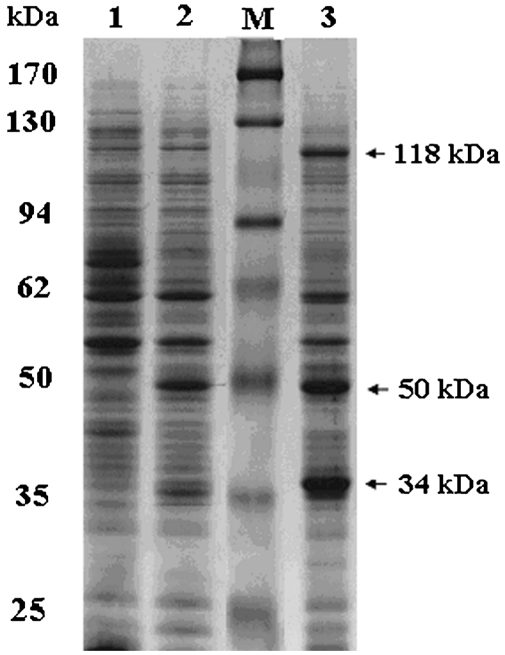

Fig. 2 SDS-PAGE analysis of overexpressing proteins in Cyt18. *M* molecular weight-sized marker; *lane 1* Cyt12; *lane 2* Cyt18, uninduced; *lane 3* Cyt18, induced with 0.1 mM IPTG. CPase, 118 and 50 kDa; ATCase, 34 kDa

shown in Fig. 3, the activities of CPS and ATCase from the lysate of IPTG-induced Cyt18 were 1.87- and 1.71-fold higher than those in Cyt12, respectively.

In nature, the pyrimidine de novo pathway enables the cells to synthesize nucleobases de novo. The de novo pathway leading to cytidine biosynthesis starts with the condensation of aspartate and carbamoylphosphate (Piette et al. 1984). This condensation reaction is catalyzed by ATCase to produce carbamoyl aspartate, which undergoes several reactions to produce UMP, the precursor for the synthesis of the cytidine. De novo biosynthesis of pyrimidine nucleotides is regulated by intracellular concentrations of various nucleotides through feedback inhibition (Caldara et al. 2008). The intracellular UMP level is tightly controlled by intracellular CTP levels through the feedback inhibition of CPS and the control of ATCase (Song et al. 2005). Moreover, UMP is converted to UDP in a reaction catalyzed by UMP kinase, UDP is converted to UTP by nucleotide diphosphate kinase, and UTP is converted to CTP by CTP synthase, which is regulated by CTP effectors through binding to specific allosteric sites on CTP synthase (Lee et al. 2004, 2009; Palmen et al. 2008). The synthesis of CTP is located at a node in the cytidine biosynthesis pathway. Because of its importance, CTP synthase is also regulated by a feedback inhibition by CTP and cytidine (Lee et al. 2009).

On the other hand, it seems that the expression of foreign CPS and ATCase in our system contributed to the enhancement of cytidine production under complex regulatory conditions. Generally, gene transcription can be enhanced by replacing a promoter sequence naturally associated with that gene with a sequence of a stronger promoter (Zhang and Switzer 2003; Choi et al. 2010). In this study, increasing the transcriptional levels of CPS and ATCase by overexpression of the *pyrB* and *pyrA* genes in *E. coli* resulted in an approximately 3-fold increase in cytidine production (Table 2), although a higher yield was expected. At this point, there are several explanations for this observation. One possibility is that the amounts of CPS and ATCase originally present in our system were sufficient to carry out the synthesis of UMP, so that increasing the copy number greatly increased cytidine production. A second possibility is that overexpression of the *pyrB* and *pyrA* genes was sufficient to increase cytidine production and that further increasing the copy numbers of CPS and ATCase may have a more positive effect on enhancing cytidine production. A third possibility is that the *pyrB* and *pyrA* genes from *Bacillus amyloliquefaciens* were not good candidates for *Escherichia coli*. Therefore, expressing foreign CPS and ATCase enzymes could potentially be used to increase the cytidine yield. Many bacteria such as *Escherichia coli, Bacillus amyloliquefaciens, Bacillus subtilis* (Zhang and Switzer 2003), *Salmonella enterica* serovar typhimurium and *Brevibacterium helvolum* (Lee et al. 2004) have been shown to contain these enzymes, which are responsible for delivering up to 70–80 % of the total UMP needed for CTP synthesis (Lee et al. 2009).

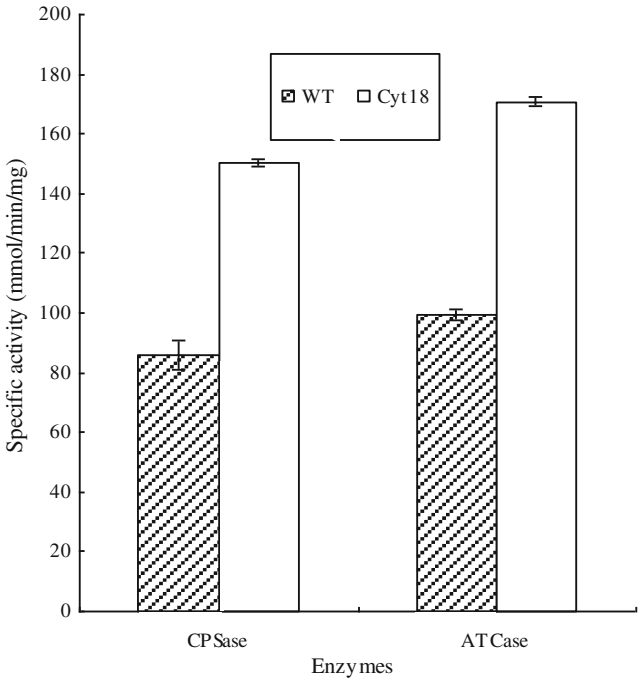

Fig. 3 In vitro-specific activity of recombinant CPS and ATCase expressed in Cyt18 and wild-type harboring the corresponding two genes. Cultures were grown at 37 °C in LB medium and induced with 0.1 mM IPTG. Data are the averages and standard deviations of three replicates

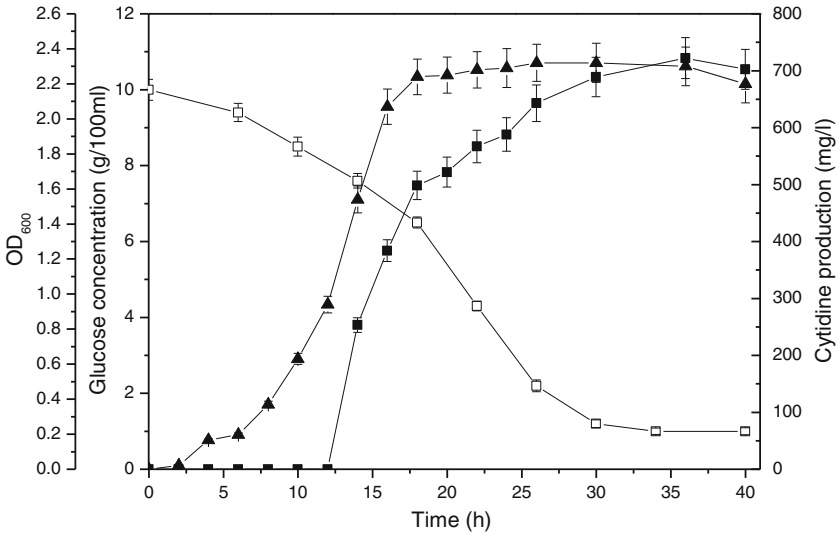

Fig. 4 Cytidine production in a 500-ml flask. Fermentation profile using Cyt18. Cultures were grown in triplicate at 37 °C in fermentation medium supplemented with 100 g/l glucose. Data points are the averages and standard deviations of the three biological replicates. ■ cytidine production; □ glucose concentration; ▲ OD_{600}

The CPS and ATCase enzyme reactions are key regulatory points of nucleotide metabolism and represent the major pathways for cytidine synthesis. It seems that the regulation of UMP synthesis by CPS and ATCase is a part of the overall regulation of CTP synthesis (Rabinowitz et al. 2008). *Bacillus amyloliquefaciens* CPS and ATCase used in this study are monofunctional enzymes that carry out only the synthesis of carbamoyl phosphate to carbamoyl aspartate. Therefore, expressing enzymes that increase the influx of UMP, such as CPS and ATCase from other organisms (Makiuchi et al. 2007), is a possibility for further enhancing cytidine production in our system. Thus, it seems reasonable to assume that the CTP for cytidine synthesis is provided via UMP from CTP through combined actions of both enzymes (Turnbough and Switzer 2008).

Enhanced cytidine production by recombinant *E. coli* strains

The general strategy used for the development of a cytidine-overproducing strain involves the alleviation of control mechanisms in key pathways. A few microorganisms have been modified for producing cytidine, including *Escherichia coli, Bacillus amyloliquefaciens* and *Bacillus subtilis* by classical mutagenesis methods, and they were selected based on their capacity to grow on toxic pyrimidine analogues (Zhang and Switzer 2003). In this study, the deletion of the *cdd* and *thrA* gened improved cytidine production to 249.3 mg/l. Moreover, *pyrB* and *pyrA* from *Bacillus amyloliquefaciens* CYTI were well co-expressed in Cyt18, by which cytidine production was surprisingly enhanced to 722.9 mg/l. The cytidine production by Cyt18 was increased by ~3-fold (722.9 vs. 249.3 mg/l) compared to the Cyt12 strain.

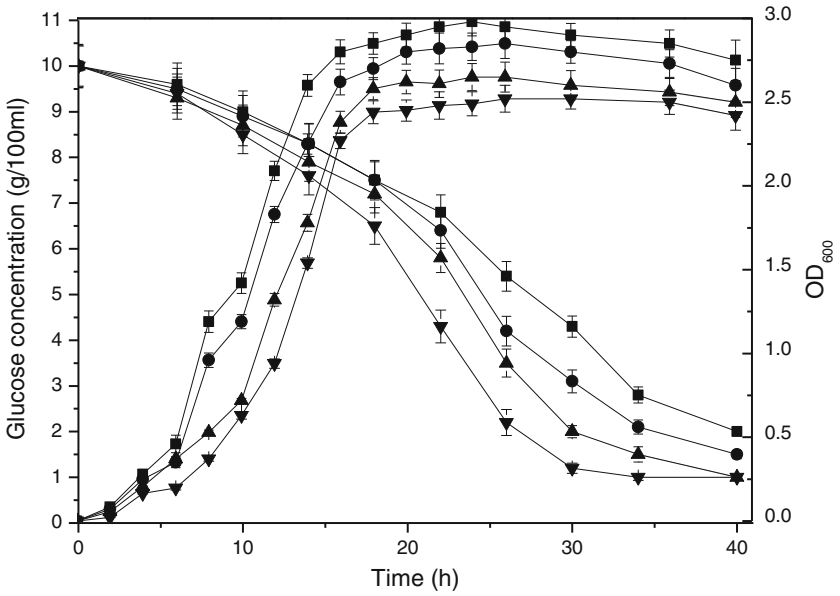

Fig. 5 Growth and consumption of glucose curve of WT (■), Cyt10 (●), Cyt12 (▲), and Cyt18 (▼)

To confirm whether genetic manipulation on *Escherichia coli* strain can increase cytidine production, fermentations were performed. As mentioned above, the cytidine production by Cty10 and Cty12 were remarkably increased. *pyrB* and *pyrA* were individually expressed in Cty12, but the cytidine production was seldom improved (data not shown). To our surprise, co-expression of *pyrB–pyrA* in Cty12 led to as much as 722.9 mg/l of cytidine production in 40 h (Fig. 4), which may be due to the higher activity of ATCase and CPS pulling the aspartate flux to the pyrimidine pathway (Turnbough and Switzer 2008).

Biomass and glucose consumption by all strains (WT, Cyt10, Cyt12, and Cyt18) were also detected during the fermentation process. Biomass of these strains ceased increasing after approximately 20 h; however, the biomass of WT was significant higher than other recombinant strains, especially than Cyt18. Lowering the biomass might be due to the burden caused by the deletion or expression of genes on the bacterium (Lee et al. 2009). And the WT strain consumed more glucose than the recombinant strains within 40 h (Fig. 5), which might be due to the higher biomass of this strain.

In conclusion, three recombinant strains (Cyt10, Cyt12, and Cyt18) have been constructed in this study with the aim of improving cytidine production. Foreign de novo biosynthetic genes were expressed to enhance cytidine production, while both the degradation pathway gene and the *thrA* gene were disrupted. Moreover, a cytidine-producing strain, Cyt18, was developed from *Escherichia coli* K-12 through 3-step genetic manipulation strategies. As a consequence of these alterations, it was possible to produce cytidine with considerable productivity by simple flask fermentation in Cyt18. Hence, the genetic manipulation of the *Escherichia coli* K-12 wild-type strain, including deletion of *cdd* and *thrA* genes by Red recombination system, and coupled with co-expression of the mutated foreign *pyrB–pyrA* operon, indeed enhanced cytidine production.

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