

Rewiring the PEP-pyruvate-oxaloacetate node through coordinated fine-tuning of *gltA* and *pykF* expression for L-threonine production in wild-type *Escherichia coli*

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Abstract

L-threonine is an essential amino acid for humans and monogastric animals, and produced industrially on a large scale by microbial fermentation. Maintaining a balanced allocation of carbon resources between cell growth and product formation is critical for achieving a high titer. Most previous studies have focused on deleting the pathways that compete with L-threonine biosynthesis, whereas few have examined fine-tuning of these pathways. Here, we rebalanced carbon distribution at the phosphoenolpyruvate (PEP)-pyruvate-oxaloacetate (PPO) node by coordinately regulating citrate synthase (*gltA*) and pyruvate kinase π (*pykF*). Growth-phase-responsive promoters maintain carbon flux through the tricarboxylic acid (TCA) cycle during exponential growth and redirected excess flux was redirected toward L-threonine biosynthesis after entry into the stationary phase. We then combined this module with reinforcement of the rate-limiting steps (*aspC*, *rhtC*, and *asd*), modulation of phosphoenolpyruvate carboxylase (*ppc*), and chromosomal integration of all expression cassettes. The resulting plasmid-free strain, WS026, required neither exogenous inducers nor antibiotics. It produced 28.3 g/L L-threonine in shake-flask culture, corresponding to a yield of 0.71 g/g glucose and an approximately 283-fold increase over the control strain. In a bioreactor, WS026 produced 101.6 g/L L-threonine. These results indicate that the fine-tuning of competing pathways, rather than deleting them, can improve flux distribution at the PPO node in *E. coli*. The strategy may also be useful for producing other aspartate-family amino acids.

Introduction

Humans and monogastric animals cannot synthesize L-threonine, so it must be supplied in the diet (Li et al., 2017). In swine diets, L-threonine is second only to L-lysine among the limiting amino acids; in poultry diets, it ranks third after L-lysine and L-methionine. Supplementation of low-protein with L-threonine can reduce feed costs and reduce nitrogen excretion (Dong et al., 2011). L-threonine is therefore produced on an industrial scale by microbial fermentation, and improving the efficiency of this bioconversion remains an active goal of metabolic engineering (Zhao et al., 2022). A central challenge is to increase product formation without compromising cell growth (Wu et al., 2023). Deletion or overexpression of individual key genes can readily disturb the intracellular metabolic network, leading to metabolic imbalance, intermediate accumulation, and byproduct formation (Ding and Liu, 2023). Balancing biomass formation, yield, and titer consequently remains difficult (Ding et al., 2020). Several methods have been developed to regulate gene expression and redirect metabolic flux, including adjustment of promoter and ribosome-binding-site strengths and expression of small regulatory RNAs (Jung et al., 2021; Xie et al., 2021).

Static regulation of gene expression cannot adapt to the changing metabolic state of a culture and may therefore produce suboptimal titers (Hartline et al., 2021). Dynamic strategies have been developed to address this limitation, including synthetic biosensors that detect pathway intermediates and genetic circuits that redirect flux. These systems have markedly improved the performance of engineered strains (Li et al., 2022; Teng et al., 2022). Most biosensor-based systems, however, require extensive

optimization of circuit components for the host strain and fermentation conditions. Temperature-sensitive regulators can reduce the metabolic burden during growth, but their industrial application is constrained by promoter leakiness and by perturbations caused by temperature shifts. Switch activation must also be timed carefully to support accumulation of the target metabolite (Fang et al., 2020).

Phosphoenolpyruvate (PEP), pyruvate, and oxaloacetate (OAA) converge at a central branch point known as the PEP-pyruvate-oxaloacetate (PPO) node. Carbon is partitioned at this node among glycolysis, the tricarboxylic acid (TCA) cycle, and the biosynthetic pathways for several amino acids (Koendjibiharie et al., 2020; Sauer and Eikmanns, 2005). PEP has the most energy-rich phosphate bond among the natural organophosphates and it is the starting material for the aromatic amino acid biosynthesis. Pyruvate is a precursor of alanine, valine, leucine, isoleucine, and lysine and must be regenerated to sustain fermentative pathways that recycle NAD(P)H. OAA is a TCA-cycle intermediate and the immediate precursor of aspartate, and thus of the aspartate-family amino acids, including L-threonine (Koendjibiharie et al., 2020). The distribution of flux at this node therefore limits L-threonine production in engineered strains (Wang et al., 2023a). L-threonine biosynthesis competes with the central metabolism for carbon and consumes NADPH and ATP, which are supplied mainly by the TCA cycle during aerobic growth (Shimizu and Matsuoka, 2022). Coordinating cellular energy and reducing power with the flux directed toward L-threonine is consequently essential.

L-threonine titers above 170 g/L have been achieved through multidimensional optimization of *E. coli* (Zhao et al., 2024). At the PPO node, however, the competing reactions are usually targeted individually or eliminated by gene deletion. Coordinated fine-tuning of multiple competing reactions in a wild-type background without exogenous inducers has received little attention. Here, we coordinately regulated the two principal competing reactions at the PPO node, citrate synthase (*gltA*) and pyruvate kinase I (*pykF*), using growth-phase-responsive promoters. We combined this module with reinforcement of the rate-limiting steps (*aspC*, *rhtC*, and *asd*), modulation of phosphoenolpyruvate carboxylase (*ppc*), and chromosomal integration of all expression cassettes. The resulting plasmid-free strain was cultivated without exogenous inducers or antibiotics. Our results show that the coordinated regulation of *gltA* and *pykF* improves L-threonine production more effectively than single-gene regulation or gene deletion. The strategy may also be applicable to the production of other aspartate-family amino acids.

Materials and methods

Strains and plasmids

Table S1 lists the strains and plasmids, and Table S2 lists the primers used in this study. Plasmids were assembled with the MultiF Seamless Assembly Mix (ABclonal Technology Co., Ltd., China) according to the supplier's instructions. Polymerase chain reaction (PCR) was performed with Phanta Flash Master DNA polymerase, and colony PCR was performed with Green Taq Mix (Vazyme Biotech Co., Ltd., China). Promoters were selected from two *E. coli* RNA-seq datasets of *E. coli*. The first dataset describes the response to different oxaloacetic acid concentrations and is available in the Genome Sequence Archive

(GSA) of the Big Data Center (<https://ngdc.cncb.ac.cn>, accession No. CRA012693). The second dataset describes different growth times and is available in the NCBI Gene Expression Omnibus (GEO, series accession No. GSE102672). Each promoter was amplified from the W3110 genome and cloned into pCDF-Duet or pACYC-Duet-1 to direct expression of the insert.

Construction of mutant strains

All mutants were constructed by CRISPR-Cas9 genome editing. The host was cultivated in Luria-Bertani (LB) medium broth or on LB-agar at either 30°C or 42°C. The editing plasmid pCas carries a constitutively expressed cas9 and lambda red recombination genes; transcription of the latter is induced by arabinose (Jiang et al., 2015). Overnight cultures were diluted to an OD₆₀₀ of 0.04 in LB broth containing arabinose (10 mM) and kanamycin (50 mg/L) to maintain the plasmid. Cultures were then incubated at 30°C and 200 rpm until the OD₆₀₀ reached 0.6. Competent cells were prepared from 50 mL of the induced culture, washed three times with ice-cold 10% glycerol, and resuspended in 2 mL of the same solution for storage at -70°C. Deletions were performed as previously described. For *gltA* deletion, pTargetF-*gltA*, which carries an N20 spacer targeting the *gltA* locus, was recovered from an overnight culture. A donor fragment containing the upstream and downstream homology arms of *gltA* was amplified by overlap-extension PCR. The plasmid and donor fragment were then electroporated into cells in which lambda red recombinase had been induced from pCas. After centrifugation, 1 mL of the culture was spread on LB-agar plates containing 50 mg/L of kanamycin and 50 mg/L spectinomycin for counterselection. Deletion of *gltA* was confirmed by colony PCR with the primers (*gltA*-up-F and *gltA*-check-R). pTargetF-*gltA* and the temperature-sensitive pCas plasmid were cured by adding 0.5 mM IPTG and incubating the cultures at 42°C with shaking. The other deletions listed in Table S1 were generated in the same manner.

Media and cultivation conditions

Shake-flask cultures were grown in 250-mL baffled flasks containing 30 mL of fermentation medium at 37°C and 200 rpm. CaCO₃ (20 g/L) was added to buffer the medium. Seed cultures were prepared by scraping cells from an LB-agar plate into 5 mL of sterile seed medium and incubating them at 37°C and 200 rpm for 8 h. Production flasks were inoculated with seed culture to an initial OD₆₀₀ of 0.2 and incubated for 48 h. The modified fermentation medium contained, per liter, 40 g glucose, 10 or 20 g (NH₄)₂SO₄, 7.46 g KH₂PO₄, 2 g citric acid, 5 g yeast extract, and 2 g MgSO₄·7H₂O, together with the trace elements 5 mg FeSO₄·7H₂O and 5 mg MnSO₄·H₂O.

Fed-batch fermentation was initiated with a two-step inoculum. The preculture was transferred at 10% (v/v) into fresh medium to generate a secondary seed culture. This culture was then used at 10% (v/v) to inoculate 1 L of fermentation medium in a 2-L bioreactor (Bailun Biotech, Shanghai, China). The pH was maintained at 7.0 by automatic addition of ammonium hydroxide. Glucose was supplied at an initial concentration of 20 g/L and was subsequently fed to maintain the concentration between 20 and 40 g/L,

with the concentration never allowed to fall 10 g/L. The temperature was maintained at 37°C, and the dissolved oxygen was kept at $\geq 30\%$ by adjusting the stirrer speed and airflow rate.

Metabolite analysis

Cell density was monitored by measuring OD₆₀₀ with a Varioskan Lux Multimode microplate reader (Thermo Fisher Scientific, USA). Supernatants were collected by centrifugation at 12,000×g for 10 min and used for analysis. Amino acids were separated by high-performance liquid chromatography (HPLC) on an Agilent 1260 series instrument (Agilent Technologies, USA) equipped with a Thermo ODS-2 Hypersil C18 column (250 mm×4.5 mm, USA). Samples were derivatized with orthophthalaldehyde (OPA) before injection (Korös et al., 2008). Residual glucose was measured with a glucose oxidase assay kit (Jian Cheng, Nanjing, China).

For analysis of the remaining metabolites, 50 mg of sample was homogenized in 500 µL of ice-cold 80% methanol in water and vortexed at 2,500 rpm for 5 min. Three freeze-thaw cycles were then performed, each consisting of 5 min in liquid nitrogen and 5 min on ice, with 2 min of vortexing between cycles. Extracts were kept at -20°C for 30 min, centrifuged at 12,000 rpm and 4°C for 10 min, and passed through a protein- precipitation plate before LC-MS analysis. Analytes were separated on a Waters ACQUITY UPLC H-Class system coupled to a Sciex QTRAP® 6500 + mass spectrometer fitted with a BEH Amide column (2.1 × 100 mm, 1.7 µm). Mobile phase A was 10 mM ammonium acetate in water containing 0.3% ammonium hydroxide, and mobile phase B of was 90% acetonitrile in water. The gradient was delivered at 0.4 mL/min, starting at 95% B from 0 to 1.2 min, stepping to 70% B at 8 min, remaining at 50% B from 9 to 11 min, and returning to 95% B over 11.1–15 min. The column was maintained at 40°C, and the injection volume was 2 µL. Metabolites were detected by electrospray ionization (ESI) in both polarities (ion-spray-voltage, + 5500 V and – 4500 V; source temperature, 550°C; curtain gas, 35 psi). Metabolites were quantified by scheduled multiple-reaction monitoring (MRM), with the declustering potential (DP) and collision energy (CE) tuned for each transition. Data were acquired and processed with Analyst® 1.6.3 and MultiQuant™ 3.0.3 software.

Statistical analysis

Values are presented as means of three biological replicates. Differences between two groups were assessed with two-tailed Student's t-tests. Biological replicates were independent cultures, each inoculated from a separate colony and grown in its own vessel.

Results

Engineering the threonine-producing chassis using wild-type *E. coli*

The presence of byproducts during microbial fermentation reduces the yield of the desired product and complicates downstream recovery. We therefore deleted three genes that divert carbon toward

byproduct formation in *E. coli* W3110: *ldhA* (lactate dehydrogenase), *adhE* (alcohol dehydrogenase), and *pflB* (pyruvate formate lyase). The *thr* operon is controlled by the leader peptide *thrL* through an attenuation mechanism that responds to the intracellular L-threonine concentration (Lee et al., 2007), which limits L-threonine production. To remove this control, we replaced the *thrL* leader region and its promoter with the $P_{trc} (\Delta LacO)$ promoter, resulting in the constitutive overexpression of *thrA*, *thrB*, and *thrC*. The pyruvate kinase isoenzymes, Pyk I and Pyk II, encoded by *pykF* and *pykA*, catalyze the final step of glycolysis. Citrate synthase, encoded by *gltA*, converts acetyl-CoA to citrate at the entry to the TCA cycle. OAA, a TCA-cycle, can instead be directed toward L-threonine biosynthesis. Thus, *gltA* and *pykF* represent the main competing reactions at the PPO node for L-threonine biosynthesis. Modulating their activities may redirect carbon toward L-threonine while preserving cell growth.

Glucose enters *E. coli* cells either through the galactose permease GalP together with the glucokinase Glk or through the glucose-specific phosphotransferase system (PTS) (Luo et al., 2014). Most glucose is taken up by the PTS, which couples transport to conversion of PEP into pyruvate. This arrangement makes glucose uptake and glycolysis efficient but leaves less PEP available for direct conversion to OAA. The PTS has therefore been modified by direct disruption, substitution, or attenuation (Zhu et al., 2019). To free PEP while maintaining efficient glucose transport, we deleted *ptsG*, which encodes the glucose-specific transporter enzyme IIBC^{Glc}, and the *crr*, which encodes enzyme IIA^{Glc}. We then placed the *glk-galP* cassette under the control of the $P_{trc} (\Delta LacO)$ and integrated it at the *adhE* locus. Three L-aspartate kinases, encoded by *thrA*, *metL*, and *lysC*, phosphorylate L-aspartate. The first two direct aspartate toward homoserine. Kinase I is the predominant isoenzyme in *E. coli* but is inhibited by L-threonine. A C-to-T substitution at position 1034 of *thrA* (*thrA**) relieves this inhibition (Lee et al., 2003). We inserted the heterologous *thrA** cassette downstream of the $P_{trc} (\Delta LacO)$ promoter and integrated it at the *ldhA* locus. The resulting threonine-producing chassis strain was designated WS000 (Fig. 1).

Coordinated fine-tuning of *gltA* and *pykF* for L-threonine production

Strains that overproduce oxaloacetate derivatives require a steady supply of the precursor. OAA is the branch point at which glycolysis, the TCA cycle, and the L-aspartate family of amino acids converge. In *E. coli*, OAA can be supplied from PEP by overexpressing phosphoenolpyruvate carboxylase (PPC) or by introducing a heterologous phosphoenolpyruvate carboxykinase (PCK) (Wang et al., 2023a). At the PPO node, the TCA cycle and L-threonine biosynthesis compete for carbon, which limits production efficiency (Wang et al., 2023b). Reducing OAA and PEP consumption by the TCA cycle and pyruvate kinase should therefore direct more carbon toward L-aspartate and L-threonine. To test this hypothesis, we deleted *gltA* and *pykF* simultaneously. As shown in Fig. 2, the OD600 of the double-deletion strain WS001 (WS000 carrying an empty plasmid) was approximately 90% lower than that of the other strains, and its L-threonine titer was only about 0.1 g/L.

We addressed this problem by developing a dynamic regulation system in which growth-phase-responsive promoters detect the cellular physiological state and redirect the distribution of carbon flux at the PPO node (Fig. 2A). During exponential growth, these promoters maintained sufficient *gltA* and

pykF expression to support carbon flux through the TCA cycle and sustain biomass formation. After the culture entered the stationary growth phase, expression decreased automatically, directing more carbon toward L-threonine production. We used this design to control *gltA* and *pykF* in WS000, and generated strains WS003-WS006 (Fig. 2B). Growth of these strains recovered to varying degrees relative to the negative control strain WS001. WS004 showed the largest increase in L-threonine titer, from 0.1 g/L to 1.9 g/L, a 19-fold increase without a detectable growth penalty. Coordinated regulation of *gltA* and *pykF* produced a substantially higher titer than regulation of *gltA* alone (WS002). To determine how this rewiring reshaped intracellular metabolism, we quantified the metabolites surrounding the PPO node by LC-MS/MS. Three groups were compared: the unregulated strain WSM1, the strain in which *gltA* alone was tuned (WSM2), and the strain in which *gltA* and *pykF* were tuned together (WSM3). As shown in Fig. 2E, several PPO-node metabolites differed markedly among them.

Combining the rate-limiting steps for L-threonine production

Beyond the flux balance at the PPO node, L-threonine synthesis depends on conversion of OAA to L-aspartate and on export of the amino acid after it accumulates. *aspC* encodes aspartate aminotransferase. In *E. coli*, *rhtA*, *rhtB*, and *rhtC* encode permeases that export amino acid. RhtC has been identified as the specific L-threonine transporter, and dynamic regulation of its activity promotes L-threonine export and production (Wang et al., 2022). *asd*, which encodes aspartate semialdehyde dehydrogenase, also contributes to L-threonine production (Zhao et al., 2018).

Although coordinated regulation of *gltA* and *pykF* improved flux balance and moderately increased L-threonine accumulation, increasing OAA utilization could further improve production further. We therefore asked whether modulating the downstream rate-limiting steps would enhance L-threonine production. Based on their roles in biosynthesis and export, *aspC*, *rhtC*, and *asd* were assembled into a single module. The expression of each gene was then tuned with plasmids carrying different promoters (Fig. 3A and B). We generated eight strains, WS007-014 with different promoter combinations (Fig. 3C), and measured their L-threonine accumulation (Fig. 3D). Strains carrying the *PgrcA* promoter showed a marked increase in L-threonine accumulation.

Combining the precursor biosynthesis gene for L-threonine production

OAA is the immediate precursor of aspartate in the TCA cycle. Studies of the aspartate-family amino acids, including homoserine, have repeatedly shown that overexpression of *ppc*, which encodes the anaplerotic enzyme phosphoenolpyruvate carboxylase, increases OAA availability. Although balanced expression of the L-threonine biosynthetic module improved production in WS013, we hypothesized, however, that production remained limited by a metabolic imbalance between cell growth and product synthesis at the PEP node.

To optimize flux distribution around the PEP node, we used different promoters to control *ppc* expression and generated a panel of strains (Fig. 4A and B). We measured L-threonine production in each strain to assess the effect of flux redistribution. As shown in Fig. 4C, only WS021, in which *ppc* overexpression was combined with the rate-limiting module under the same P_{grcA} promoter, produced more L-threonine than the parent strain WS013. Growth of the *ppc*-overexpressing strain was nevertheless clearly impaired (Fig. 4D).

Engineering chromosomal expression for L-threonine production

To control gene expression more precisely, we integrated the P_{tdcA} -*gltA-pykF* cassette at the *iclR* locus and the P_{grcA} -*aspC-rhtC-asd* cassette at the *acrA* and/or *poxB* loci. A second copy of the P_{tdcA} -*gltA-pykF* cassette was integrated at the *adiA* locus, and a third copy was integrated at the *speC* locus. We therefore obtained plasmid-free strains WS022-WS027. As shown in Fig. 5, the optimal chromosomal dosage was two copies of P_{tdcA} -*gltA-pykF* and one copy of P_{grcA} -*aspC-rhtC-asd*. The plasmid-free strain WS026 produced up to 19.4 g/L L-threonine, a 3.5-fold over the plasmid-bearing strain WS021. When WS026 was cultivated in the fermentation medium supplemented with 20 g/L $(\text{NH}_4)_2\text{SO}_4$, production increased substantially (Fig. 6A and B). The titer reached 28.3 g/L, approximately 50% higher than in the original medium, with a yield of 0.71 g/g glucose (Fig. 6C).

Fed-batch fermentation for L-threonine production

We assessed the production performance of the final strain, WS026, in fed-batch fermentation in a 2-L bioreactor. The pH was maintained at 7.0 to support rapid growth. L-threonine remained at a low concentration during the early fermentation phase, presumably because biomass formation preceded product synthesis. We monitored cell density and the residual glucose throughout the process. L-Threonine accumulation accelerated after the culture entered the stationary phase. The maximum cell density reached an OD_{600} of 49, and the residual glucose concentration was 1.5 g/L. After 72 h, WS026 had accumulated 101.6 g/L L-threonine (Fig. 6D), corresponding to an overall yield of 0.64 g/g glucose. This titer was approximately 3.6-fold than that obtained in shake flasks, and the process required neither exogenous inducers nor antibiotics.

Discussion

Excess of pyruvate is preferentially directed into the TCA cycle, where it is oxidized to CO_2 while generating reducing power (NADH/NADPH) and ATP (Sauer and Eikmanns, 2005). Pyruvate kinase (PYK) releases most intracellular pyruvate by dephosphorylating PEP. Additional pyruvate is generated by the phosphotransferase system during glucose uptake and, to a lesser extent, by the Entner-Doudoroff (ED) pathway (Fang et al., 2020). Carbon entering the TCA cycle under these conditions is therefore committed to oxidation and energy generation rather than to L-threonine biosynthesis. Accordingly, the

simultaneous deletion of *gltA* and *pykF* in strain WS001 did not redirect flux toward L-threonine; instead, both growth and production collapsed. Complete inhibition of the TCA cycle therefore disrupts cellular homeostasis and compromises L-threonine synthesis, consistent with previous research work (Fang et al., 2020). The competing reactions at the PPO node must therefore be modulated rather than eliminated. This balance allows available carbon to support both growth and L-threonine biosynthesis.

Deletion of *pykF* reduces flux through the Embden-Meyerhof-Parnas (EMP) pathway from 65% to 20% and increases flux through the pentose phosphate pathway rises from 34% to 79% (Siddiquee et al., 2004). This redistribution away from glycolysis, together with the associated reduction in acetate overflow, suggests that *pykF* deletion may decrease acetate production and improve L-threonine synthesis (Xie et al., 2014). These findings support the selection of *pykF* and *gltA* as targets for flux redistribution at the PPO node rather than as genes for complete deletion. We therefore downregulated both genes only after the culture had accumulated sufficient biomass. Fine-tuning key genes in *E. coli* has also been more effective than complete deletion for improving L-threonine production (Zhao et al., 2020).

Because static deletion cannot resolve the competition at the PPO node, we used growth-phase-responsive promoters to allow the *gltA-pykF* module to sense the cellular physiological state and redirect carbon automatically. Flux through the TCA cycle was maintained during exponential growth and redirected toward L-threonine after the culture entered the stationary phase. This system requires neither the temperature shifts nor the chemical inducers, which are required by metabolic toggle switches. Conditional inactivation of *gltA* has similarly been used to redirect carbon flux toward isopropanol production in *E. coli* (Soma et al., 2014). Our strategy also avoids the two-stage inducer-dependent processes that depend on an inducer, in which glycolytic flux is first directed to the TCA cycle and then redirected toward threonine by inducing *aspC*, *fdh*, *gdhA*, and *pntAB* (Liu et al., 2019).

The behaviour of the *aspC-rhtC-asd* module indicates that the expression levels of downstream steps must match the flux supplied by the upstream redistribution module. When the module was expressed too strongly, L-threonine decreased rather than increased. This result is consistent with reports that *RhtC* is the specific L-threonine transporter and that dynamic regulation of its activity promotes export and production (Wang et al., 2022). The contribution of aspartate semialdehyde dehydrogenase (*asd*) to L-threonine synthesis supports the same conclusion (Zhao et al., 2018). The capacity of the L-aspartate branch must therefore be matched to precursor supply. Excess capacity in the transamination and export steps may impose a burden that outweighs the benefit of increased export.

Adequate OAA supply is a required for flux through the L-aspartate branch, and *ppc* overexpression is a common way to increase it. Replacing the native *ppc* promoter with the strong P_{trc} promoter improved homoserine production in the W-H10 (Vo and Park, 2022). However, *ppc* overexpression may impair growth by reducing flux entering the TCA cycle at the PEP node (Sun et al., 2023). This growth penalty can also offset the benefit of increased precursor supply, leaving L-threonine production unchanged. The promoter series tested here supports this trade-off: *ppc* overexpression alone had little effect on L-

threonine accumulation, consistent with earlier reports (Du et al., 2020; Zhao et al., 2018). Production improved only when *ppc* was expressed together with the rate-limiting module under the same P_{grcA} promoter. An optimal, *ppc* expression level, rather than the highest possible level, is therefore required for maximum L-threonine production.

Plasmids are widely used to express and analyse functional genes and pathways. because they are easy to manipulate provide a broad range of expression levels (Li et al., 2019). Chromosomal integration, however, offers greater stability, lower cell-to-cell variability, and eliminates the need for antibiotics to maintain plasmids (Ou et al., 2018). Maximum expression is not always beneficial in metabolic engineering because it can impose a substantial stress response (Saleski et al., 2021). In the present study, chromosomal expression outperformed plasmid-based overexpression, consistent with previous reports (Wang et al., 2016). The optimum was reached at two copies of the *gltA-pykF* cassette and one copy of the *aspC-rhtC-asd* cassette. Precise control of the gene expression was therefore required to achieve a favourable flux distribution at the PEP node. Increasing the $(\text{NH}_4)_2\text{SO}_4$ concentration produced a 50% increase in titer, likely because of the greater ammonium supply. Amino acid biosynthesis requires nitrogen for transamination and precursor formation. The higher $(\text{NH}_4)_2\text{SO}_4$ concentration presumably met the cellular demand for ammonium ions (NH_4^+), which serve as nitrogen donors in central metabolism and support L-threonine formation (Zhang et al., 2025).

The highest reported L-threonine titer is 170.3 g/L, with a yield of 0.625 g/g glucose, achieved by the multidimensionally optimized strain THRH16. This strain was constructed through extensive protein and global-regulatory engineering of an already high-yielding parent (Zhao et al., 2024). WS026, by contrast, was developed from wild-type *E. coli* W3110 with a limited set of targeted modifications. Its shake-flask titer of 28.3 g/L nevertheless exceeded that of a strain engineered from wild-type MG1655, which produced 17.98 g/L (Zhu et al., 2019). Under fed-batch conditions, WS026 reached a yield of 0.64 g/g glucose, higher than those reported for most L-threonine-producing *E. coli* strains reported date. These include TWF083, which produced 116.62 g/L at a yield of 0.486 g/g (Zhao et al., 2020), and THRH16, which reached a yield of 0.625 g/g (Zhao et al., 2024). Fermentation of WS026 required neither exogenous inducers nor antibiotics, unlike processes that rely on IPTG induction (Liu et al., 2019) or plasmid-based expression (Song et al., 2024). Its titer remains below the highest reported values, which may reflect the limited number of engineering steps applied to the wild-type parent. Combining the coordinated fine-tuning of the PPO node with additional targets identified in high-yielding backgrounds may narrow this gap. Overall, these results support fine-tuning of competing reactions at the PPO node as a route to to high-yield L-threonine production from a wild-type chassis.

Conclusions

We developed a system that rewires the PPO node through the coordinated fine-tuning of *gltA* and *pykF*, enabling growth-phase-dependent control of metabolic flux. Flux into the TCA cycle was maintained during exponential growth and redirected toward L-threonine biosynthesis after the culture reached

sufficient cell density and entered the stationary phase after reaching an adequate cell density. Because the switch responds to the cellular physiological state, the strategy links the growth and production phases without external induction. It may be applicable to other bioproduction processes that require carbon redirection toward a synthetic pathway while limiting the burden on cell growth and maintenance.

Declarations

Competing Interests

The authors declare that they have no relevant financial or non-financial interests to disclose.

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Author Contribution

All authors contributed to the study conception and design of the study. Material preparation, data collection, and data analysis were performed by Buli Su, Peixuan Lai, Xiong Xiong, Yonghong Wang, and Man Li. Buli Su wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript and were responsible for funding acquisition and writing, review, and editing. All authors read and approved the final manuscript.

Data availability

Data will be made available upon reasonable request.

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Figures

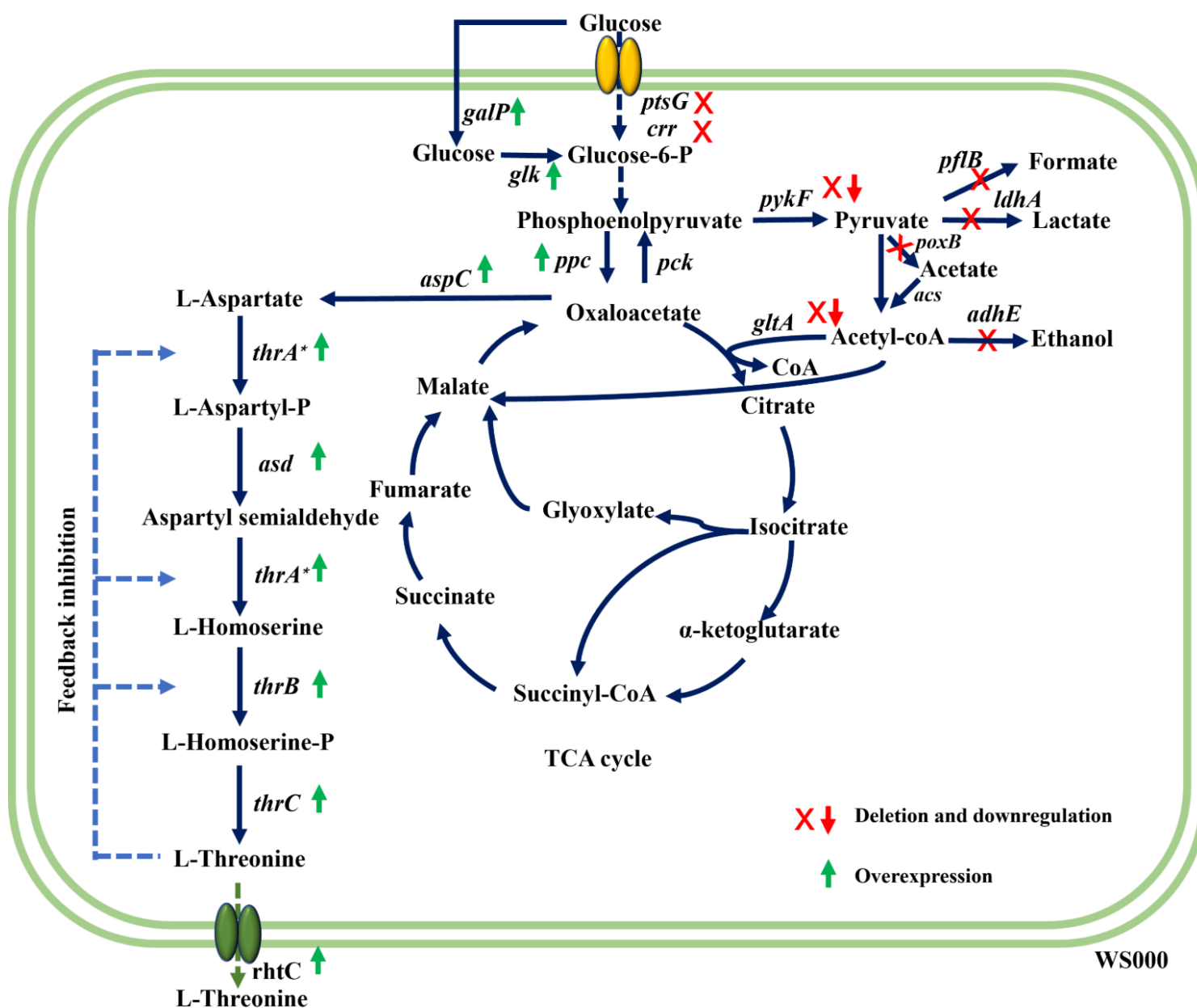


Figure 1

Schematic diagram of the strategies used to engineer the L-threonine-producing chassis.

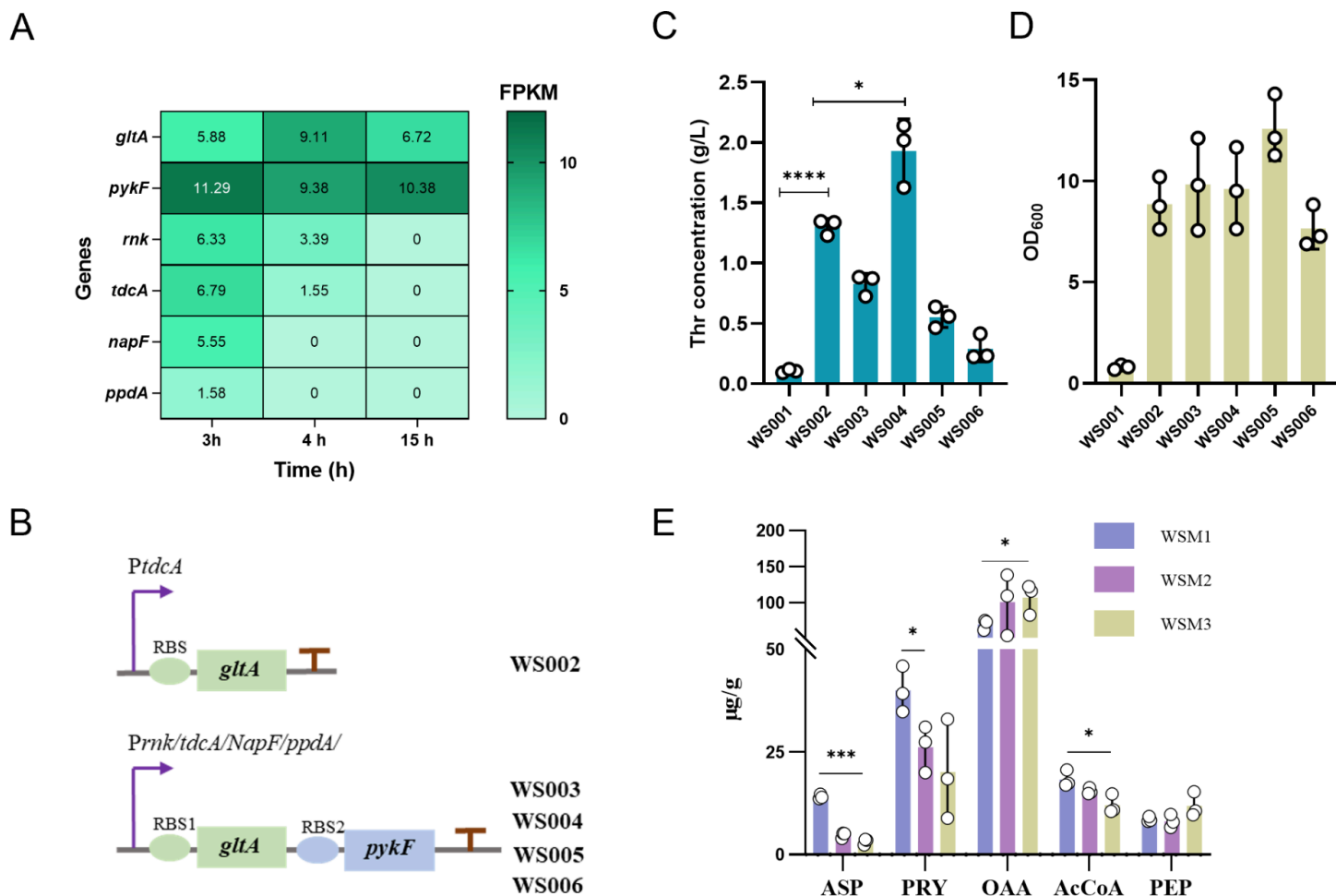


Figure 2

Coordinated fine-tuning of *gltA* and *pykF* for L-threonine production. (A) Expression patterns of the *gltA* and *pykF* and of the genes whose promoters were selected to regulate the *gltA-pykF* cassette. Times (h), RNA-seq measurements at 3, 4, and 15 h in *E. coli*. (B) Schematic representations of the cassette P_{tdcA} -*gltA* cassette and the *gltA-pykF* cassettes driven by different promoters. (C) L-threonine (Thr) concentrations (g/L) and (D) cell growth (OD₆₀₀) of strains WS001-WS006. (E) Metabolites surrounding the PPO node by LC-MS/MS for different strains. L-ASP, Aspartate; PRY, Pyruvic acid; OAA, Oxaloacetate; AcCoA, Acetyl-CoA; PEP, Phosphoenolpyruvic-acid. Data are means $\pm$ standard deviations of three biological replicates. *: $p < 0.05$ and ***: $p < 0.001$ by two-tailed Student's t-test.

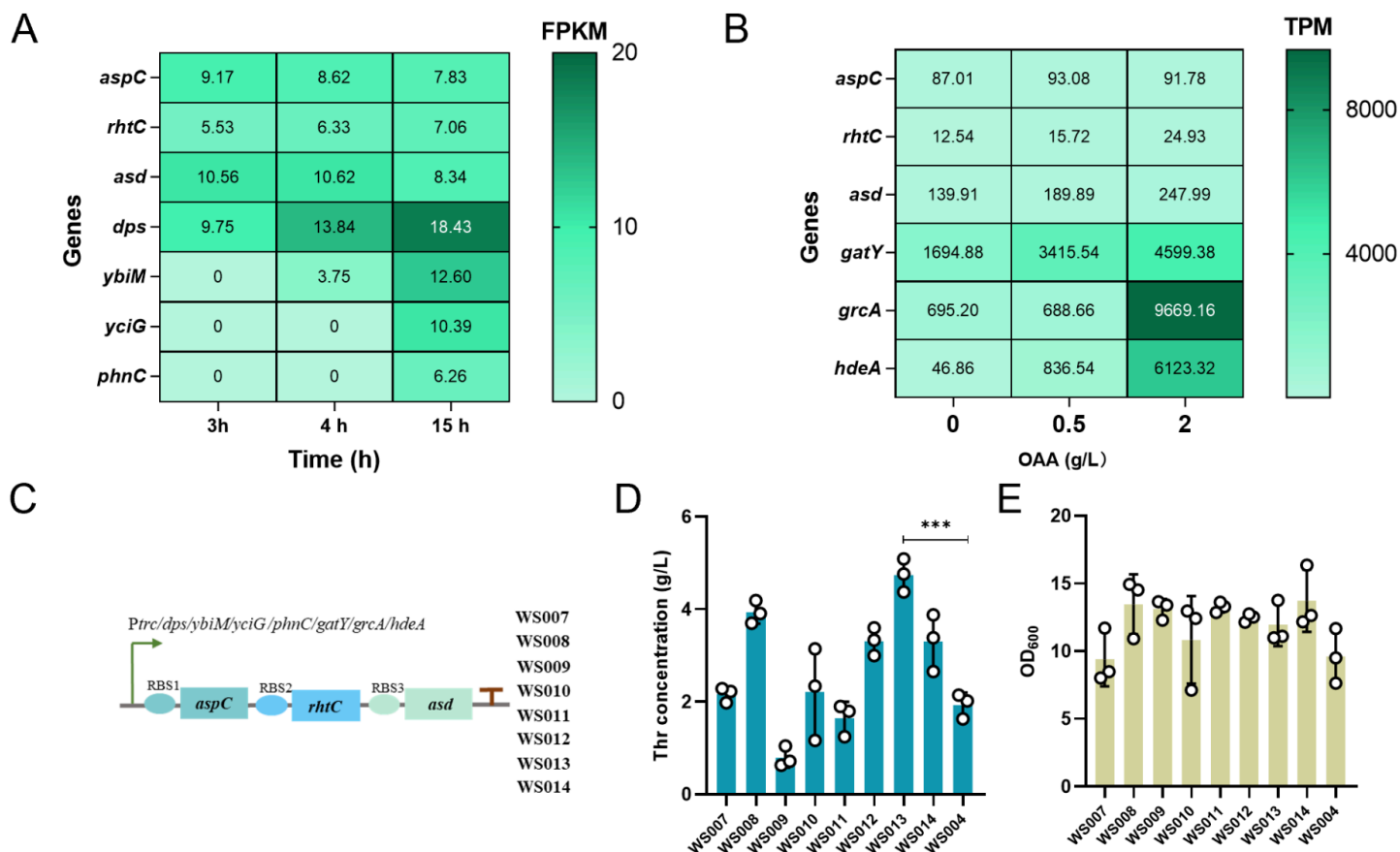


Figure 3

Regulation of *aspC*, *rhtC*, and *asd* for L-threonine production. (A, B) Expression patterns of the *aspC*, *rhtC*, and *asd* and of the genes whose promoters were selected to regulate the *aspC-rhtC-asd* cassette. Times (h), RNA-seq at 3, 4, and 15 h in *E. coli*. OAA (g/L), RNA-seq measurements in *E. coli* under different oxaloacetic acid concentrations. (C) Schematic representations of the *aspC-rhtC-asd* cassette under different promoters. (D) L-threonine (Thr) concentrations (g/L) and (E) cell growth (OD_{600}) of strains WS007-WS014. Data are means $\pm$ standard deviations of three biological replicates. ***: $p < 0.001$ by two-tailed Student's t-test.

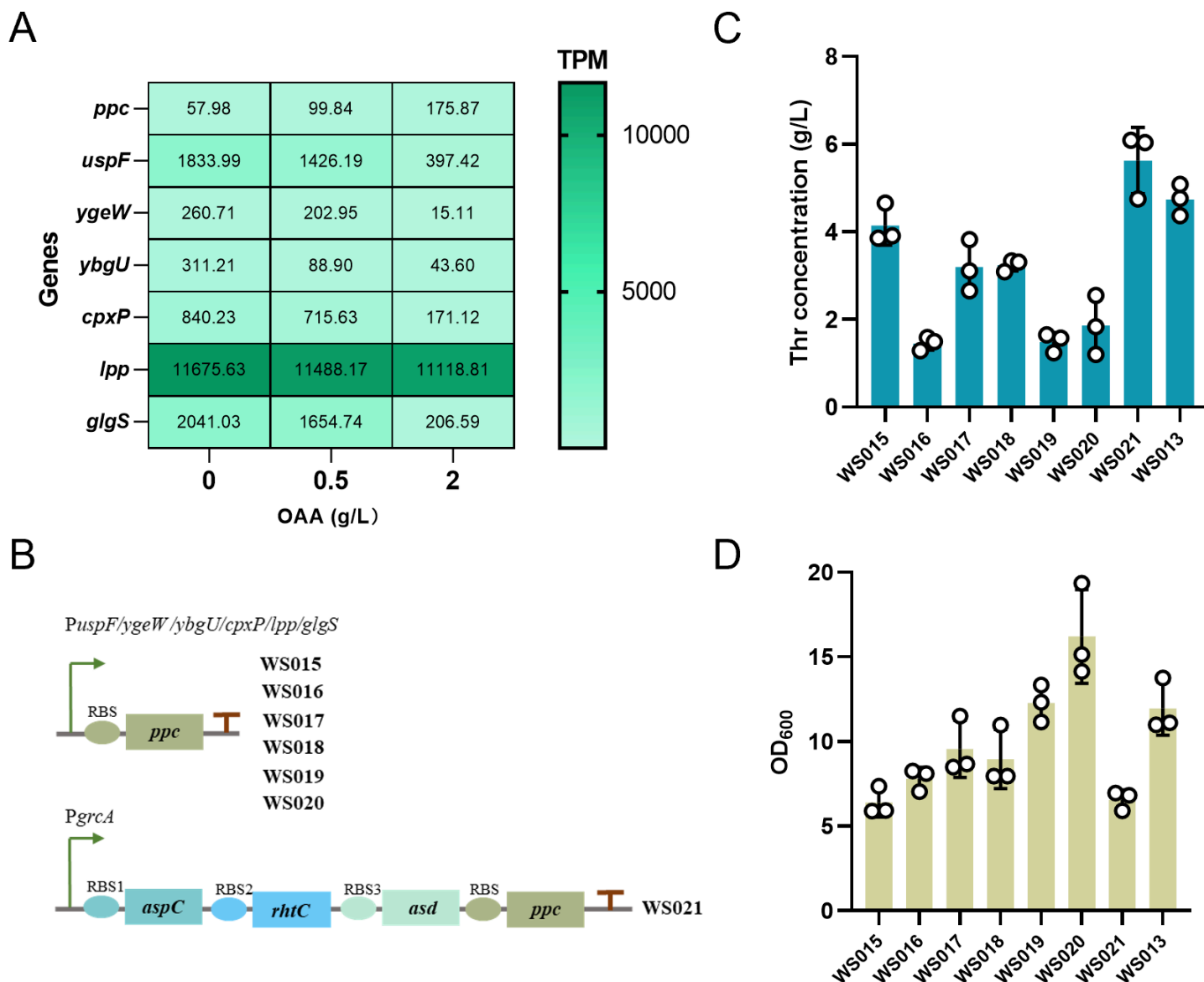


Figure 4

Regulation of *ppc* for L-threonine production. (A) Expression patterns of *ppc* and of the genes whose promoters were selected to regulate *ppc*. OAA (g/L), RNA-seq measurements in *E. coli* under different oxaloacetic acid concentrations. (B) Schematic representations of the *ppc* gene under different promoters. (C) L-threonine (Thr) concentrations (g/L) and (D) cell growth (OD₆₀₀) of strains WS015-WS021. Data are means $\pm$ standard deviations of three biological replicates.

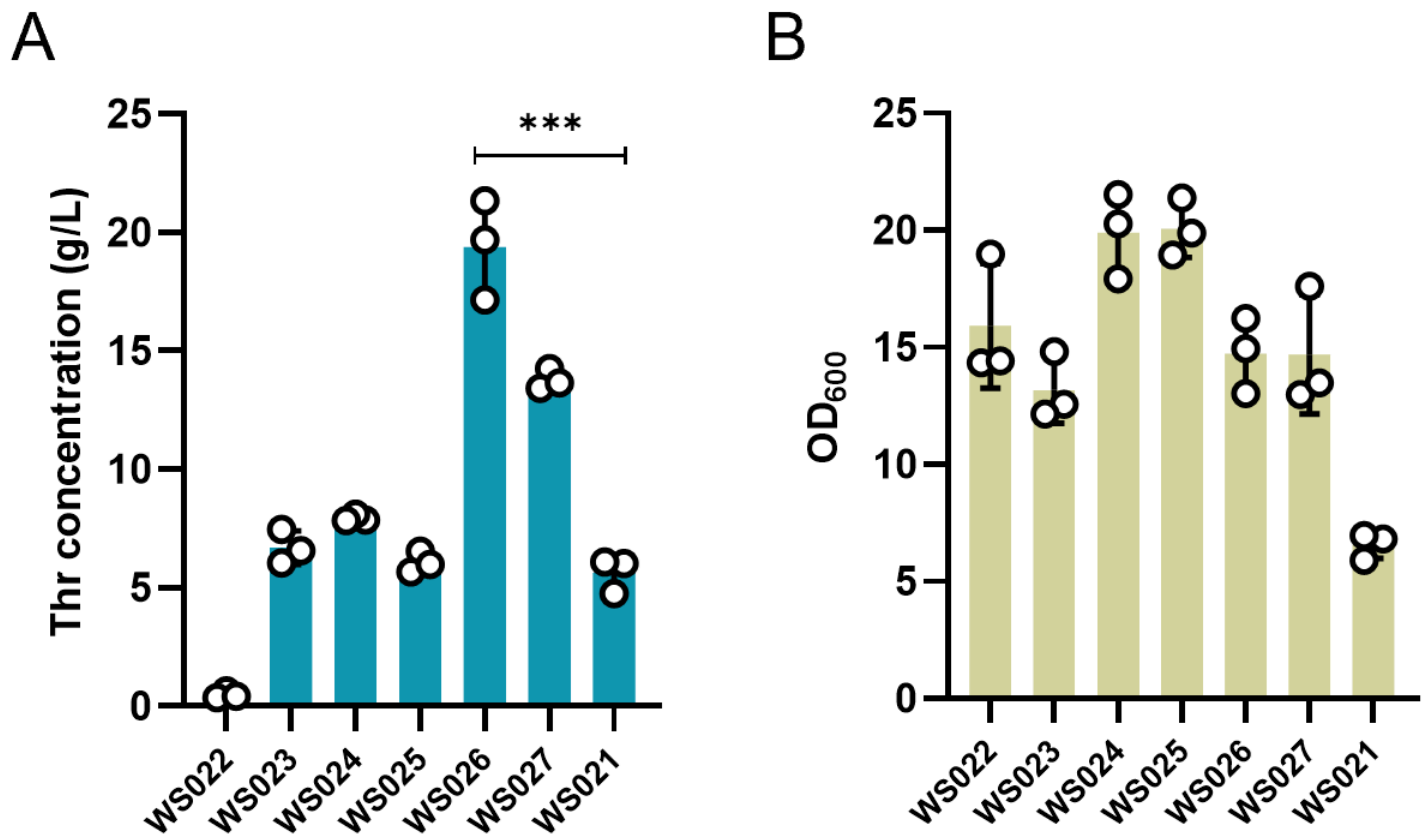


Figure 5

Chromosomal expression engineering for L-threonine production. (A) L-threonine (Thr) concentrations (g/L) and (B) cell growth (OD₆₀₀) of strains WS022-WS027. Data are means $\pm$ standard deviations of three biological replicates. ***: $p < 0.001$ by two-tailed Student's t-test.

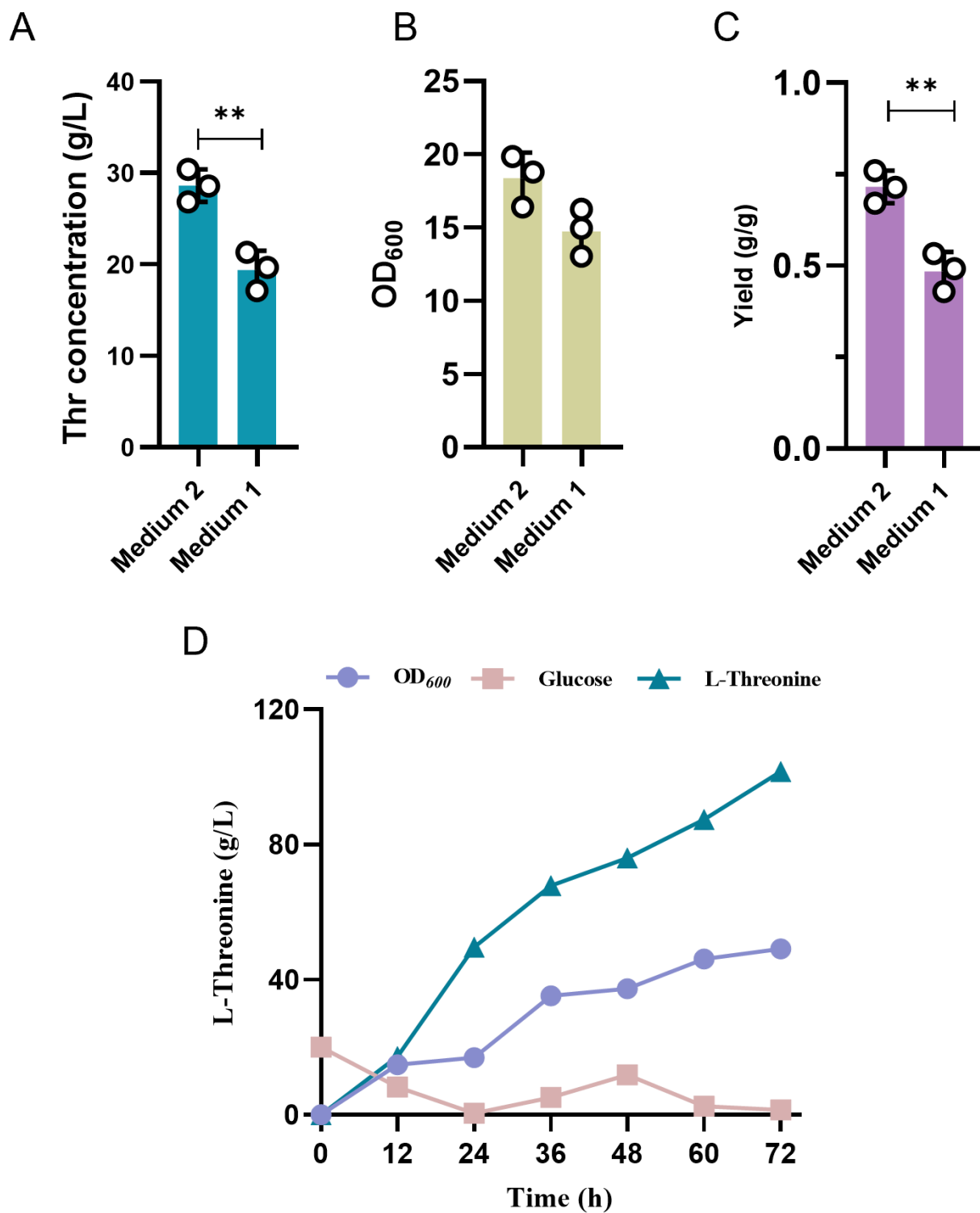


Figure 6

The L-threonine production by WS026 under different media and during fed-batch fermentation in a bioreactor. The L-threonine concentration (g/L) (A), cell growth (B) and L-threonine yield under different media (C); (D) time courses of L-threonine concentration (g/L), residual glucose (g/L), and cell density (OD₆₀₀) in the bioreactor.

Supplementary Files

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