Multiplex design of metabolic network for production of L-homoserine in *Escherichia coli*

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Abstract

L-homoserine, which is one of the few amino acids that is not produced on a large scale by microbial fermentation, plays a significant role in the synthesis of a series of valuable chemicals. In this study, systematic metabolic engineering was applied to target *E. coli* W3110 for production of L-homoserine.

Initially, the basic L-homoserine producer was engineered through the strategies overexpressing *thrA* (encoding homoserine dehydrogenase), removing the degradative and competitive pathways by knocking out *metA* (encoding homoserine O-succinyltransferase) and *thrB* (encoding homoserine kinase), reinforcing the transport system and redirecting the carbon flux by deleting *iclR* (encoding isocitrate lyase regulator). The resulted strain constructed by these strategies yielded 3.21 g/L of L-homoserine in batch cultures. Moreover, based on CRISPR-Cas9/dCas9 mediated gene repression for 50 genes, the iterative genetic modifications of biosynthesis pathways stepwise improved the L-homoserine yield. The rational integration of glucose uptake and recovery of L-glutamate increased L-homoserine production to 7.25 g/L in shake-flask cultivation. Furthermore, the intracellular metabolic analysis further provided target for strain modification by introducing the anaplerotic route afforded by pyruvate carboxylase to oxaloacetate formation, which resulted in accumulating 8.54 g/L L-homoserine (0.33 g/g glucose, 62.4% of the maximum theoretical yield) in shake-flask cultivation.

Finally, rationally designed strain gave 37.57 g/L L-homoserine under fed-batch fermentation, with a yield of 0.31 g/g glucose.

Importance

In this study, the bottlenecks that sequentially limited the L-homoserine biosynthesis were identified and resolved, based on the rational and efficient metabolic engineering strategies coupled with CRISPRi-based systematic analysis. The metabolomics data largely expanded the understanding of
metabolic effects and revealed relevant targets for further modification to achieve a preferable
performance. The systematic analysis strategy as well as metabolomics analysis can be used to
rationally design cell factories for the production of highly valuable chemicals.

Keywords
L-homoserine; Metabolic engineering; CRISPR interference system; Intracellular metabolite profiling;
Microbial cell factory
Introduction

L-homoserine (α-amino-γ-hydroxybutyric acid), the nonessential amino acid for the biosynthesis of L-threonine and L-methionine (1, 2), was firstly synthesized in 1907 by Fischer and Blumenthal. L-homoserine is also an important precursor for production of isobutanol, 1,4-butanediol (3), L-phosphinothricin (4), 2,4-dihydroxybutyrate (5) and 1,3-propanediol (6). Therefore, maximizing the productivity of L-homoserine based on the new genetic engineering tools would further extend its potentially chemical and biological applications (3, 7). The biosynthesis of L-homoserine was classified as the linear biosynthetic pathway consisting of glycolysis, TCA cycle and aspartate metabolic pathway (Fig. 1). The accumulation of L-homoserine was derived from the L-threonine- or L-methionine-producing strain. It’s found that most efforts were focused on regulating genes expression of L-aspartate metabolic pathway (8, 9). Generally, three aspartokinases isoenzymes (AKI, AKII, and AKIII) which catalyze the first step in L-homoserine biosynthesis were overexpressed to enhance the carbon flux to L-homoserine production. In addition, the transporter system was also enhanced to improve the survivability under the stress of L-homoserine (10). Due to lack of optimizing upstream of glycolysis as well as branched pathways (11), L-homoserine was produced with low yield by mutant and metabolically engineered strains including Corynebacterium glutamicum (12) and Escherichia coli (13, 14). Thus far, the optimization of each gene participating in different pathways and the identification of bottlenecks limiting the L-homoserine biosynthesis in E. coli have rarely been reported.

Currently, pathway design and optimization are essential to obtain microbial cell factories with excellent performance for industrial production of commercial chemicals (15, 16). A series of attempts have been employed to construct novel producers for biobased chemicals as the technologies in support...
of synthesizing new materials and manipulating genes matured (17–19). Indeed, some strategies have been applied to optimize the biosynthetic pathways which consist of large number of genes or reprogram gene expression to manipulate complex phenotypes. Prominent examples of such methods include multidimensional heuristic process (MHP) (20), multiplex navigation of global regulatory networks (MINR) (21) and multibranched and multilevel regulated biosynthetic pathways (MBMRPs) (22), which rapidly engineer organisms with desired capabilities without trial-and-error of iterative experimentation. On the other hand, “-omics” profiling technologies such as metabolomics has facilitated an overview of cell metabolism, hence allowing for a more in-depth insight into intracellular mechanisms in modified organisms analysis (23).

Herein, the initial L-homoserine producing strain was obtained through blocking the degradative and competitive pathways and overexpressing thrA (encoding homoserine dehydrogenase) based on O-succinyl homoserine producing strain (24). Then the rational designs, including reinforcement of transport system, redirection the carbon flux, CRISPRi system and module integration strategy, were used to construct an L-homoserine-producing strain. The iteratively modified strain HS33 could produce 7.25 g/L L-homoserine in a shake flask from glucose. Metabolomics analysis further revealed the intracellular behaviors (metabolic differences) in response to genetic modifications for directing the carbon to L-homoserine formation. Based on the metabolomics analysis, the anaplerotic route afforded by pyruvate carboxylase was introduced to direct carbon toward L-homoserine, which resulted in accumulating 8.54 g/L L-homoserine (0.33 g/g glucose, 62.4% of the maximum theoretical yield) in shake-flask cultivation. Finally, 37.57 g/L L-homoserine was produced under fed-batch fermentation, with a yield of 0.31 g/g glucose.

**Results**
Construction of an initial L-homoserine producing strain

The “pull-push-block” strategy is an efficient method to engineer microorganism in biosynthesizing target products by modifying metabolic networks (25, 26). In our previous study, the strain E. coli \( \Delta JIB^* \) Tm

\[ \text{metL} \]

with overexpression of \( \text{metL} \) and removal of \( \text{metJ}, \text{metI} \) and \( \text{metB} \) genes was constructed to produce O-succinyl homoserine from L-homoserine and succinyl-CoA (24). To construct the basic L-homoserine producing strain HS1, L-homoserine converting pathways related genes (\( \text{thrB} \) encoding homoserine kinase and \( \text{metA} \) encoding homoserine O-succinyltransferase) were successively deleted to “block” L-homoserine degradation. Then the L-homoserine converting pathways was further strengthened by overexpression of \( \text{thrA} \) to “push” the carbon flux to L-homoserine production. Subsequently, the lysine-auxotrophic strain HS2 was generated by deleting \( \text{lysA} \) to investigate the effect of eliminating precursor competing metabolic pathway on L-homoserine production. As shown in Fig. 2, HS1 could accumulate 1.85 g/L of L-homoserine in MS medium. The disruption of L-lysine biosynthesis increased the production of HS2 to 2.01 g/L with the same specific production of 0.33 g/g CDW when adding L-lysine (0.025 g/L, optimized amount) (Fig. S1).

Modification of transport system for the improvement of L-homoserine titer

From the observations that L-homoserine inhibits the activity of aspartokinase (encoded by \( \text{metL} \)) and L-glutamate dehydrogenase (encoded by \( \text{gdhA} \)) (27, 28), toxicity stress arising from gradual accumulation of product thus results in repressing cell growth and product yield (29, 30). Therefore, strengthening the ability of L-homoserine transport system and transformation of other toxic intermediate metabolites is a top priority. \( \text{rhtA} \), encoding the inner membrane transporter which is involved in the export of L-homoserine (31), was overexpressed chromosomally by replacing the native promoter with trc promoter to obtain the strain HS3 (Trc-\( \text{rhtA} \)). The strain HS3 increased the
production of L-homoserine by 30.9% to 2.63 g/L (Fig. 3). It is reported that overexpression of eamA confers resistance to the toxic chemicals (32). In order to further increase the L-homoserine export capacity and release the growth burden of homoserine-producing strains to afford survival, the native promoter of eamA gene was replaced by trc promoter in the strain HS4 (Trc-eamA). Batch cultivation of HS4 resulted in the production of 2.17 g/L L-homoserine. Moreover, two rhtA gene copies (the native rhtA gene and replacement of lacI gene) and eamA were overexpressed under trc promoter in chromosome to construct strain HS5 (ΔlacI:Trc-rhtA Trc-rhtA Trc-eamA). Under batch culture, the strain HS5 with modification of the transport system and construction of constitutive expression system could produce 3.14 g/L L-homoserine, which was 54.2% higher than strain HS2. In addition, the specific production of the strain HS5 was also increased (Fig. 3), which confirmed that enhancing transport system was beneficial for the improvement of L-homoserine productivity.

**Effect of glyoxylate shunt on L-homoserine production**

The deletion of iclR gene can active the glyoxylate shunt, which increases the availability of malate as the precursor of oxaloacetate (2, 33). To further direct the carbon flux to L-homoserine, the transcriptional regulator IclR was removed to construct strain HS6. On the other hand, the AspA-catalyzed reaction is an efficient alternative to the AspC-catalyzed one on basis of fumarate and oxaloacetate respectively, to produce aspartic acid derived products (34, 35). Then the deletion of fumarase genes (fumA, fumC and fumB) and overexpression of aspA were implemented respectively. As shown in Fig. 4, the final L-homoserine concentration of iclR-deleted strain HS6 obtained was equal to the control strain. Under batch culture, both HS7 (fumAC-deleted) and HS8 (aspA-overexpressed) strains showed lower L-homoserine production compared to the HS6.

**Repression of candidate genes by CRISPRi system for further enhancing L-homoserine**
To identify the target genes to be further manipulated, CRISPR interference (CRISPRi) system for sequence-specific control of gene expression was performed on basis of L-homoserine production strain HS6. Specifically, the homoserine-forming pathway were partitioned into three modules, which were separated at GAP (D-glyceraldehyde 3-phosphate) and OAA (Oxaloacetate). These metabolic nodes involved in glycolytic pathway, by-products production and amino acid biosynthesis. The results of strains with target gene downregulation were measured and compared with the control harboring the pTarget-null plasmid without an N20 sequence (HS6/(pdCas9 + pTarget-null)). As shown in Fig. 5a, 39 genes were chosen to study the effects of target genes repression on L-homoserine production. Compared with the control group HS6/(pdCas9 + pTarget-null), the strains of sgRNAs targeting genes exhibited difference in the accumulation of L-homoserine. The L-homoserine productions of sgRNAs interfered strains with down-regulation of ptsH, ptsI, crr, ptsG, tktA, rpe, talB, argA, argG, proB and gadA in three modules were increased by more than 100%. On the other hand, the sgRNAs targeting zwf, pta and poxB also increased the accumulation of L-homoserine by 50% - 100% (Fig. 5a).

On basis of these results, the candidate genes with promising positive targets on L-homoserine yields were selected for further individual deletion. As such, ptsG, ptsH, ptsI, crr, tktA, rpe, talB, zwf, ackA, pta, ilvA, poxB, argG, gadA and proB were removed individually based on strain HS6 to construct a series of novel L-homoserine producers (strains HS9 to HS23). As shown in Figure 5b, the cell growth of these engineered strains was variant, especially that the growth of the strain with deletion of ptsI was inhibited seriously. Additionally, the results showed that the strains with individual deletion of ptsG, ptsH, crr, tktA, rpe, pta, argG and proB accumulated higher L-homoserine content than the control strain. Especially, the strains HS9(ΔptsG) and HS12(Δcrr) with perturbation of the
PTS accumulated L-homoserine production to 5.27 g/L and 5.52 g/L respectively, which were higher than other mutations. These positive genes which were screened out by CRISPRi system would be further investigated to identify the rate-limiting steps blocking the L-homoserine biosynthesis.

Restoration of glucose uptake by non-PTS sugar transporter modification on basis of HS9

On basis of above results, the effects of the iterative genetic modifications of the selected genes not participating in L-homoserine biosynthesis were investigated. In this study, combinational genetic perturbation targeting PTS and pentose phosphate pathway was performed. To provide ample upstream pathway strength, new set of five strains were engineered (HS24 to HS28) on basis of perturbation of the PTS equilibrium, which iterative genetic modifications was carried out between PTS and pentose phosphate pathway. Unexpectedly, the results showed that L-homoserine yield was reduced in these further engineered strains (Fig. 6a). Since the differences of cell growth and major by-products accumulation between HS9 and HS12, the ΔptsG strain (HS9) was chosen as the candidate for further investigation (Fig. 6b). Given the unsatisfied glucose uptake caused by the ptsG gene deletion, the promoter region of the glk gene (encoding glucokinase) was replaced with the trc promoter, and inactivation of galR lead to constitutive expression of non-PTS sugar transporter to restore glucose transport capacity for efficient L-homoserine production (Fig. S2). The L-homoserine titer observed with the HS29 strain was 6.27 g/L, which increased 19.0% compared to the HS9 strain. The major byproducts including acetic acid and α-ketoglutarate, which were produced to 1.86 g/L and 2.39 g/L in flask fermentation, respectively.

Flux reinforcement through enhancing circulation of amino group donor

Glutamate functioning as the amino donor for the transformation of oxaloacetate to L-aspartate, the accumulation of α-ketoglutarate from L-glutamate in conditions of nitrogen limitation should be
unneglectable. Not only is it responsible for catalyzing the first reaction in ammonia assimilation, but coordinates carbon and nitrogen utilization by rapid modulation of glycolytic flux to alter import of glucose and consumption of PEP (11). Therefore, in order to convert α-ketoglutarate to L-glutamate, the strain HS30 was constructed by replacing the wild promoter of gltBD operon (encoding L-glutamate synthase) with a strong trc promoter on basis of strain HS6, and L-homoserine yield was increased by 23.1% to 3.95 g/L. It showed that the amino group donor (L-glutamate) supply was considered as another bottleneck in biosynthesis of L-homoserine. Additionally, another set of two strains (HS31 and HS32) were constructed by deleting argG and proB based on gltBD gene up-regulation strain HS30 for increasing availability of intracellular L-aspartate and L-glutamate, respectively. However, not only were the cell growth and glucose consumption severely repressed, but lower accumulation of L-homoserine was observed especially for the strain HS32 (Fig. 7a).

The observations prompted the construction of the strain HS33, which was iteratively modified by upregulating the expression of L-glutamate synthase based on strain HS29 to remove the deeper rate-limiting step. The successful overexpression of selected genes was subsequently verified by RT-qPCR (Fig. S3). Notably, the observed L-homoserine production by strain HS33 was increased with 15.6% up to 7.25 g/L without affecting the cell growth and consumption of glucose (Figure 7b). As well, the concentration of byproduct α-ketoglutarate was further decreased to 1.63 g/L (Figure 7b), indicating that the up-regulation of gltBD operon could effectively facilitate the conversion of α-ketoglutarate into L-glutamate. It is concluded that the multi-directional control of metabolic pathways associating with metabolic strategies and CRISPR-based transcriptional regulators enabled efficient carbon utilization and recovery of amino group donor for production of L-homoserine.

**Metabolic variation response to the iterative genetic modifications**
The intracellular metabolite profiling study was performed in *E. coli* W3110 and HS33 to further investigate the effectiveness of the above iterative genetic modifications and identify important metabolic pathways that are closely associated with overproduction of L-homoserine (Fig. 8a). In strain HS33, the intracellular levels of glycolytic intermediates such as glucose-6-phosphate (G6P), pyruvate and acetyl-CoA increased for 2.3-, 2.2- and 18.2-fold, respectively. Additionally, a 3.3-fold increase in AMP was also detected in this strain. These results indicated that in the strain HS33, the disruption of PTS system (deletion of *ptsG*) effectively reduced consumption of phosphoenolpyruvate (PEP), which was beneficial to increase the formation of precursor for L-homoserine biosynthesis. Moreover, the activation of non-PTS system by *glk* gene promoter replacement and *galR* gene deletion increased the consumption of ATP, resulting in the elevation of AMP. 2.3- and 2.2-fold increase for fumarate and malate as well as 35.0% decrease for isocitrate were observed in strain HS33, which indicated the activation of glyoxylate shunt in TCA cycle. The concentrations of α-ketoglutarate and L-glutamate increased for 5.9- and 3.2-fold, respectively, in the strain HS33. In terms of two metabolites, the biosynthesis of asparate and α-ketoglutarate is through transamination between oxaloacetate and L-glutamate. And L-glutamate, as the donor of an α-amino group for the biosynthesis of amino acids, can be synthesized from α-ketoglutarate by transamination reaction. The increased L-glutamate and α-ketoglutarate pools indicated that the overexpression of *gliBD* cluster could effectively provide amino donor for L-homoserine biosynthetic pathway and adjust circulation of their pools within the proper range. With the increasing of L-homoserine pool, the decrease of aspartate was also observed. These results demonstrated that the enhancement of L-aspartate metabolism pathway associating with disruption of competitive pathways could effectively activate the biosynthesis of L-homoserine from L-aspartate. The concentration of the byproduct 2,6-diaminopimelate (DAP) was increased 2.2-fold due
to knocking out *lysA*. Based on the metabolome analyses, the better understanding is needed on the new limitations such as carbon redirection and L-homoserine efflux, which would facilitate flux reinforcement through introducing anaplerotic pathway or other regulatory mechanisms for further valid and fast transport of L-homoserine.

**Replenishment of oxaloacetate from pyruvate towards further improvement based on metabolomics analysis**

While obtaining a global overview of metabolic variation, we found that intracellular levels of acetyl-CoA for strain HS33 was higher (more than 18-fold) as compared to the wide-type strain, suggesting the carbon flux was not efficiently diverted to oxaloacetate or citrate for production. To address this new bottleneck, two strategies were investigated: 1) introducing the pyruvate carboxylase gene into the engineered strain to direct pyruvate to the oxaloacetate rather than the acetyl-CoA; 2) introducing the citrate synthase gene into the engineered strain to direct acetyl-CoA to the TCA cycle.

Since *E. coli* lacks the anaplerotic enzyme pyruvate carboxylase, pyruvate accumulation in the strain could not be directly altered by directing pyruvate to the aspartate branch (Fig. 9a). Although the *thrA* gene was previously overexpressed by replacing the trc promoter, the transcription level was increased by about 2-fold (Fig S3). To further enhance the flux of L-aspartate branch, we overexpressed an aspartate-insensitive pyruvate carboxylase (encoded by *pycP438S*) from *C. glutamicum*, threonine-insensitive bifunctional aspartokinase/homoserine dehydrogenase (encoded by *thrA*G433R) and aspartokinase (encoded by *lysC*) from *E. coli* in plasmid pACYC 184. Additionally, the *E. coli* citrate synthase functions as a trimer of dimeric subunits and its activity is allosterically inhibited by NADH and 2-oxoglutarate (36). By contrast, citrate synthase from Gram-positive bacteria and all eukaryotes is a simple dimer that is not allosterically regulated (37). Therefore, we overexpressed various citrate
synthase genes including *citA*<sub>bs</sub> (*B. subtilis*), *gltA*<sub>cg</sub> (*C. glutamicum*) and *gltA*<sub>ec</sub> (*E. coli*) genes in plasmid pTrc99A, respectively. Four plasmids were transformed into strain HS33 respectively, resulting in strain HS33/pACYC-*pyc<sup>P458S</sup>-*thr<sup>G433R</sup>*lysC*, HS33/pTrc-*citA*<sub>bs</sub>, HS33/pTrc-*gltA*<sub>cg</sub> and HS33/pTrc-*gltA*<sub>ec</sub> (see Table 1).

Five strains including HS33 were cultivated in MS medium for 48 h. Among the tested strains, HS33/pACYC-*pyc<sup>P458S</sup>-*thr<sup>G433R</sup>*lysC* can produce 8.54 g/L L-homoserine (0.33 g/g glucose), which was increased by 17.8% compared to the strain HS33. Pyruvate carboxylase serves as an anaplerotic role for carbon redistribution by directing pyruvate to the aspartate branch, which was shown to be efficient to reduce the accumulation of α-ketoglutarate (0.26 g/L) and acetate (1.35 g/L). However, the other three strains that overexpressing citrate synthase produced lower amounts of L-homoserine than the corresponding strain HS33 (Fig. 9b). The overexpression of citrate synthase from *B. subtilis* resulted in reducing the accumulation of α-ketoglutarate, but the L-homoserine yield was higher than the control (0.30 vs 0.23 g/g glucose). By contrast, the overexpression of citrate synthase from *C. glutamicum* and *E. coli* showed improved growth and reduced by-product (acetate) accumulation. However, the α-ketoglutarate was significantly accumulated, which was probably due to that the flux was altered by directing oxaloacetate to the citrate branch with this enzyme.

**Fed-batch production of L-homoserine**

In order to scale up for L-homoserine production of HS33/pACYC-*pyc<sup>P458S</sup>-*thr<sup>G433R</sup>*lysC* from glucose, fed-batch culture was implemented in a 5 L bioreactor containing 2 L of media. The cell growth, L-homoserine production and residual glucose during the fed-batch culture were shown in Figure 10. A total of 450 mL feeding medium containing 500 g/L glucose was added into the bioreactor during the cultivation, and the residual glucose concentration was maintained below 10 g/L. The cell...
growth entered stationary phase within 40 h, and the OD600 value fluctuated around 30 till the end of
cultivation. Within 108 h of the fermentation, the L-homoserine production continuously increased and
was independent of cell growth. And the maximum titer achieved was 37.57 g/L with yield of 0.31 g/g
glucose, representing a 4.4-fold increase compared to the titer achieved in flask-shake cultivation.

By-products of amino acids were almost undetectable. However, further effort is needed to increase the
titer and shorten fermentation time, such as optimizing the expression of the recognized genes and the
culture conditions.

Discussion

This work shows that a basic homoserine producer was constructed through the combination of the
traditional “pull-push-block” effects of metabolic engineering and rational genetic modifications. In
accordance with the results reported previously, the disruption of competitive and degradative
pathways (L-threonine, L-lysine and L-methionine pathways) contributed to increasing the production
of L-homoserine (13). When it comes to the accumulation of target products which are toxic to cells or
inhibit activity of enzymes in the metabolic pathway, strengthening the exportation of these metabolites
is of great importance to release cell burden and enhance the production (32, 38–40). Here,
strengthening transport system and construction of constitutive expression system not only allowed
constitutive expression of modified genes with sparing the addition of inducer, but avoided suppressing
cell growth caused by inducer and L-homoserine (32). Additionally, the observations of deleting the
transcriptional regulator IclR indicated that the TCA cycle could operate smoothly when activating the
glyoxylate shunt flux. However, the diversion of flux away from forming oxaloacetate from fumaric
acid by deletion of fumarase genes or overexpression of aspA dramatically reduced the production of
L-homoserine, which may attribute to the toxicity of fumaric acids (41).
In addition to above rather obvious genetic modifications, we also tested the impact of other different genes on L-homoserine production by using CRISPRi-based gene repression methodology. The results of gene repression indicated that CRISPRi system was a feasible method to regulate gene expression with less labor and time consumption. Specifically, down-regulation of the phosphotransferase system (PTS) is widely approved for reinforcing the biosynthesis of phosphoenolpyruvate (PEP)-derived products (42, 43). The repression of zwf, tktA, rpe and talB disrupted the pentose phosphate pathway, which can direct more carbon to the synthesis of precursors in glycolytic pathway. argA, argG, proB and gadA repressing-regulation may contribute to accumulate L-glutamate and L-aspartate and therefore provided more amino donors for L-homoserine biosynthesis (44). And the repression of ackA, pta and poxB contributed to reducing secretion of acetate, which blocked the byproducts formation pathway and would release the acid stress. Moreover, validation of screened candidate genes by individual gene deletion further indicated that sequence-specific control of gene expression on a genome-wide scale was a practical tool for engineering genetic regulatory systems in L-homoserine biosynthesis (45). However, we observed that deletions of some genes led to the decrease in L-homoserine production, which were not consist with the results that these genes were repressed by CRISPR-dCas9. Because the possibility of completely inhibiting gene expression with CRISPRi is low. In some cases, even a low expression level of certain genes located in metabolic pathways could potentially contribute enough enzyme activity to maintain its flux and thus cover the effect of targeting such genes (46).

Based on CRISPRi-based systematic analysis, integration of glucose uptake and recovery of L-glutamate was adopted to develop a stable strain with better performance. In the metabolism of the carbon skeletons of L-homoserine, PTS disruption is beneficial to improve the intracellular
phosphoenolpyruvate (PEP) pool size, which is direct precursor of L-aspartate family amino acids (47). However, the observed phenotype of PTS disruption mutations other than ptsG mutation was in accord with the reports that partial or complete inactivation of PTS forced itself to actuate ATP-dependent glucose import system (48). Therefore, restoration of glucose transport capacity could efficiently improve L-homoserine production. In terms of engineering amino acid supply pathways, enhancing circulation of amino group donor through converting α-ketoglutarate to L-glutamate could also efficiently enhance L-homoserine production. However, increasing the availability of intracellular amino acids L-aspartate and L-glutamate by blocking the metabolic pathways failed to increase the accumulation of L-homoserine. The results indicated that the regulation of L-aspartate and L-glutamate metabolism was complicated as they are precursors involved in more than 10 pathways, which made it difficult to identify the major bottleneck for L-homoserine accumulation.

The metabolomics analysis revealed that the multiplex design of metabolic network caused less flexibility of flux distribution on the metabolome level. The significant variations of strain HS33 directed more carbon toward the L-homoserine biosynthesis after the multiple genetic modifications. Additionally, it facilitated understanding of the underlying metabolic effects and provided relevant targets for strain modification to achieve a preferable performance. The results demonstrated that introducing the anaplerotic pathway to direct pyruvate to the oxaloacetate was superior to the strategy by directing acetyl-CoA to the TCA cycle. The metabolic bottleneck for the production of L-homoserine could be efficiently relieved by modification of metabolic flux at both oxaloacetate and pyruvate nodes. Directing pyruvate to the L-aspartate branch could directly realize carbon redistribution while regulation of the TCA cycle was quite complex as it is a crucial component of metabolism in organisms (49).
In conclusion, 5 levels of bottlenecks including transport system, transcriptional regulation, carbon utilization, amino group donor circulation and anaplerotic path, which sequentially limited the L-homoserine biosynthesis, were identified and resolved after systematically searching and multiplex design of L-homoserine biosynthesis pathway. The L-homoserine production of the optimal strain HS33/pACYC- pyc^{P458S}-thrA^{G433R}-lysC reached 8.54 g/L (0.33 g/g glucose, 62.4% of the maximum theoretical yield) and 37.57 g/L (0.31 g/g glucose, 58.6% of the maximum theoretical yield) in shake-flask cultivation and fed-batch fermentation without optimizing the expression of the selected genes or the culture conditions (Table 2). Further, it is expected that the L-homoserine production by the optimal strain constructed in this study can be further enhanced through optimizing the expression of the positive genes and fermentation process, which would unlock the potential of the strain for the production of L-homoserine and its derivatives on a large scale.

Materials and Methods

Strains, plasmids and culture conditions

All strains and plasmids used in this study were listed in Table 1. Escherichia coli BL21 was used for plasmid construction, and the OSH-producing strain (24) was used as original strain. Low-copy plasmid pACYC184 was used as backbone for overexpressing mutated thrA gene (G433R) and lysC gene from Escherichia coli W3110 and mutated pyc gene (P458S) from Corynebacterium glutamicum ATCC 13032. Plasmid pTrc99A was used for overexpressing citrate synthase genes from E. coli W3110, Bacillus subtilis 168 and C. glutamicum ATCC 13032 respectively. Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) was used for cell cultivation. When needed, kanamycin and chloramphenicol were supplemented with final concentrations of 50 μg/mL.

Plasmid construction
To construct plasmid pACYC-pycP458S-thrA(G433R)-lysC, the mutated pyc gene (P458S) (NC_006958.1) was amplified from *C. glutamicum* and thrA gene (G433R) and lysC gene were amplified from *E. coli*, respectively (50). The three fragments were ligated into the pACYC184 by the standard protocol of *Gibson* assembly (51).

For expression of citrate synthase, the corresponding genes gltAec (NC_007779.1), citAbs (NC_000964.3) and gltAec (NC_006958.1) were amplified from the genomic DNA of *E. coli*, *Bacillus subtilis* and *C. glutamicum* using the primers GltAec_F/R, CitAbs_F/R and GltAec_F/R. Then the three fragments were assembled with the pTrc99A backbone fragment obtained by the primers pTrc99A_F/R to yield pTrc-gltAec, pTrc-citAbs and pTrc-gltAec, respectively. The primers used for plasmid construction are listed in Table 3.

**Construction of sgRNA-expressing plasmid for CRISPRi system**

For expression of citrate synthase, the corresponding genes gltAec (NC_007779.1), citAbs (NC_000964.3) and gltAec (NC_006958.1) were amplified from the genomic DNA of *E. coli*, *Bacillus subtilis* and *C. glutamicum* using the primers GltAec_F/R, CitAbs_F/R and GltAec_F/R. Then the three fragments were assembled with the pTrc99A backbone fragment obtained by the primers pTrc99A_F/R to yield pTrc-gltAec, pTrc-citAbs and pTrc-gltAec, respectively. The primers used for plasmid construction are listed in Table 3.

**Construction of sgRNA-expressing plasmid for CRISPRi system**

The pdCas9 plasmid with a catalytically dead Cas9 mutant was stored in our collection (45). The original plasmid pCas and pTargetF containing the Cas9 gene (52) and single-guide RNA (sgRNA) were donated by Dr. Sheng Yang from Institute of Plant Physiology and Ecology (Chinese Academy of Sciences Shanghai, China). The sgRNA-expressing plasmid library targeting selected genes was constructed according to the previously reported methods (22). Briefly, sgRNA cassettes were generated through PCR-based site-directed mutagenesis using original pTarget as template. Primer sequences used for generation of sgRNA cassettes and the corresponding sgRNA expression vectors are listed in Table 4. Every sgRNA-expressing plasmid for downregulation of target gene was transformed into electrocompetent cells containing pdCas9 plasmid to obtain a series of strains HS6/(pdCas9 + pTarget-X). X refers to the genes in central metabolism and selected amino acid biosynthetic pathways.

**Genetic manipulation of E. coli derivatives using CRISPR-Cas9**

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Gene insertion, deletion and chromosomal promoter replacement were achieved by using the CRISPR-Cas9 system as previously described (24). The detailed protocol of gene editing is described in the Supporting Information. Primers used in this study are listed in Table 5. Electrocompetent cells were prepared as previously described (52).

**Culture conditions**

The MS medium was used for L-homoserine production by *E. coli* W3110-derived strains, consisting of (per liter): 40 g glucose, 17 g (NH₄)₂SO₄, 4 g yeast extract, 1 g KH₂PO₄, 1 g MgSO₄, 25 g CaCO₃, 5 mg FeSO₄·7H₂O, 5 mg MnSO₄·7H₂O and 5 mg ZnSO₄. Shake-flask fermentation was performed in 500 mL flasks containing 20 mL MS medium at 28 °C for 48 h at 150 rpm. When necessary, the medium was supplemented with 50 μg/mL chloramphenicol or kanamycin. L-methionine and L-threonine were supplemented to the final concentrations of 0.05 and 0.2 g/L. Additionally, L-lysine was added to MS medium with a final concentration ranging from 0 to 0.5 g/L (0, 0.0125, 0.025, 0.05, 0.125, 0.25 and 0.5 g/L), depending on the experiments described in the text. For expression of CRISPRi system, strains were induced with 0.1 mM IPTG when the optical density at 600 nm (OD₆₀₀) reached 0.4-0.6. The cells were cultivated aerobically at 28 °C in a shaking incubator at 150 rpm, and 50 μg/mL antibiotics were supplemented when necessary.

In fed-batch fermentation, 5-L bioreactor (Baoxing, Shanghai, China) was used for L-homoserine with an effective working volume of 2.0 L. The medium for fed-batch fermentations consisted of (per liter) 15 g glucose, 14 g (NH₄)₂SO₄, 2 g yeast extract, 2 g KH₂PO₄, 0.5 g MgSO₄, 0.5g L-threonine, 0.2g L-methionine, 0.1g L-lysine, 5 mg FeSO₄·7H₂O, 5 mg MnSO₄·7H₂O and 5 mg ZnSO₄. The feeding medium contained 500 g/L glucose, 12.5 g/L KH₂PO₄, 10.0 g/L NaHCO₃, 4 g/L L-threonine, 1 g/L L-methionine, 0.5 g/L L-lysine, and the feed was started when the residual glucose concentration
was below 10 g/L. The fermentation temperature was maintained at 28 °C, and the pH was adjusted to 6.8 with 40 % ammonia water throughout the process. The dissolved oxygen level was maintained above 20 % by adjusting aeration and agitation rates.

**Analytical method**

**OD**

OD$_{600}$ was measured using the Eppendorf BioPhotometer D30 (Amersham Biosciences, Uppsala, Sweden). Concentrations of glucose were determined by using a glucose analyzer (YSI model 2300, Xylem Inc., NY, USA) with an IC Sep Ion-300 column (Transgenomic, San Jose, CA, USA).

Sykam S-433D amino acid analyzer (Sykam, Munich, Germany) was used to determine the concentration of amino acids. The by-products measurement was performed with Aminex HPX-87H Column (300 × 7.8 mm) using 5 mM H$_2$SO$_4$ as the mobile phase with a flow rate of 0.6 mL/min and detected via refractive index or UV absorption at 210 nm.

**RT-qPCR for mRNA Quantification**

The strains, *E. coli* W3110 and HS33, were cultured for 18 h in 20 mL MS medium at 150 rpm and 28 °C. Samples were collected during the exponential growth phase prepared by centrifugation and flash freezing in liquid nitrogen, and were send to TSINGKE Biological Technology (Wuhan, China) for RT-qPCR analysis with 16S rRNA as a control for normalization between samples. Fold changes of target genes were calculated as $2^{-\Delta\Delta CT}$ according to Schmittgen and Livak (53).

**Metabolome Analysis**

Metabolomics was applied to characterize the biological variation of the intercellular metabolites between *E. coli* W3110 and HS33. The samples were collected from cultures grown in 6 technical replicates per strain during the exponential growth phase. The cells were washed twice with ice-cold PBS and flash frozen in liquid nitrogen for further metabolite extraction. Statistical analysis of
metabolite profiles was performed by BGI TechSolutions Co., Ltd (Shenzhen, China). The intracellular metabolites concentrations of *E. coli* W3110 were used as a control for normalization between samples. The detail protocol of LC-MS system is described in the Supporting Information. The screened metabolomics data was subjected to Partial Least Square Discrimination Analysis (PLS-DA). Significantly changed metabolites (SCM; defined by based VIP >1.0, fold-change >1.20 or < 0.83 and q < 0.05) were selected for subsequent chemical structure identification.

**Statistical analysis**

All experiments were conducted in triplicate. The data were averaged and presented as mean ± standard deviation (SD). P values of <0.01 were considered statistically significant. All the figures were prepared using the Origin Software version 8.0 (OriginLab Corp., Northampton, MA, USA).

**Data availability**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

**Author Contributions**

ZQL, YGZ and BZ initiated and supervised the project. PL and ZHY carried out all the experiments and data analyses. PL and ZQL are responsible for the preparation and revision of the manuscript. All authors read and approved the final manuscript.

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The authors declare that they have no conflict of interest.

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Table 1 Bacterial strains and plasmid used in this study

| Strains or plasmids | Characteristics | Source |
|---------------------|-----------------|--------|
| **Strains**         |                 |        |
| *E. coli* BL21      | *E. coli* B F- dcm ompT hsdStrB-mB- gal [malB+] K-12(λ, S) | Trans BL21* |
| *E. coli* W3110     | Wild type, F-, λ, IN (rnmD-rrnE)1, rpl-1 | CGSC® |
| *E. coli* ΔJIB* TrcmetL | W3110, ΔmetI ΔmetL ΔmetB Trc-metL | (24) |
| HS1                 | *E. coli* ΔJIB* TrcmetL, ΔthrB ΔmetA Trc-thrA | This study |
| HS2                 | HS1, ΔlysA      | This study |
| HS3                 | HS2, Trc-rhtA   | This study |
| HS4                 | HS2, Trc-eamA   | This study |
| HS5                 | HS2, ΔlacI-2Trc-rhtA Trc-rhtA Trc-eamA | This study |
| HS6                 | HS5, ΔiclR      | This study |
| HS7                 | HS6, ΔjamAC     | This study |
| HS8                 | HS7, Trc-aspa   | This study |
| HS9                 | HS6, ΔptsG      | This study |
| HS10                | HS6, ΔptsH      | This study |
| HS11                | HS6, ΔptsI      | This study |
| HS12                | HS6, ΔacrA      | This study |
| HS13                | HS6, ΔaktA      | This study |
| HS14                | HS6, Δarpe      | This study |
| HS15                | HS6, ΔtalB      | This study |
| HS16                | HS6, Δawf       | This study |
| HS17                | HS6, ΔackA      | This study |
| HS18                | HS6, Δpta       | This study |
| HS19                | HS6, ΔsilA      | This study |
| HS20                | HS6, ΔpoxB      | This study |
| HS21                | HS6, ΔargG      | This study |
| HS22                | HS6, ΔgadA      | This study |
| HS23                | HS6, ΔproB      | This study |
| HS24                | HS6, Δacr ΔptsH | This study |
| HS25                | HS6, Δacr ΔptsI | This study |
| HS26                | HS6, Δacr ΔptsG | This study |
| HS27                | HS6, Δacr Δarpe | This study |
| HS28                | HS6, Δacr ΔaktA | This study |
| HS29                | HS9, ΔgalR Trc-glk | This study |
| HS30                | HS6, Trc-glb    | This study |
| HS31                | HS30, ΔargG     | This study |
| HS32                | HS30, ΔproB     | This study |
| HS33                | HS9, ΔgalR Trc-glk Trc-glb | This study |
| **Plasmids**        |                 |        |
| pTarget-X           | A plasmid used to transcript sgRNA targeting the gene X in genome. X refers to the genes in central metabolism and | This study |
selected amino acid biosynthetic pathways.

| Expression | Description |
|------------|-------------|
| pACYC-\(\text{pyc}^{\text{P458S}}-\text{thrA}^{\text{G433R}}-\text{lysC}}\) \(\text{Cm}^R\) | pACYC184 containing \(\text{pyc}^{\text{P458S}}\), \(\text{thrA}^{\text{G433R}}\) and \(\text{lysC}\) |
| pTrc-gltA\(_{\text{ec}}\) | pTrc99A containing \(\text{gltA}\) from \(\text{E. coli}\) |
| pTrc-citA\(_{\text{bs}}\) | pTrc99A containing \(\text{citA}\) from \(\text{Bacillus subtilis}\) |
| pTrc-gltA\(_{\text{cg}}\) | pTrc99A containing \(\text{gltA}\) from \(\text{Corynebacterium glutamicum}\) |

This study

\(^a\) TransGen Biotech, Shanghai, China.

\(^b\) \text{E. coli} \ Genetic Resource Center.
Table 2 The L-homoserine production and relevant fermentation parameters of the engineered strains.

| Strains                          | L-homoserine titer (g/L) | Cell growth (OD<sub>600</sub>) | Cultivation mode | Analytical methods          | Source        |
|----------------------------------|--------------------------|---------------------------------|------------------|-----------------------------|---------------|
| E. coli NZ10                     | 10.6                     | --                              | Shake-flask (glucose) | HPLC (o-phthalaldehyde)    | (54)          |
| C. glutamicum 9366-EMS           | 14.5                     | --                              | Batch cultivation (sucrose) | Amino analyzer | (55)          |
| C. glutamicum MH20-22B           | 3.0                      | --                              | Shake-flask (glucose) | HPLC (phenylisothiocyanate) | (12)         |
| HM5(pBRmetL–pNrhtA)              | 39.54 ± 1.10             | 38.99 ± 0.55                    | Fed-batch (glucose) | HPLC (o-phthalaldehyde) | (13)          |
| HS1                              | 1.85 ± 0.11              | 14.01 ± 0.14                    | Shake-flask (glucose) | Amino analyzer | This study |
| HS2                              | 2.01 ± 0.11              | 15.21 ± 0.11                    | Shake-flask (glucose) | Amino analyzer | This study |
| HS5                              | 3.14 ± 0.01              | 15.17 ± 0.27                    | Shake-flask (glucose) | Amino analyzer | This study |
| HS6                              | 3.26 ± 0.13              | 14.67 ± 0.44                    | Shake-flask (glucose) | Amino analyzer | This study |
| HS9                              | 5.28 ± 0.10              | 17.65 ± 0.27                    | Shake-flask (glucose) | Amino analyzer | This study |
| HS12                             | 5.52 ± 0.04              | 12.06 ± 0.47                    | Shake-flask (glucose) | Amino analyzer | This study |
| HS29                             | 6.27 ± 0.05              | 17.32 ± 0.44                    | Shake-flask (glucose) | Amino analyzer | This study |
| HS30                             | 3.95 ± 0.07              | 17.07 ± 0.35                    | Shake-flask (glucose) | Amino analyzer | This study |
| HS33                             | 7.25 ± 0.20              | 15.58 ± 0.55                    | Shake-flask (glucose) | Amino analyzer | This study |
| HS33/pACYC-pyc<sup>PS8S</sup>-<i>thrA</i>G148R,<i>lysC</i> | 8.54 ± 0.10              | 15.48 ± 0.34                    | Shake-flask (glucose) | Amino analyzer | This study |
| HS33/pACYC-pyc<sup>PS8S</sup>-<i>thrA</i>G148R,<i>lysC</i> | 37.57 ± 0.66             | 29.3 ± 0.14                     | Fed-batch (glucose) | Amino analyzer | This study |
Table 3 Primers used for plasmid construction

| Primer     | Sequence (5’-3’)                                      | Source |
|------------|-------------------------------------------------------|--------|
| pACYC-F    | CCTGATGAATGCTCATCCGG                                  | This   |
| pACYC-R    | GCAAATATTACGCAAGG                                     | This   |
| Pyc-F      | CCTTGCGTATAATTTTGCATGTCGACTCACACATCTTC               | This   |
| Pyc-R      | ACACTCGCATGTATATCTCTTTTTAGAAACGACGACGATCA            | This   |
| ThrA-F     | AAGGAGATATACATCGAGTGTTGAGTTCGG                       | This   |
| ThrA-R     | TTTTCAGACATGTATATCTCTTCTTTAGACTCCTAATCTTTCCATG       | This   |
| LysC-F     | AGGAGTCTGAAAGAGATATACATGTCTGAAATTGTTGTTCTC           | This   |
| LysC-R     | CCTGATGAATGCTCATCCGGTTACTCAAACAAATTACTAT             | This   |
| pTrc99A-F  | TCTGTTTCCTGTGGAATTT                                   | This   |
| pTrc99A-R  | TCTAGAGTCGACCTGCAG                                    | This   |
| GltAc_F    | AATTTTCACACAGGAAACAGAATGGCTGATAAAAAAGCAA             | This   |
| GltAc_R    | GCCTGCAGGTCGACTCTAGATTAACGCTGATATCGCTTT             | This   |
| CitAc_F    | AATTTTCACACAGGAAACAGAATGGTACATTACCGATTTA             | This   |
| CitAc_R    | GCCTGCAGGTCGACTCTAGATTAACGCTGATATCGCTG              | This   |
| GltAcg_F   | AATTTTCACACAGGAAACAGAATGGTATGGAAGGATATCGT            | This   |
| GltAcg_R   | GCCTGCAGGTCGACTCTAGATTAGGCGCTCCTCGGAGGA             | This   |
### Table 4 Primers used for CRISPRi

| Target | Primers | Sequence (5’-3’) |
|--------|---------|------------------|
| ptsH   | pTarget-ptsH | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| ptsG   | pTarget-ptsG | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| ptsI   | pTarget-ptsI | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| crr    | pTarget-crr | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| tktA   | pTarget-tktA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| tktB   | pTarget-tktB | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| rpe    | pTarget-rpe | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| rpiA   | pTarget-rpiA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| rpiB   | pTarget-rpiB | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| talA   | pTarget-talA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| talB   | pTarget-talB | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| zwf    | pTarget-zwf | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| ackA   | pTarget-ackA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| pta    | pTarget-pta | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| poxB   | pTarget-poxB | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| ldhA   | pTarget-ldhA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| dld    | pTarget-dld | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| adhE   | pTarget-adhE | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| mdh    | pTarget-mdh | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| serA   | pTarget-serA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| pck    | pTarget-pck | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| pps    | pTarget-pps | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| maeB   | pTarget-maeB | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| ilvA   | pTarget-ilvA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| tdcB   | pTarget-tdcB | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| pyrL   | pTarget-pyrL | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| pyrB   | pTarget-pyrB | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| asA    | pTarget-asA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| asnB   | pTarget-asnB | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| purA   | pTarget-purA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| putA   | pTarget-putA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| argG   | pTarget-argG | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| glnA   | pTarget-glnA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| ltaE   | pTarget-ltaE | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| tdh    | pTarget-tdh | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| argA   | pTarget-argA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| proB   | pTarget-proB | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| aspA   | pTarget-aspA | ATACTAGTGTTGCAGACCGTGCTCGAGGTATTTGAGCTAGAAATAGC |
| Primer                      | Sequence                  |
|----------------------------|---------------------------|
| gadA pTarget-gadA          | ATACTAGTGTGAATCGAGTAGTTCTGAGGTTTTAGAGCTAGAAATAGC |
| pTarget-null1              | ATACTAGTGTATTTAGAGCTAGAAATAGC |
| pTarget R-common          | ACTAGTATTACCTAGGACTGAGC |

1. Each pTarget primer shares the same reverse primer pTarget R-common (at the end of the Table).

2. pTarget-null represents the primer to construct pTarget-null without N20 sequence used as negative control.

The blue letters represent the Restriction Enzyme Cutting site (ScaI) and the red letters represent the 20-bp region complementary to the targeting region of selected genes.
Table 5 Primers used for genome editing

| Target   | Primers used for gene knock out | Sequence (5'-3') |
|----------|---------------------------------|-----------------|
| **metA** | pTarget-metA                    | TAAATACAGTTCTGCGAGAGCAGGGAAGAGTT |
|          | L-metA-F                        | TTAGAGCTAGAAATAGC |
|          | L-metA-R                        | TCACAGAGAGAATCCGTTGAGATACCTACATA |
|          | R-metA-F                        | TAAATAGGTTCCTTCGTAGATGTCCGAGT |
|          | R-metA-R                        | ATAAAGACTTACATTGGCAGGTT |
|          | T-metA-F                        | TGCTTAAGCAGAAATACG |
|          | T-metA-R                        | GTAAAATTTGCTGCTTTAGC |
| **lysA** | pTarget-lysA                    | TAAATGCTACAGACAGCCAGGGAATAGTG |
|          | L-lysA-F                        | CGTTCTGTCCTCCGAGGCTATGGA |
|          | L-lysA-R                        | CTAAACGCAGAAAATCCAGATAGTG |
|          | R-lysA-F                        | GGAATGTTTCGCTGGTTAGTTCGCTGGTG |
|          | R-lysA-R                        | ATCCTGCTGCTTTTAAACGTT |
|          | T-lysA-F                        | ATAGCTGTCAGACGAGAAGA |
|          | T-lysA-R                        | TTACCAACAGAAAGAAAGA |
| **ΔlacI::Trc-rhtA** | pTarget-lacI                    | GCTTAAAGCAATGAGTGAGCTAAGCTCACAAAC |
|          | L-lacI-F                        | AAGTGTTATGCGCTGACGAT |
|          | L-lacI-R                        | TAATTGTCTAACACCTGAGATAG |
|          | lacI::Trc-metA-F                | GGAATGTTAATGTTATGCTAGAT |
|          | lacI::Trc-metA-R                | CATTAAATACATTACATTATAGCTCCG |
|          | R-lacI-F                        | GGTGATGTTAATGTTAATGCTAGAT |
|          | R-lacI-R                        | TTAATATACATTACATTATAGCTCCG |
|          | T-lacI-F                        | ATGATAATTGGTACCAG |
|          | T-lacI-R                        | AAAAGATAATTTATAGCG |
| **iclR** | pTarget-iclR                    | TAATACTAGTCTAAACACGAGATCAAGCAGT |
|          | L-iclR-F                        | GTGGTGTAGCAGTCGGAGAAAATAGA |
|          | L-iclR-R                        | CCAGAAAAGGACAGTCTTCCTTCAGT |
|          | R-iclR-F                        | AGAGACTGTCCCTTTCCTGCGCGACGAGG |
|          | R-iclR-R                        | AGTGTTGTTTCTGGTTTTTAAT |
|          | T-iclR-F                        | GTGGTGTAGCAGTCGGAG |
|          | T-iclR-R                        | TCGGCATACATACGTTT |
| **fumB** | pTarget-fumB                    | GCTCTAAAACAATGAGTGAGCTAAGCTCACAAAC |
|          | L-fumB-F                        | TTAATATAGCGACAGTCGGAG |
|          | L-fumB-R                        | GAAGAGGTATAGCAGTCGGAG |
pTarget-galR  TAAATACGAGCGGTAGGAAACCATGAAAGATGC
L-galR-F   CTCTGATTCAGTAAAAGCGA
L-galR-R   TCTGGGTGTTACATGAAAATACCTTAGA
R-galR-F   TAACCGCAGAAACACTCCAGATAAGTTGCT
R-galR-R   TTYTTAGAGCTAGAAATAGC
T-galR-F   TAAATACGAGCGGTAGGAAACCATGAAAGATGC
T-galR-R   TCTGGGTGTTACATGAAAATACCTTAGA
pTarget-ikzA  TAGAGCTAGAAATAGC
L-ikzA-F   TTGTACACAGTATCGGTATTG
L-ikzA-R   ATGCTTTTCCAGATGAAAGTTG
R-ikzA-F   GAACCCGATGACAGATGCTTTG
R-ikzA-R   GCCAGATGATGAAAGTTG
T-ikzA-F   ATGCTTTTCCAGATGAAAGTTG
T-ikzA-R   GCCAGATGATGAAAGTTG
pTarget-rpe  AGAGCTAGAAATAGC
L-rpe-F   TAAATTTTAGCCTACGCTAC
L-rpe-R   TTGTACACAGTATCGGTATTG
R-rpe-F   GAACCCGATGACAGATGCTTTG
R-rpe-R   GCCAGATGATGAAAGTTG
T-rpe-F   ATGCTTTTCCAGATGAAAGTTG
T-rpe-R   GCCAGATGATGAAAGTTG
pTarget-talB  AGAGCTAGAAATAGC
L-talB-F   ACTGTACACAGGACCTCTCTA
L-talB-R   AGATGATATTGAGATAGTATTTTCTCTT
R-talB-F   TAAATTTTAGCCTACGCTAC
R-talB-R   TTGTACACAGTATCGGTATTG
T-talB-F   ATGCTTTTCCAGATGAAAGTTG
T-talB-R   GCCAGATGATGAAAGTTG
pTarget-zwf  TAGAGCTAGAAATAGC
L-zwf-F   TTGTACACAGGACCTCTCTA
L-zwf-R   AGATGATATTGAGATAGTATTTTCTCTT
R-zwf-F   TAAATTTTAGCCTACGCTAC
R-zwf-R   TTGTACACAGGACCTCTCTA
T-zwf-F   ATGCTTTTCCAGATGAAAGTTG
T-zwf-R   GCCAGATGATGAAAGTTG
pTarget-ackA  TAGAGCTAGAAATAGC
L-ackA-F   CATAAAACAGGATGACGTTTTA
L-ackA-R   TAAATTTTAGCCTACGCTAC
T-ackA-F   ATGCTTTTCCAGATGAAAGTTG
T-ackA-R   GCCAGATGATGAAAGTTG
R-ackA-F  TACTTCCATGTGATTTCACACCGCCAGCTC
R-ackA-R  TGATGGTTGGTTTTTGGA
T-ackA-F  CTTCATAAAACCAGTTAAGG
T-ackA-R  ACTTTAGCTTTGGAAAGATGC
pTarget-pta  ATACTAGTTACCGAAACCAGCTCGTCGTTTT
L-pta-F  TAAATGCGTGACACCTCTA
L-pta-R  GACGAGATTACCGGAACCAGCGTCGGTGTTT
R-pta-F  ATAAACGGTAAATCTCGTACATCCGCA
R-pta-R  TACCGTTATCAATGGTTCTCT
T-pta-F  CTTACAACCTGTACAAAGA
T-pta-R  ATTTGCGTGGCAATATAGGT

pta

R-gadA-F  ATACTAGTTACCGAAACCAGCTCGTCGTTTT
R-gadA-R  TGATGGTTGGTTTTTGGA
T-gadA-F  ACTGATGCCATTGCTGAATT
T-gadA-R  TCTCTACTACAGTGATGAAC

gadA

R-argG-F  ATACTAGTTACCGAAACCAGCTCGTCGTTTT
R-argG-R  TGATGGTTGGTTTTTGGA
T-argG-F  CCTCTGACACGAATACAAA
T-argG-R  ACTGATGCCATTGCTGAATT

argG

R-poxB-F  ATACTAGTTACCGAAACCAGCTCGTCGTTTT
R-poxB-R  TGATGGTTGGTTTTTGGA
T-poxB-F  ACTGATGCCATTGCTGAATT
T-poxB-R  TCTCTACTACAGTGATGAAC

poxB

R-ilvA-F  ATACTAGTTACCGAAACCAGCTCGTCGTTTT
R-ilvA-R  TGATGGTTGGTTTTTGGA
T-ilvA-F  ACTGATGCCATTGCTGAATT
T-ilvA-R  TCTCTACTACAGTGATGAAC

ilvA

R-target-poxB-F  ATACTAGTTACCGAAACCAGCTCGTCGTTTT
R-target-poxB-R  TGATGGTTGGTTTTTGGA
T-target-poxB-F  ACTGATGCCATTGCTGAATT
T-target-poxB-R  TCTCTACTACAGTGATGAAC

poxB

R-target-argG-F  ATACTAGTTACCGAAACCAGCTCGTCGTTTT
R-target-argG-R  TGATGGTTGGTTTTTGGA
T-target-argG-F  ACTGATGCCATTGCTGAATT
T-target-argG-R  TCTCTACTACAGTGATGAAC

argG

R-target-gadA-F  ATACTAGTTACCGAAACCAGCTCGTCGTTTT
R-target-gadA-R  TGATGGTTGGTTTTTGGA
T-target-gadA-F  ACTGATGCCATTGCTGAATT
T-target-gadA-R  TCTCTACTACAGTGATGAAC

gadA
Primers used for in situ promoter replacement

**pTarget-proB**
- **pTarget**-**proB**: ATACTAGTGCAGTTTTACCACCAGCGTCGTTTT
- **L-proB-F**: CAGTATCACCTGCTGCTTTTA
- **L-proB-R**: CTGCTCCCTACATGATTTCTGCCCATTCAA
- **R-proB-F**: GAGAATCATGTAAGGAGCAGGCTGATGCTG
- **R-proB-R**: TTAGTGCGACACGTTTCTTT
- **T-proB-F**: AGGTACGCCATAATCGAA
- **T-proB-R**: TTATCCATACGACCATTTTC

**proB**

**rhtA**
- **pTarget**-**rhtA**: TAATACTAGTGCAGTTTTACCACCAGCGTCGTTTT
- **L-rhtA-F**: TGGATGTGAATGTTAGTCCGGTGATTTCC
- **L-rhtA-R**: ACCACACATTATACGAGCCGATGATTTG
- **R-rhtA-F**: ATACATGCGCTGCCATTTACATGAAATTTCC
- **R-rhtA-R**: ACTATAAATGTAAATAGCCAAATTTCC
- **T-rhtA-F**: AAAGTGATTAGAAGGGTTAAGGC
- **T-rhtA-R**: TGCTGCTTTACCCAAATTTGA

**eamA**
- **pTarget**-**eamA**: TAATACTAGTGCAGTTTTACCACCAGCGTCGTTTT
- **L-eamA-F**: TGGCGTTTACCCAAAATTTGA
- **L-eamA-R**: ACCACACATTATACGAGCCGATGATTTG
- **R-eamA-F**: ATACATGCGCTGCCATTTACATGAAATTTCC
- **R-eamA-R**: ACTATAAATGTAAATAGCCAAATTTCC
- **T-eamA-F**: AAAGTGATTAGAAGGGTTAAGGC
- **T-eamA-R**: TGCTGCTTTACCCAAATTTGA

**asPA**
- **pTarget**-**asPA**: TAATACTAGTGCAGTTTTACCACCAGCGTCGTTTT
- **L-asPA-F**: TGGATGTGAATGTTAGTCCGGTGATTTCC
- **L-asPA-R**: ACCACACATTATACGAGCCGATGATTTG
- **R-asPA-F**: ATACATGCGCTGCCATTTACATGAAATTTCC
- **R-asPA-R**: ACTATAAATGTAAATAGCCAAATTTCC
- **T-asPA-F**: AAAGTGATTAGAAGGGTTAAGGC
- **T-asPA-R**: TGCTGCTTTACCCAAATTTGA

**glk**
- **pTarget**-**glk**: TAATACTAGTGCAGTTTTACCACCAGCGTCGTTTT
- **L-glk-F**: TGGATGTGAATGTTAGTCCGGTGATTTCC
- **L-glk-R**: ACCACACATTATACGAGCCGATGATTTG
- **R-glk-F**: ATACATGCGCTGCCATTTACATGAAATTTCC
- **R-glk-R**: ACTATAAATGTAAATAGCCAAATTTCC

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| Primer          | Sequence                        |
|----------------|---------------------------------|
| R-\textit{gltB}-R | ATAATGGCCTCTTCTTCACT             |
| T-\textit{gltB}-F  | TTTGCGAAAATATCAACGCC            |
| p\textit{Target}-\textit{gltB} | TTTAGAGCTAGAAATAGC              |
| L-\textit{gltB}-F  | TTTGCCCATAACGACGGGT             |
| L-\textit{gltB}-R  | CATTATACGAGCCGGATGATAATTGCAAAT |
| R-\textit{gltB}-F  | GGCAAGCTTATTGGTACA              |
| R-\textit{gltB}-R  | GTATAATGTGTGTCACAAAAGGATATAAC  |
| T-\textit{gltB}-F  | ATGACACGCAAACCCCGTACG          |
| R-\textit{gltB}-R  | TTTGCCTATGCGTGGCAGA             |
| T-\textit{gltB}-F  | AAACGAGGAACACATTACAGA           |
| Tr\textit{cV}^2  | GTGACCACACATTATAACGAGCCGGATGA  |

\(^1\)Each p\textit{Target} primer shares the same reverse primer p\textit{Target} R-common listed in Table 4.

\(^2\)Tr\textit{cV} represents a primer complementary to the trc promoter.
Fig. 1 The key metabolic pathway for the construction of inducer free L-homoserine-producing strain. The red crosses indicate that the genes are disrupted. The green arrows indicate that pathways are overexpressed. Abbreviations: Glu, glucose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F1.6P, fructose-1,6-diphosphate; 6PG, 6-phosphogluconolactone; X5P, xylulose 5-phosphate; GAP, glyceraldehyde 3-phosphate; G3P, 3-phosphate-glycerate; G2P, 2-phosphate-glycerate; SER, serine; PEP, phosphoenolpyruvate; Ace-CoA, acetyl-CoA; PRY, pyruvate; LAC, lactate; ACE, acetate; CIT, citrate; ICL, isocitrate; α-KG, α-ketoglutarate; SUCC, succinyl-CoA; SUC, succinate; FUM, fumarate; MAL, L-malate; OAA, oxaloacetate; ASP, L-aspartate; ASP-P, L-aspartate 4-semialdehyde; ASP-SA, L-aspartate 4-semialdehyde; HS, L-homoserine; LYS, L-lysine; THR, L-threonine; OSH, O-succinylhomoserine.

Fig. 2 Comparison of the fermentation performances of the engineered strains. Cells were cultured in MS media at 28 °C for 48 h.

Fig. 3 Effect of transport system (RhtA and EamA) modification on L-homoserine production. Cells were cultured in MS media at 28 °C for 48 h.

Fig. 4 Effect of TCA cycle perturbation on fermentation performances of different engineered strains.

Cells were cultured in MS media at 28 °C for 48 h.

Fig. 5 CRISPR-dCas9-based strategy for systematic screening and fine-tuning of gene expression for L-homoserine overproduction. (a) Schematic of CRISPR-dCas9-based gene regulation in the L-homoserine biosynthesis pathway. The 39 target genes including glycolysis, by-products and branch pathways were investigated in this study. The results were drawn from a combined data of the single gene target among the genes. Relative changes in L-homoserine yield compared with the negative
control (HS5 harboring a pTarget-null plasmid without N20 sequence) are represented as colored circles. Blue circles represent no significant change (-20% to 5%) of L-homoserine production compared to the negative control. Targets that increased the L-homoserine production are shown in red.

(b) Cell growth and homoserine production in deletion of positive targets. The control refers to the strain HS6. Cells were cultured in MS media at 28 °C for 48 h.

Fig. 6 Modification targets for enhancement of glycolysis pathway. (a) The effects of disrupting PTS system and pentose phosphate pathway on L-homoserine production and cell growth. (b) The effects of modification of non-PTS glucose uptake system on cell growth and homoserine production. Cells were cultured in MS media at 28 °C for 48 h.

Fig. 7 Modification of metabolic flux at glutamate node. (a) The L-homoserine production, cell growth and glucose consumption in different engineered strains were compared. (b) Effects of the gltBD operon overexpression on L-homoserine and by-products production.

Fig. 8 Systems metabolic engineering of *Escherichia coli* for homoserine production. The data comprise relative concentrations of intracellular intermediates and metabolic flux prediction in the central metabolic pathways as well as the genomic traits engineered into the strain. The thickness of the reaction arrows represents the predicted flux amount. The relative pool sizes of selected metabolites within two strains are given as REL values in the table, with concentrations in *E. coli* W3110 taken as the reference for normalization between samples. The genomic traits include deletion of the transcriptional repressor (ΔmetJ), deletion of O-succinylhomoserine lyase (ΔmetB), overexpression of aspartate kinase (PmetL), deletion of homoserine kinase (ΔthrB), deletion of homoserine O-succinyltransferase (ΔmetA), overexpression of homoserine dehydrogenase (PthrA), deletion of diaminopimelate decarboxylase (ΔlysA), insertion of rhtA into the *lacI* locus (ΔlacI::rhtA),
overexpression of the L-homoserine exporter (Ptrc rhtA), overexpression of the cysteine/O-acetylserine exporter (Ptrc eamA), deletion of the isocitrate lyase regulator (ΔiclR), deletion of the glucose-specific PTS enzyme IIBC component (ΔptsG), deletion of the galactose operon repressor (ΔgalR), overexpression of glucokinase (Ptrc glk), overexpression of the glutamate synthase operon (Ptrc gltBD).

Abbreviations: REL, relative values; Glu, L-glutamate; α-KG, α-ketoglutarate; Asp-SA, L-aspartate 4-semialdehyde; DAP, diaminopimelic acid; GOX, glyoxylate cycle.

The screened metabolomics data was subjected to Partial Least Square Discrimination Analysis (PLS-DA). Significantly changed metabolites (SCM; defined by based VIP >1, fold-change >1.2 or < 0.8333 and q < 0.05) were selected for subsequent chemical structure identification.

Fig. 9 Introducing the anaplerotic route afforded by pyruvate carboxylase further improved the production of homoserine. (a) The effects of overexpression of pyruvate carboxylase or citrate synthase on fermentation performance of L-homoserine. gltAec, encoding citrate synthase from E. coli; citAbs, encoding citrate synthase from Bacillus subtilis; gltAcg, encoding citrate synthase from Corynebacterium glutamicum; (b) An overview of central metabolic pathway on the strategy for diverting carbon to homoserine from acetyl-CoA. Blue arrows represent the paths (genes) introduced in the construction of further improved strain.

Fig. 10 Fed-batch fermentation profile of HS33/pACYC-pycP438S-thrA<sup>G433R</sup>-lysC in a 5-L bioreactor. Time profiles of L-homoserine production, glucose concentration and cell growth during the fed-batch cultivation are shown. Circle, L-homoserine; square, glucose; triangle, cell growth.
1. Glucose-6P
2. Fructose-6P
3. Glyceraldehyde-3P
4. Glycerate 3P
5. Phosphoenolpyruvate
6. Pyruvate
7. Acetyl-CoA
8. Citriate
9. α-ketoglutarate
10. Succinate
11. Fumarate
12. Oxaloacetate
