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**REPORT**

# Systems metabolic engineering of *Escherichia coli* for L-threonine production

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**Amino-acid producers have traditionally been developed by repeated random mutagenesis owing to the difficulty in rationally engineering the complex and highly regulated metabolic network. Here, we report the development of the genetically defined L-threonine overproducing *Escherichia coli* strain by systems metabolic engineering. Feedback inhibitions of aspartokinase I and III (encoded by *thrA* and *lysC*, respectively) and transcriptional attenuation regulations (located in *thrL*) were removed. Pathways for Thr degradation were removed by deleting *tdh* and mutating *ilvA*. The *metA* and *lysA* genes were deleted to make more precursors available for Thr biosynthesis. Further target genes to be engineered were identified by transcriptome profiling combined with *in silico* flux response analysis, and their expression levels were manipulated accordingly. The final engineered *E. coli* strain was able to produce Thr with a high yield of 0.393 g per gram of glucose, and 82.4 g/l Thr by fed-batch culture. The systems metabolic engineering strategy reported here may be broadly employed for developing genetically defined organisms for the efficient production of various bioproducts.**
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*Subject Categories:* metabolic and regulatory networks; synthetic biology
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## Introduction

Amino acids are important fermentation products that have been used in food, animal feed, pharmaceutical and cosmetic industries with annual growth rate of 5–7% (Leuchtenberger *et al*, 2005). All of the traditional amino-acid producing strains have been constructed by multiple rounds of random mutation and selection. Despite the successful development of industrial amino-acid producers by such approaches, they have several disadvantages. Further strain development is often required to improve the productivity and yield, and to cope with changing fermentation such as the necessity for utilizing alternative raw materials. However, undesirable random mutations may cause growth retardation and by-product formation. Furthermore, unidentified mutations make it difficult to understand the production mechanism, which hinders subsequent strain improvement. Other industrial microorganisms producing various metabolites that have been developed by random mutagenesis share the same problems.

Recent advances in genomics and other omics technology combined with computational analysis are now opening a new avenue toward the development of genetically defined production strains (Lee *et al*, 2005a; Park *et al*, 2007). Although Thr is one of the three major amino acids produced by fermentation processes (Takors *et al*, 2007), there has been no report on the construction of genetically defined Thr-overproducing strain. It was thus our goal to engineer *Escherichia coli* to make a Thr overproducer with completely known genotype by systems-level metabolic engineering.

## Results and discussion

### Metabolic engineering of E. coli for Thr production

We first constructed a Thr-producing *E. coli* base strain from WL3110, a *lacI*-mutant strain of W3110, using the metabolic and regulatory information available in the literature (Patte, 1996). Thr belongs to the aspartate family of amino acids and its biosynthetic pathway consists of five enzymatic steps from L-aspartate (Figure 1). Aspartokinase isozymes are key enzymes. Aspartokinase I encoded by *thrA* is inhibited by Thr, and its synthesis is repressed by Thr and L-isoleucine. Aspartokinase II is encoded by *metL* and its synthesis is repressed by L-methionine. In *E. coli* K-12, the amount of aspartokinase II is much less than that of isozyme I (Patte, 1996). Aspartokinase III encoded by *lysC* is inhibited by L-lysine and also inhibits its synthesis. Based on the previously published data (Ogawa-Miyata *et al*, 2001; Lee *et al*, 2003), feedback inhibitions of aspartokinase I and III were removed by replacing the 1034th base C with T (Ser 345→Phe) in the *thrA* gene and the 1055th base C with T (Thr 342→Ile) in the *lysC* gene. Transcriptional attenuation was removed by replacing the native promoter of the *thrABC* operon with the *tac* promoter, which allows constitutive expression in *lacI*-mutant strain. Next, the pathways that compete with Thr formation and degrade Thr were removed by deleting the *lysA* (encoding diaminopimelate decarboxylase), *metA* (encoding homoserine succinyltransferase), and *tdh* (encoding threonine dehydrogenase) genes. Furthermore, a point mutation replacing the 290th C with T (Ser 97→Phe) was introduced into the *ilvA* gene to decrease the activity of threonine dehydratase (Lee *et al*, 2003). This base strain was named TH07 strain (Figure 1).

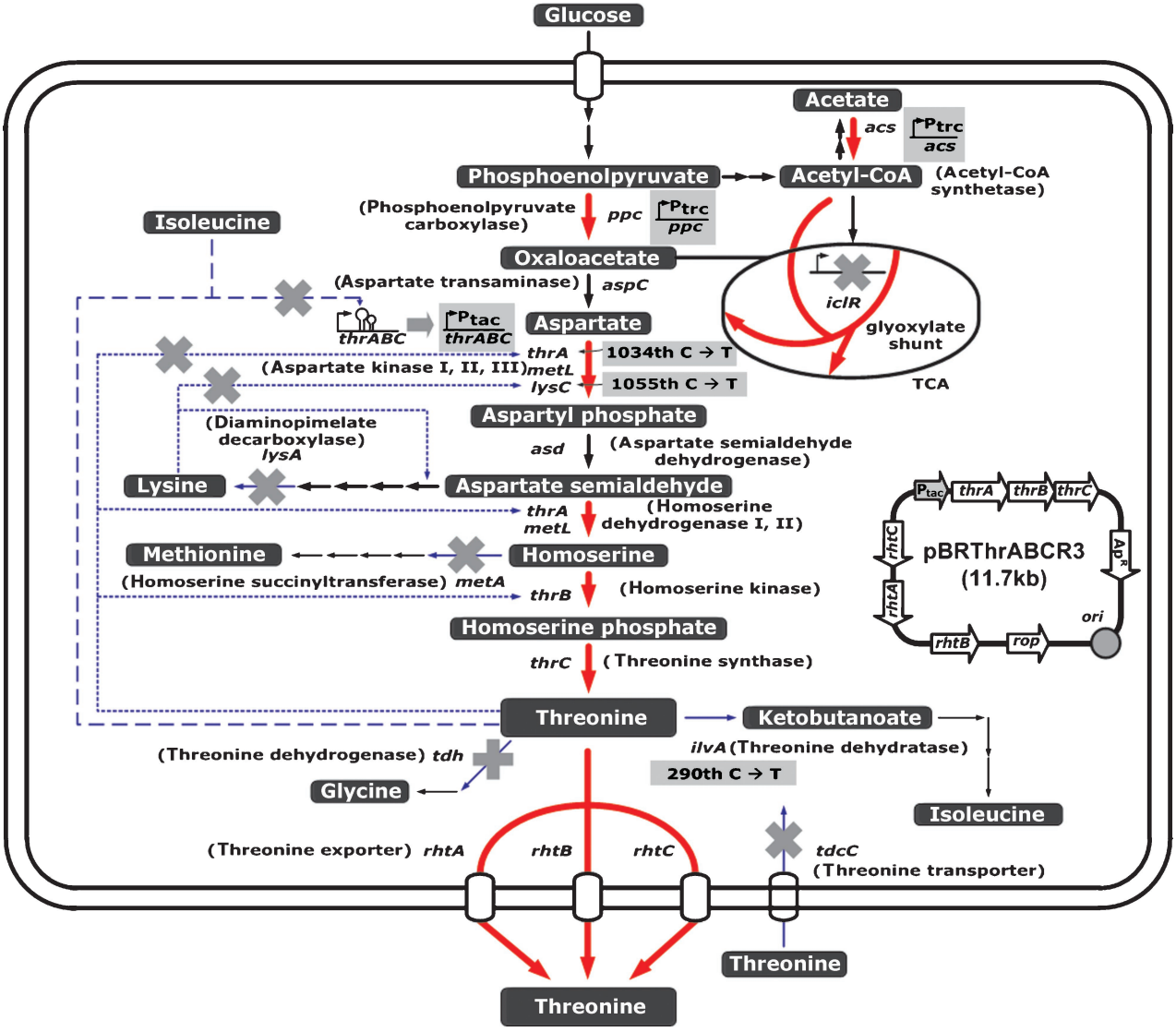


**Figure 1** Overall systems metabolic engineering strategies employed for the development of a genetically defined Thr-overproducing *E. coli* strain. Central metabolic pathways that lead to biosynthesis of Thr together with the regulatory circuits and competing pathways are shown. The shaded boxes represent the targeted mutations introduced into the genome. The gray Xs indicate that the genes are knocked out or the inhibition/repression is removed. Thick red arrows indicate the increased flux or activity by directly overexpressing the corresponding genes. Dashed line indicates repression regulation. Dotted lines indicate feedback inhibition.


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Plasmid pBRThrABC was constructed to increase the carbon flux from L-aspartate to Thr by the amplification of the mutated *thrA*$^{C1034T}$*BC* operon. When recombinant TH07 (pBRThrABC) strain was flask cultured in TPM1 medium containing 50 g/l glucose for 48 h, the final Thr concentration of 10.1 g/l (a yield of 0.202 g Thr per gram of glucose) was obtained. After this initial success in metabolic engineering of *E. coli* for Thr production, further stepwise improvements were made based on combined transcriptome analysis and *in silico* metabolic simulation.

## Transcriptome analysis of the Thr production strain

To identify the target genes to be manipulated, transcriptome profiling was carried out during Thr production. Transcriptome profiles of TH07 (pBRThrABC) were compared with those of the WL3110 (pBR322) control strain (Supplementary information; Supplementary Figure 3; Supplementary Tables V, VI and VII). In the TH07 (pBRThrABC) strain, the expression of the *thrABC* genes was significantly upregulated by 43.60 (*thrA*), 39.05 (*thrB*) and 23.55-fold (*thrC*), reflecting their successful overexpression.

## Effect of phosphoenolpyruvate carboxylase on Thr production

Among the genes that were downregulated during Thr production (Supplementary Table VI), the *ppc* gene (downregulated to 0.43-fold) encoding the phosphoenolpyruvate carboxylase (PPC) was selected as a target gene to be manipulated. The *ppc* gene was cloned into pACYC177 to make pACYCppc, which was transformed into the TH07 (pBRThrABC) strain. To examine Thr production, batch cultures were carried out in TPM2 medium containing 10 g/l glucose. The specific activity of PPC in the *ppc*-amplified strain was 9.3-fold higher than that of the control strain (Supplementary Table IV). To our surprise, however, Thr production was rather decreased by 17.1% compared to the control strain TH07 (pBRThrABC, pACYC177). When the *ppc*-deleted strain TH11C (pBRThrABC, pACYC177) was cultured under the same condition, Thr production significantly decreased by 87%, which suggested that PPC is necessary for Thr production (Supplementary Figure 4).

Thus, it was reasoned that there is an optimal level of PPC for efficient production of Thr. To find the relationship between the PPC flux and Thr production rate, we performed flux response analysis; the response of Thr production rate to the varying PPC flux was examined in TH07 (pBRThrABC) strain. As shown in Figure 2A, Thr production rate increased as the PPC flux increased to 12.2 mmol/gDCW/h (gram dry cell weight (gDCW)), but decreased with further increase of the PPC flux. Instead, increased PPC flux was predicted to enhance biomass formation. The *in vivo* PPC flux experimentally measured for *E. coli* growing in a glucose minimal medium was reported to be 4 mmol/gDCW/h (Fischer *et al*, 2004). Thus, these simulation results suggested that the expression of the *ppc* gene should be increased but not to a too high level. To implement this, we replaced the native promoter of the *ppc* gene with the *trc* promoter in the chromosome to make TH19C strain, which should provide higher PPC flux than the wild type but lower than the plasmid-based overexpression. The TH19C (pBRThrABC) strain showed a 27.7% increase in Thr production compared with the control TH07 (pBRThrABC) strain (Supplementary Figure 4F).

## Effect of glyoxylate shunt on Thr production

To find next modification targets, we went through the list of significantly upregulated genes from the transcriptome analysis. It was notable that the *aceBA* genes involved in glyoxylate shunt were upregulated by 2.23- and 1.80-fold, respectively (Supplementary Table V), which is contradictory to our knowledge that they are not activated in aerobic condition using glucose as carbon source (Yamamoto and Ishihama, 2003). It was reasoned that this might be due to the increased demand for oxaloacetate in Thr-producing strain, which made the glyoxylate shunt operate under this condition. Thus, to further increase the carbon flux through the glyoxylate shunt, the *iclR*-deleted TH09C strain was constructed; the transcriptional regulator IclR negatively regulates the expression of the *aceBA* genes encoding isocitrate lyase (ICL) and malate synthase (Yamamoto and Ishihama, 2003). Batch culture of TH09C (pBRThrABC) resulted in the production of 1.93 g/l Thr (a yield of 0.186 g Thr per gram of glucose), which is 30.4% higher than that obtained with the control TH07C (pBRThrABC) strain (Supplementary Figure 4). This result indicates that the increased expression of the *aceBA* genes indeed improves Thr production (see Supplementary information). To combine the positive effects of the *ppc* overexpression and *iclR* deletion, the *iclR* gene in the chromosome of the TH19C strain was deleted to make TH20C strain. Batch culture of TH20C (pBRThrABC) resulted in the production of 2.24 g/l Thr (a yield of 0.213 g Thr per gram of glucose), which is 51.4% higher than that obtained with the control TH07 (pBRThrABC) strain (Supplementary Figure 4; Supplementary Table III).

## Enhanced production of Thr by engineering transport system

Efficient export of amino acid is also important to further enhance its production. Transcriptome profiling suggested several transporter targets to be engineered. The expression of the *tdcC* gene encoding a Thr transporter (Sumantran *et al*, 1990) was found to be upregulated by 1.7-fold (Supplementary Table V). Since this transporter uptakes extracellular Thr into the cell, we deleted the *tdcC* gene to prevent it. When the resulting TH27C (pBRThrABC) strain was batch cultured in TPM2 medium containing 30 g/l glucose, 7.4 g/l of Thr (a yield of 0.246 g Thr per gram of glucose) was produced. This is 15.6% higher than that obtained with TH20C (pBRThrABC) under the same culture condition. This positive effect was not expected, as the *tdcC* gene has been known to be induced under anaerobic conditions (Sumantran *et al*, 1990). Thus, it would have not been possible to select the *tdcC* gene for deletion if transcriptome analysis was not performed.

Next, we searched for the Thr exporters to engineer. The transcriptome data showed that the *rhtC* gene encoding a

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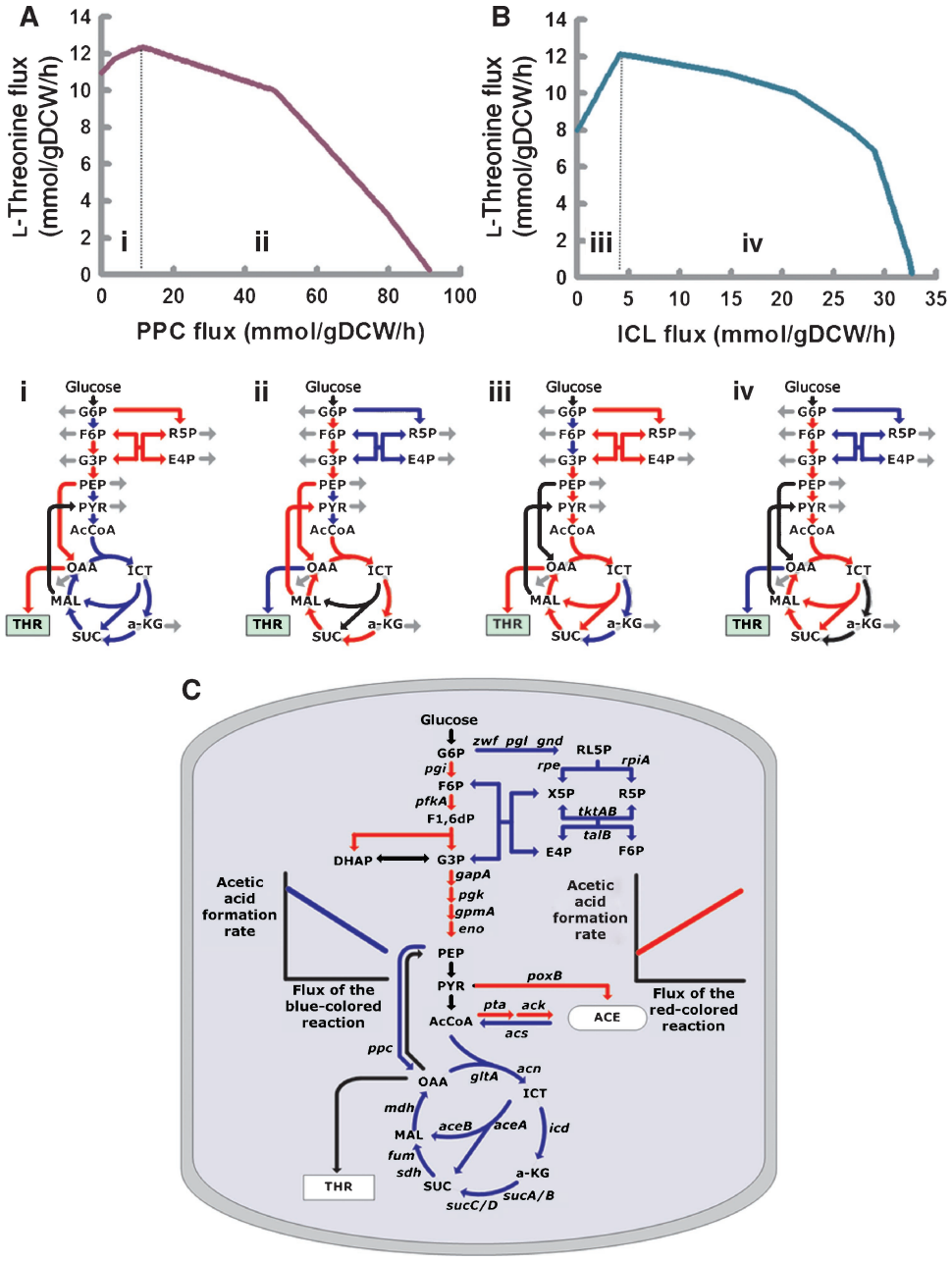


**Figure 2** Flux response analysis during production of Thr using the *in silico* genome-scale metabolic model. (**A**) The response of Thr production rate to varying PPC flux. (**B**) The response of Thr production rate to varying ICL flux. The predicted flux distributions belonging to the region i, ii, iii, and iv are described with colored arrows; red, blue, and black arrows indicate the fluxes that are predicted to be increased, decreased, and remain unchanged, respectively, in each region. (**C**) The response of acetic acid formation rate to the varying flux of the individual central metabolic reaction. Those reactions that upon increasing their fluxes result in decreasing the acetic acid formation rate are shown in blue. In contrast, those reactions that upon increasing their fluxes result in increasing the acetic acid formation rate are shown in red. In the two graphs inside, the *x*-axis denotes the normalized flux of one of the color-coded reactions, whereas the *y*-axis denotes the normalized acetic acid formation rate. Abbreviations are as follows: G6P, glucose-6-phosphate; RL5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; F1,6dP, fructose-1, 6-bisphosphate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetonephosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; ICT, isocitrate; α-KG, α-ketoglutarate; SUC, succinate; MAL, malate; OAA, oxaloacetate; THR, L-threonine; ACE, acetate.

threonine exporter (Kruse *et al*, 2002) was upregulated by 2.99-fold (Supplementary Table V). It is likely that the *rhtC* gene was induced to protect cells from toxic effects of intracellular Thr accumulation by exporting Thr out of the cell. In order to further increase the Thr export capacity, a recombinant TH27C (pBRThrABCR) strain, which overexpresses the *rhtC* gene, was constructed (see Supplementary information). Batch culture of this strain in TPM2 medium containing 30 g/l glucose resulted in the production of 11.1 g/l Thr (a yield of 0.370 g Thr per gram of glucose), which is 50.2% higher than that obtained without *rhtC* amplification (Supplementary Figure 5). Further amplification of the *rhtA* and *rhtB* genes,


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which are involved in the export of Thr and homoserine (Zakataeva *et al*, 1999), resulted in the small increase in Thr production. Batch culture of the final engineered strain TH27C (pBRThrABCR3) allowed production of 11.8 g/l Thr with a high yield of 0.393 g Thr per gram of glucose (Supplementary Table III). Thus, systems metabolic engineering of *E. coli* by targeted genome engineering, manipulation of target genes based on transcriptome profiling and *in silico* simulation, and transporter engineering successfully yielded a genetically-defined Thr overproducer.

## Metabolic engineering based on in silico flux response analysis

Fed-batch fermentation is the cultivation method of choice in industry for the high level production of amino acids. We thus performed fed-batch culture of TH27C (pBRThrABCR3) strain with intermittent glucose feeding (see Materials and methods), which allowed production of 77.1 g/l Thr in 56.1 h, resulting in a volumetric Thr productivity of 1.37 g/l/h (Figure 3A; Supplementary Table III). Acetic acid started to accumulate considerably after consuming 180 g/l of glucose, and it retarded cell growth and lowered the rates of glucose uptake and Thr production in the later stages of fermentation. To solve this problem, the flux that can most effectively reduce acetic acid production was examined by *in silico* flux response analysis; the response of the acetic acid production rate to the varying individual flux of central metabolic pathway was evaluated (Figure 2C). Knocking out the genes in the acetate pathway (*pta-ack* or *poxB*) was not considered because it can retard growth and/or increase pyruvate excretion (Causey *et al*, 2004). The strategy of amplifying the *acs* gene encoding acetyl-CoA synthetase was selected to reduce acetic acid production (Supplementary information). The resulting TH28C strain, which constitutively expresses the *acs* gene, was transformed with pBRThrABCR3 and fed-batch fermentation was performed (Figure 3B; Supplementary Figure 6). As shown in Figure 3B, acetic acid production decreased considerably to 2.35 g/l. The final Thr concentration reached 82.4 g/l in 50 h, with a volumetric productivity of 1.65 g/l/h (Supplementary Table III), which is 20.4% higher than that obtained with TH27C (pBRThrABCR3) strain, and is comparable to a value typically obtained with a randomly mutagenized industrial strain.

In this paper, we reported the strategies for systems metabolic engineering of *E. coli* to develop a genetically defined Thr overproducer. Regulatory and metabolic circuits were rewired to develop an initial Thr producer, which was further engineered based on transcriptome profiling and *in silico* flux response analysis. Additional metabolic engineering was performed to enhance Thr export and to reduce acetic acid formation. The final engineered strain was able to produce Thr with high efficiency. As this engineered strain is completely defined with respect to its genome traits, it is possible to further engineer the metabolic and regulatory circuits according to the changing demands. The maximum achievable theoretical yield (Hong *et al*, 2004) of L-threonine was calculated to be 0.81 g Thr per gram of glucose, which can be achieved if there is no biomass formation. In the case of batch culture of TH27C (pBRThrABCR3), the biomass yield and Thr yield were 0.366 and 0.393 g per gram, respectively. These two yields add to 0.759 g per gram, which is close to the maximum achievable yield of Thr if there was no biomass formation. This analysis also suggests that there is still a room for further improvement in Thr production. Taken together, the systems metabolic engineering strategy reported here can be broadly employed for developing the genetically defined strains for the efficient production of other amino acids and various bioproducts.

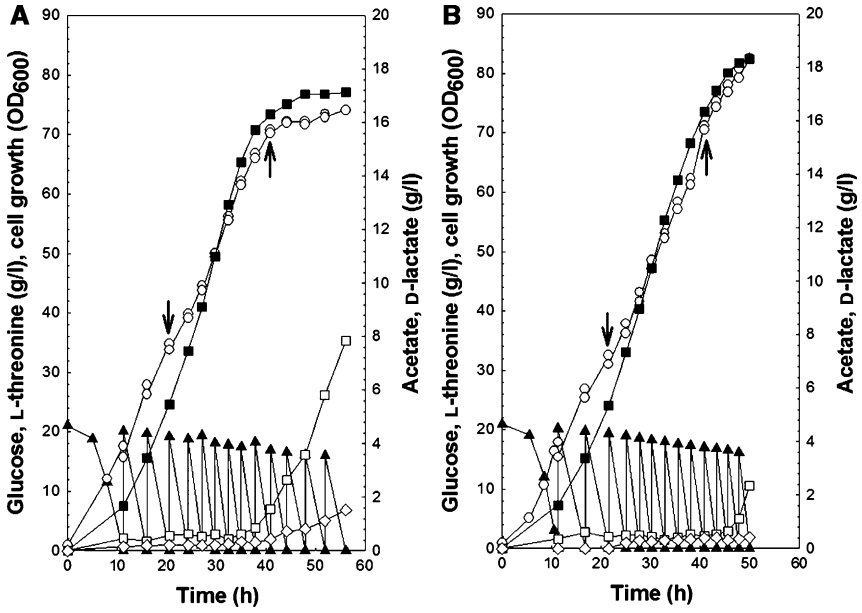


**Figure 3** Time profiles of cell growth, Thr production, acetic acid accumulation during the fed-batch culture of (**A**) TH27C (pBRThrABCR3), and (**B**) TH28C (pBRThrABCR3). The arrows indicate the sampling points for real time RT–PCR analysis. Symbols are as follows: ○, cell growth ($OD_{600}$); ■, L-threonine (g/l); ▲, glucose (g/l); □, acetic acid (g/l); ◇, lactic acid (g/l).

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# Materials and methods

## Strains and plasmids used in this study

The strains and plasmids constructed and used in this study and the primers used for gene cloning are listed in Supplementary Table I. Detailed procedures for the construction of plasmids are shown in Supplementary information and Supplementary Figure 1. All restriction enzymes and pfu DNA polymerase for PCR used in this work were purchased from New England Biolabs (Ipswich, MA) and Solgent (Daejeon, Korea), respectively.

## Genome engineering: promoter replacement and site-directed mutagenesis

The primers used for chromosomal manipulation are listed in Supplementary Table I. Replacement of native promoter in threonine operon with the strong *tac* promoter, removal of feedback inhibitions of aspartokinase I and III, and reduction of threonine dehydratase activity were performed using *sacB* homologous recombination system (Schweizer, 1992). Substitution of the endogenous promoters of the *ppc* gene and that of the *acs* gene by the *trc* promoter were performed by PCR-mediated λ-Red recombination. Detailed procedures for genome engineering are provided in Supplementary information and Supplementary Figure 1.

## Knocking out chromosomal genes

Deletion of the *lysA*, *metA*, *tdh*, *ppc*, and *iclR* genes was performed using one-step inactivation method (Datsenko and Wanner, 2000). The schematic procedure of gene inactivation is shown in Supplementary Figure 1F. The λ-Red recombinase expression plasmid pKD46 was used to disrupt the genes in the chromosome of *E. coli* W3110 with appropriate antibiotic markers. Detailed procedures for gene inactivation are provided in Supplementary information.

## Batch and fed-batch cultivation

Batch and fed-batch cultures were carried out using the TPM2 medium containing the indicated amount of glucose at 31°C. The TPM2 medium contains the following per liter: yeast extract, 2 g; $MgSO_4 7H_2O$, 2 g; $KH_2PO_4$, 2 g; $(NH_4)_2SO_4$, 10 g; L-methionine, 0.2 g; L-lysine, 0.2 g; L-isoleucine, 0.05 g; and trace metal solution, 10 ml. Glucose was added to give the final concentration of 10 or 30 g/l depending on the experiments described in the text. Chloramphenicol (35 μg/ml), kanamycin (40 μg/ml), and ampicillin (50 μg/ml) were added to the medium when necessary. Seed cultures were prepared by transferring 500 μl of 10 ml overnight cultures prepared in Luria–Bertani (LB) medium into 250 ml Erlenmeyer flasks containing 50 ml LB medium and cultured at 31°C with 250 r.p.m. Cultured cells were used to inoculate the fermentor containing 2 l of TPM2 medium. Batch fermentations were carried out in a 6.6-l Bioflo 3000 fermentor (New Brunswick Scientific Co., Edison, NJ). The pH was controlled at 6.5 by automatic feeding of 25% (v/v) $NH_4OH$. The dissolved oxygen concentration was maintained above 40% of air saturation by supplying air at 1 vvm (air volume/working volume/minute) and by automatically controlling the agitation speed up to 1000 rpm.

Fed-batch cultures were carried out in a 6.6-l Bioflo 3000 fermentor containing 2 l of TPM2 medium containing 20 g/l glucose at 31°C. Feeding solution was made of 500 g/l glucose, 12.5 g/l $KH_2PO_4$, 3 g/l L-methionine, and 4.357 g/l L-lysine. When the glucose concentration in the culture broth fell to 1 g/l, 80 ml of feeding solution (thus, equivalent to 40 g glucose, 1 g $KH_2PO_4$, 0.24 g L-methionine, and 0.35 g L-lysine) was added. The dissolved oxygen concentration was controlled at 40% of air saturation by automatically increasing the agitation speed up to 1000 r.p.m. and by changing the percentage of pure oxygen added. The pH was controlled at 6.5 by automatic feeding of 25% (v/v) $NH_4OH$. The pH-stat fed-batch fermentation was carried out similarly. When glucose becomes exhausted, the pH increases. When the pH becomes higher than 6.59, 6 ml of feeding solution composed of 565 g/l glucose, 12.9 g/l $KH_2PO_4$, 3.59 g/l L-methionine, and 5.27 g/l L-lysine was automatically added.

## Enzyme assays

To determine enzyme activities, exponentially growing cells were harvested by centrifugation at 5000 *g* and 4°C for 10 min. After centrifugation, the supernatant was decanted and the cell pellet was washed twice with 50 mM potassium phosphate buffer containing 1 mM dithiothreitol (DTT, pH 7). The washed cell pellet was resuspended in the same buffer, rapidly frozen, and stored at −70°C until required. Cell disruption was performed using an ultrasonic homogenizer (VCX-600; Sonics and Materials Inc., Newtown, CT), equipped with a titanium probe 40 T (4-mm diameter and 127-mm length; Sonics and Materials Inc.) in a conical tube in ice water. Sonication was carried out at 20 kHz for five cycles with 30 s of resting time between each cycle; each cycle was composed of 10 rounds of 3-s sonication and 5 s rest. The disrupted cells were centrifuged for 10 min at 13 000 *g* and 4°C. The supernatant was immediately used or stored at −70°C for future use. Protein concentration in the cell-free extracts was determined by Bradford assay using bovine serum albumin as a standard. Enzyme activities were determined spectrophotometrically in a temperature-controlled spectrophotometer (SpectraMax M2; Molecular Devices Co., Sunnyvale, CA) in triplicates. Reactions were monitored by following the NADH at 340 nm. The extinction coefficient for NADH of 6.23/mM · cm at 340 nm was used. One unit (U) of enzyme activity was defined as the amount of enzyme necessary to catalyze the conversion of 1 nmol of substrate per minute into specific products. The specific activity was defined as units per mg of protein. The activity of PPC was measured in a reaction mixture containing 66 mM Tris–HCl (pH 9), 5 mM PEP, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.15 mM NADH, 2 U malate dehydrogenase, and the cell-free extract (Terada *et al*, 1991). The activity of ICL was measured as described by Van der Werf *et al* (1997) in a reaction mixture containing 0.1 M potassium phosphate (pH 7), 5 mM $MgCl_2$, 1 mM DTT, 0.3 mM NADH, 5 mM isocitrate, 20 U rabbit muscle L-lactate dehydrogenase, and the cell-free extract.

## RNA purification and labeling of cDNA

Samples for transcriptome analysis were taken at the exponential phase (Supplementary Figure 2). Total RNA was purified using the RNeasy Mini kit (Qiagen, Chatsworth, CA) following the manufacturer's protocol. Fluorescent labeled cDNA was prepared by an indirect labeling method. For this purpose, aminoallyl-modified nucleotides (aa-dUTP; Sigma) were incorporated during the reverse transcription of the first strand. The aminoallyl-modified first-strand cDNA was synthesized from 20 to 50 μg of total RNA by reverse transcription using random hexamer primers, SuperScriptII reverse transcriptase (Invitrogen Corp., Carlsbad, CA) and a dNTP plus aa-dUTP mixture (25 mM dATP, dCTP, dGTP, and 7.5 mM dTTP, aa-dUTP). After hydrolysis and cleanup using Microcon YM-30 filters (Millipore, Bedford, MA), the *N*-hydroxysuccinimidyl ester dyes (Cy3- and Cy5-NHS esters; Amersham Pharmacia, Piscataway, NJ) were coupled to the aminoallyl-labeled first-strand cDNA. Uncoupled dyes were removed using Qiaquick PCR purification columns (Qiagen).

## Transcriptome profiling

Transcriptome profiling was performed using the TwinChip *E. coli*-6K oligo chip (Digital Genomics, Seoul, Korea), following the manufacturer's protocol. In this oligo chip, 5978 of 70-mer probes that cover the genomes of *E. coli* K12, O157:H7 (EDL933) and O157:H7 (Sakai) are spotted in duplicates. Cy3- and Cy5-labelled cDNA probes were concentrated by vacuum drying, dissolved in 20 μl of hybridization solution (30% formamide, 5 × SSC, 0.1% SDS, 0.1 mg/ml salmon sperm DNA), and combined. The microarray was incubated with prehybridization solution at 42°C for 1 h, washed in water for 5 min, and finally centrifuged at 650 r.p.m. for 5 min. The combined hybridization probes were denatured by incubation at 95°C for 5 min. Hybridization was performed under a coverslip, with a final


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volume of 20 μl inside a hybridization chamber (Genomic Tree, Daejeon, Korea). After hybridization at 42°C for 16 h, the microarray was washed for 5 min in 2 × SSC and 0.1% SDS at 42°C, 10 min in 0.1 × SSC and 0.1% SDS at room temperature, and four times for 1 min in 0.1 × SSC at room temperature. Finally, the microarray was dried by centrifugation for 5 min at 650 r.p.m. The mean signal intensity values of the duplicate spots were averaged and then normalized by the global normalization method. The genes showing the *P*-values lower than 0.01 were considered significant.

The gene expression data from this study have been submitted to Gene Expression Omnibus database under accession numbers GSE9434.

### Real-time RT–PCR analysis

The expression level of acetyl-CoA synthetase was determined by real-time RT–PCR analysis, because enzyme assay of acetyl-CoA synthetase can be interfered by activities of acetate kinase and phosphotransacetylase. Samples were taken during fed-batch fermentations at $OD_{600}$ values of 34.9 and 70.8 for TH27C (pBRThrABCR3), and at $OD_{600}$ values of 32.5 and 71.2 for TH28C (pBRThrABCR3). Total RNA was purified using the RNeasy Mini kit (Qiagen, Chatsworth, CA) following the manufacturer's protocol. Real-time RT–PCR was carried out with the iCycler iQ (Bio-Rad, Hercules, CA), using the iQ SYBR Green Supermix (Bio-Rad), following the manufacturer's protocol. PCR conditions were as follows: 3 min at 95°C, and three-step cycle at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for a total of 40 cycles followed by melting curve analysis. PCR was performed with 1 ng of cDNA template and 0.1 pM of primers. The *rrsB* gene was used as a reference gene for normalization. The data were analyzed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The primers used were as follows: acsf (5′-CATTCCTGCCAACATCGCA-3′), acsr (5′-TTCGCCCCAGAAGGTATCA-3′), rrsBf (5′-GTGGCGGACGGGTGAGTAAT-3′), and rrsBr (5′-CCTCTTTGGTCTTGCGACG-3′). The primer pairs were designed to yield PCR products of 100 bp in length.

### *In silico* flux response analysis

The *in silico* flux response analyses were performed using the genome-scale metabolic model *E. coli* MBEL979 consisting of 979 metabolic reactions and 814 metabolites (144 extracellular metabolites and 670 intermediates), which is a slightly modified network of *i*JR904 reported by Palsson and co-workers (Reed *et al*, 2003; Lee *et al*, 2005b). The steady-state flux values for *in silico* mutants were calculated by linear programming-based optimization of an objective function as follows.

*Step 1*: To construct the *in silico* mutant strains, the knockout of the genes was reflected in the model by setting the corresponding fluxes to zero.

*Step 2*: In order to examine the effect of PPC or ICL flux on threonine production, the PPC or ICL flux was perturbed from the minimum value to the maximum value, with maximization of the Thr production rate as an objective function. To investigate the effect of the central metabolic flux on acetic acid formation rate, each flux was perturbed from the minimum value to the maximum value, with the maximization of the acetic acid production rate as an objective function.

*Step 3*: The flux profile graph is generated by plotting and comparing the threonine production rate or acetic acid production rate on *y*-axis at different rates of the corresponding flux perturbed.

In order to investigate the effect of PPC or ICL flux on Thr production, constraint-based flux analysis was performed on the rationally engineered TH07C (pBRThrABC) strain. The glucose uptake rate was set to be 3.457 mmol/gDCW/h, which is the experimentally measured value at the exponential growth phase (7.5 ~ 10.75 h). In order to investigate the most effective flux change for reducing the acetic acid production rate, constraint-based flux analysis was performed on the rationally engineered TH27C (pBRThrABCR3) strain. The glucose uptake rate was set to be 0.9 mmol/gDCW/h, which is the experimentally measured value at 51.9 ~ 56.1 h. During simulation, the uptake rates of L-methionine and L-lysine were set at 5 mmol/gDCW/h, which is large enough to ensure cell growth.

### Analytical procedures

Cell growth was monitored by measuring absorbance at 600 nm ($OD_{600}$) using an Ultrospec3000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Cell concentration defined as gram dry cell weight (gDCW) per liter was calculated from the predetermined standard curve relating $OD_{600}$ to dry weight (1 $OD_{600}$=0.431 gDCW/l). Glucose concentration was measured using a glucose analyzer (model 2700 STAT; Yellow Springs Instrument, Yellow Springs, OH). For analysis of amino acids, the supernatant obtained by centrifugation and filtration of the sampled culture broth (5 ml) was analyzed at Science Lab Center Co. (Daejeon, Korea). The appropriately diluted supernatant (100 ~ 500-fold diluted) was injected into the cation seperation column (LCA K06/Na 1.6 × 150 mm; Sykam GmbH, Eresing, Germany) and analyzed with the Sykam S433 amino-acid analyzer.

### Supplementary information

Supplementary information is available at the Molecular Systems Biology website (www.nature.com/msb).

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The gene expression data from this study have been submitted to Gene Expression Omnibus database under accession number GSE9434.

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